From lab to forest and orchard

The Final CopyTree Conference

Book of Proceedings

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Tirana, Albania

Hosted by
Academy of Sciences of Albania

The 4th Conference of
Cost Action CA21157 - COPYTREE

From Lab to Forest and Orchard: The Final Conference of CopyTree

Organized by

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From Lab to Forest and Orchard: The Final Conference of CopyTree

Book editing and layout: Prof. Valbona SOTA
Science Communication Coordinator of CA21157
University of Tirana, Albania
valbona.sota@fshn.edu.al

Prof. Stefaan WERBROUCK
Chair of CA21157
Ghent University, Belgium
Stefaan.werbrouck@ugent.be

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SCIENTIFIC COMMITTEE

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Branislav Cvjetković – University of Banja Luka, Bosnia and Herzegovina; *Co-Leader of WG5*

Dicle Dönmez – Cukurova University, Turkey; *Scientific Innovation Network Coordinator of CopyTree*

Tobias Breugmann – Technical University Freiberg, Germany, formerly Thünen Institute; *Webinar Coordinator of CopyTree*

LOCAL ORGANIZING COMMITTEE

- **Valbona Sota –** [1]Research Center of Biotechnology and Genetics, Academy of Sciences of Albania; [2]University of Tirana, Albania
- **Efigjeni Kongjika –** Research Center of Biotechnology and Genetics, Academy of Sciences of Albania
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- **Eljora Sema** – Administrative Sector, Academy of Sciences of Albania
- **Blerina Shkreta** – Administrative Sector, Academy of Sciences of Albania

Contact:

valbona.sota@fshn.edu.al

copytree21157@gmail.com

RESEARCH TOPICS

WG1: Recalcitrance

Insights in genetics, epigenetics, and protein networks. New chemical and physical tools that might break recalcitrance. Bio-imaging systems.

WG2: Diagnosis, sanitation, and conservation

Diagnosis of viruses, viroids, and bacteria, sanitation protocols for clean stock production, mid- and long-term storage of clean stocks, and germplasm.

WG3: Scaling up and automation

Initiation, multiplication, preservation, germination, and conversion of somatic embryos of new species. Temporary immersion systems. Fully automatic micropropagation systems combining robotics, laser cutting, machine learning, and artificial intelligence.

WG4: Technological risk assessment, public acceptance, legislation, and commercialization

Risk assessment of cloning plants, strategy to stimulating technology awareness and acceptance of stakeholders, stimulating investment and commercialization.

WG5: Communication, dissemination, and technology transfer

Communication, dissemination, and technology transfer.

Conference Opening Speech (September, 2026)

Good morning, dear colleagues, dear friends. It is both a great pleasure and a privilege to welcome you to Tirana for the final conference of the COST Action CopyTree, entitled "From Lab to Forest and Orchard."

I would first like to express our sincere gratitude to the Academy of Sciences of Albania for hosting this conference, and to Acad. Prof. Dr. Skënder Gjinushi, President of the Academy, under whose auspices this event is being held. I also warmly thank Valbona Sota and the entire local organizing team for their commitment, hospitality, and considerable efforts in bringing us together for these three important days.

When CopyTree began, our ambition was clear: to bring together a fragmented but highly skilled community working on the cloning, propagation, conservation, and sustainable use of woody plants. We wanted to create a network in which researchers, commercial producers, policymakers, students, and other stakeholders could exchange knowledge, share facilities and expertise, and develop solutions that no institution or country could achieve alone. Today, as we meet for our final conference, we can look back with pride on how that ambition has developed into a dynamic and collaborative international community.

Woody plants are fundamental to our societies and ecosystems. Their importance is increasing as we face climate change, emerging diseases, the erosion of genetic resources, and a growing demand for resilient and high-quality planting material. Yet woody plants are often among the most difficult species to propagate under *in vitro* conditions. This challenge has been at the heart of CopyTree.

Our work begins in the laboratory, where we study cells, tissues, microorganisms, genes, hormones, culture media, and developmental processes. However, it cannot end there. Scientific advances must ultimately produce healthy plants, resilient forests, productive orchards, sustainable businesses, conserved genetic resources, and benefits for society.

During this conference, we will hear about new approaches to overcoming recalcitrance, the use of omics and genome-editing technologies, pathogen diagnosis and sanitation, cryopreservation, somatic embryogenesis, temporary immersion systems, digital phenotyping, robotics, machine learning, and artificial intelligence. We will also examine quality assurance, commercialization, regulation, public perception, communication, and the transfer of research results into practice.

CopyTree has also been a network of people. Its most valuable results are not limited to publications, protocols, databases, conferences, or scientific missions. They can also be found in the professional relationships that have been created: between established scientists and young researchers, between laboratories and companies, and between colleagues from different countries who might otherwise never have worked together.

I am particularly proud of the opportunities that the Action has provided for young researchers. Through conferences, training activities, working-group meetings, and Short-Term Scientific Missions, they have gained access to new techniques, laboratories, mentors, and international collaborations. Their energy and scientific curiosity give us confidence

that the work initiated through CopyTree will continue well beyond the formal lifetime of the Action.

Our most important knowledge output is the Springer book volume 'Next-Generation *In vitro* Strategies in Woody Plant Cloning'. It will help preserve and extend the knowledge generated through our collaboration, and it will serve as a lasting memory. We extend our thanks to Valbona Sota for being the driving force behind the editorial team.

I would like to thank our complete Core Group. Your enthusiasm, leadership, patience, creativity, and voluntary commitment have sustained this Action. My sincere thanks also go to COST and to the officers and administrative colleagues who have guided us throughout this journey.

Above all, I thank every active CopyTree participant. Whether you organized an event, hosted a scientific mission, contributed to a publication, reviewed a manuscript, presented a poster, shared a protocol, mentored a young colleague, or simply participated actively in our discussions, you have helped shape this network.

Although this is called our final conference, it should not be understood as an ending. A successful network does not disappear when its official funding period concludes. It continues through joint projects, publications, exchanges, friendships, new consortia, and a shared scientific vision.

Let us therefore use these three days not only to present what we have achieved, but also to decide what comes next. Let us identify the questions that remain unanswered, the technologies that need further development, and the partnerships required to carry our work from experimental systems into forests, orchards, nurseries, conservation programs, and commercial production.

I wish you all an inspiring, productive, and enjoyable conference. May our discussions be open, our collaborations ambitious, and our shared commitment to the future of woody plants stronger than ever.

It is my great pleasure to declare the Final Conference of CopyTree officially open.

Thank you.

Stefaan Werbrouck,
Chair of CopyTree

WORKING GROUP 1

Recalcitrance

CKX inhibitor 3TFM,5Cl-2HE as an effective booster for shoot formation in wild service tree (*Sorbus torminalis* L.) explants

Eva Pokorná[1], Pavlína Máchová[1], Jaroslav Dostál[1], Jaroslav Nisler[2]

[1]Department of Forest Tree Species Biology and Breeding, Forestry and Game Management Research Institute, Strnady 136, 252 02 Jíloviště, Czech Republic;

[2]Isotope Laboratory, Institute of Experimental Botany, Czech Academy of Sciences, Rozvojová 263, 165 02 Prague 6, Czech Republic;

pokorna@vulhm.cz

Abstract

The wild service tree (*Sorbus torminalis* (L.) Crantz) is a valuable deciduous tree species with a high potential in forestry and forest ecology. In the Czech Republic, high-quality individuals in terms of morphology and timber production are used as a source of reproductive material for forestry. Outstanding genotypes are often represented by old trees that are difficult to propagate, making *in vitro* techniques important for their conservation. Although a micropropagation protocol was developed and valuable genotypes were established for the National Bank of Forest Tree Explants (FGMRI, CZ), some genotypes show low shoot proliferation, requiring further optimization of the protocol to ensure stable *in vitro* cultures with sufficient microcuttings. This study evaluated the effects of the isoprenoid cytokinin N^6-(Δ^2-isopentenyl)-adenine (iP) combined with the inhibitor of cytokinin oxidase/dehydrogenase, 1-(3-chloro-5-(trifluoromethoxy)phenyl)-3-(2-(2-ethyl)phenyl)urea compound "20" (3TFM,5Cl-2HE), tested at a 5 µM concentration. In the first subculture, 5 µM iP + 5 µM 3TFM,5Cl-2HE increased shoot formation by 1.5-fold compared with explants routinely cultured on medium supplemented with BAP. However, this effect was not sustained in subsequent subcultures, in which lateral shoot formation ceased. Our results indicate that replacing BAP with isoprenoid cytokinin combined with 3TFM,5Cl-2HE can transiently stimulate shoot formation in wild service tree, suggesting a beneficial „booster" effect in long-term BAP-maintained cultures.

Keywords: *Sorbus torminalis, explants, phytohormones,* 3TFM,5Cl-2HE *treatment, shoot formation.*

1. INTRODUCTION

The wild service tree (*Sorbus torminalis* (L.) Crantz) is a minor scattered broadleaf deciduous species widely distributed across Europe, with an environmentally important role in stabilization and enrichment of forest and woodland ecosystems. Ancient wild populations still survive with a mean of considerable conservation significance (Máchová *et al.*, 2025, More and White, 2003). In the Czech Republic, the exceptional characteristics of wild service trees in terms of, e.g., production of very high-quality wood, drought resistance and biodiversity conservation are often found in valuable genetic resources that cannot always be effectively propagated (Malá *et al.*, 2009, Buriánek and Novotný, 2019). Improved protocols for *in vitro* reproduction are among the techniques for the vegetative propagation of woody plant species that may ensure a fast and high rate of clonal production (Sota *et al.*, 2025). However, the success of organogenesis and micropropagation is influenced by several significant factors such as the age; genotype; physiological condition and health status of the donor plant; the time of collection; surface

sterilization; and appropriate culture conditions related, for example, to the chemical composition of the culture medium as well as the concentrations and ratios of phytohormones (Chowdhury 2025, Gupta *et al.,* 2020, Guru *et al.,* 2026).

In plant tissue cultures of endemic rowan species, two groups of phytohormones are commonly used: cytokinins (CKs) and auxins represented by 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) (Máchová *et al.,* 2013). While BAP is an active aromatic CK with a stimulatory role in cell division and shoot formation, IBA, as a naturally occurring auxin, plays a role in both shoot and root morphogenesis, and promotes adventitious root formation (Gaspar *et al.,* 1996, Ludwig-Müller 2000, Strnad 1997). In plants, besides aromatic CKs are present an isoprenoid types in which several derivatives of N^6-(Δ^2-isopentenyl)-adenine (iP), *trans*- and *cis*-zeatins (*t*Z and *c*Z) and dihydrozeatin (DZ) are frequently found (Sakakibara 2006). According to the results of CK activity determined in various bioassays, the isoprenoid type of CKs appears to have a more potent effect on growth processes, while the aromatic CKs have a stronger influence on developmental processes. Although the results between different bioassays were not always consistent, generally *t*Z and iP exhibited higher activities than *c*Z or BAP (Sakakibara 2006, Holub *et al.,* 1998, Gajdošová *et al.,* 2011, Pokorná *et al.,* 2020, Manos and Goldthwaite 1976). Irreversible CK degradation is catalyzed by cytokinin oxidase/dehydrogenase (CKX) enzymes, which selectively cleave unsaturated isoprenoid side chains of *t*Z, iP and, to a lesser extent *c*Z and their ribosides, resulting in the formation of adenine/adenosine and the corresponding side chain aldehyde. Unfortunately, the aromatic CK degradation pathways remain to be elucidated (Strnad 1997, Werner *et al.,* 2006, Stirk *et al.,* 2012). To improve plant productivity, CKX inhibitors were developed and tested. Nowadays, CKX inhibitors are synthesized as attractive compounds for commercial use in plant biotechnology and agriculture (Aremu *et al.,* 2014, Voko *et al.,* 2024, Nisler *et al.,* 2018 and 2020).

Due to the long-lasting low multiplication coefficient observed in wild service tree explants cultivated on nutrient medium containing BAP, we aim in this study to characterize the effects of isoprenoid CK treatment applied together with a CKX inhibitor, 3TFM, 5Cl-2HE, on shoot formation.

2. MATERIALS AND METHODS

2.1. Materials

Plant tissue culture of wild service tree (*Sorbus torminalis* (L.) Crantz), genotype H17 from the collection of FGMRI (CZ) was used in the study.

2.2. Methods

For shoot induction, the multiplication MS medium (Murashige and Skoog 1962) was prepared according to Malá *et al.* (2009) containing 1.76 µM BAP and 0.49 µM IBA (Sigma™). The explants were long-term maintained (approximately 8 years) on this type of medium. The testing multiplication medium contained iP and CKX inhibitor, 1-(3-chloro-5-(trifluoromethoxy)phenyl)-3-(2-(2-ethyl)phenyl)urea compound "20" (3TFM,5Cl-2HE, gifted by Dr. Jaroslav Nisler), both at 5 µM concentrations. All phytohormones, except IBA, were dissolved in dimethyl sulfoxide (DMSO), diluted with water and added after autoclaving at 120°C for 20 min into the cooled medium. DMSO concentration was kept

consistent across all the media. To avoid plant tissue culture contamination 1mL L^{-1} of Plant Preservation Mixture (PPM, Carolina Biosystems) was added to the medium before autoclaving. The 40 mL of prepared medium was dispensed into a 200 mL Erlenmeyer flask and two young shoots were transferred into the tested medium, including five replicates per treatment and subcultured for 6 weeks. After that, the number of new shoots was counted and the effects of the tested compounds were evaluated. The experiment was repeated three times (1. – 3. subculturing).

2.3. Statistical analysis

Statistical analyses were performed using the NCSS 10 Statistical Software (LLC, Kaysville, Utah, USA, 2015). Based on the measured data representing a non-normal distribution, a nonparametric one-way Kruskal-Wallis ANOVA ($P < 0.05$) was performed, followed by a Kruskal-Wallis (Dunn) multiple comparison test. The experiment was conducted with five replicates; each comprises 10 explants per treatment.

3. RESULTS

The use of the isoprenoid cytokinin iP in combination with the 3TFM,5Cl-2HE, a compound that inhibits CKs degradation, in culture media for explants of wild service tree resulted in an increased formation of lateral shoots during the first subculturing compared with the MS medium containing BAP (Fig. 1). As shown in Fig. 1, a reduction in explant biomass was observed during the second and third subculturing, particularly on MS medium containing an isoprenoid-type cytokinin. In addition, a high concentration of iP+3TFM,5Cl-2HE in the culture medium led to phytotoxicity, which was obvious on the leaves of explants after the second and third subculturing (Fig. 1).

The mean number of newly formed shoots during the first subculturing of wild service tree was 2.8 ± 1.94 on BAP-containing medium, compared with the 4.2 ± 1.17 shoots growing on the medium containing 5 µM iP and 5 µM 3TFM,5Cl-2HE (Fig. 2). However, during the second subculturing, a similarly pronounced decrease in lateral shoot formation was observed on both types of media containing either an isoprenoid or an aromatic CK. In the third subculturing, a significant increase in lateral shoot formation (1.2 ± 0.75) was observed only *in vitro* cultures grown on BAP-containing medium, in contrast to any of the new shoots detected in explants growing on the medium containing 5 µM iP and 5 µM 3TFM,5Cl-2HE (Fig. 2). In addition, our results confirmed that DMSO itself, when present in the culture medium, did not affect lateral shoot formation (Fig. 2).

Our results indicate that the tested 5 µM concentration of iP, combined with a CK degradation inhibitor, positively affects shoot formation compared with BAP during first subculturing. We found that the first application of iP with a CKX inhibitor led to more efficient microcutting production of wild service tree during the multiplication phase; however, this effect significantly decreased in long-term *in vitro* cultures.

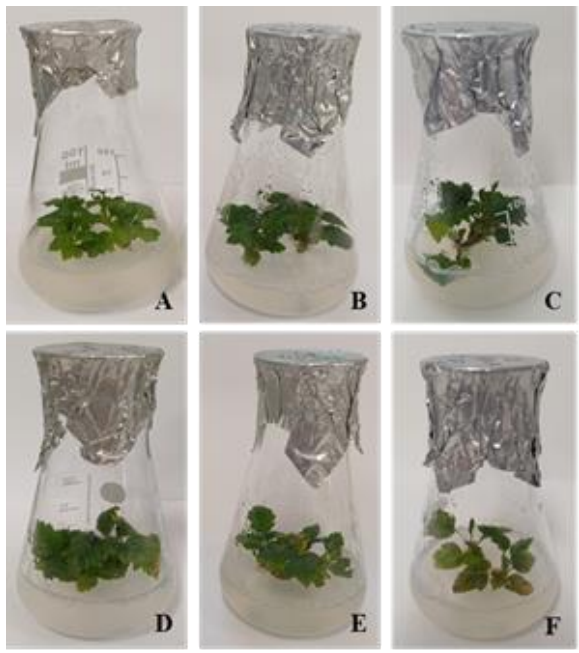


Fig. 1. Shoot induction in wild service tree (*Sorbus torminalis* (L.) Crantz) during three independent experiments. Explant cultures of wild service tree cultivated on MS medium augmented by 1.76 µM BAP (A, B, C) and 5 µM iP with 5 µM 3TFM,5Cl-2HE (D, E, F) were subcultured every 6 weeks/ three times (A, D - 1. subculturing; B, E–2. subculturing; C, E–3. subculturing).

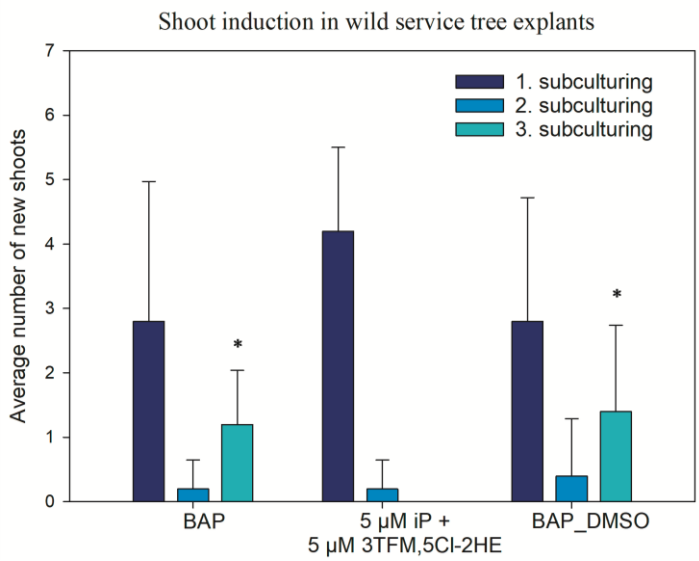


Fig. 2. Average number of new shoots of wild service tree (*Sorbus torminalis* (L.) Crantz) explants. *In vitro* culture of wild service tree was established on MS medium supplemented with different types of cytokinin and subcultured every 6 weeks/ three times (1.–3. subculturing). Significant differences between the aromatic and isoprenoid-type CK treatments were observed using Kruskal-Wallis One-Way ANOVA *P < 0.05.

4. DISCUSSION

For the micropropagation of *Sorbus* species, BAP, an aromatic cytokinin (CK), is preferentially utilized CK in the culture media. BAP is the most effective cytokinin for stimulating lateral shoot formation, not only in wild service tree but also in other *Sorbus* species, compared with other types of aromatic CKs (Malá *et al.,* 2009, Máchová *et al.,* 2013, Šedivá *et al.,* 2023). In contrast, culture media containing isoprenoid CKs are used less frequently in tissue cultures because their physiological effects are adversely affected by the activity of CKX enzymes, which catalyze their irreversible degradation (Werner *et al.,* 2006, Nisler 2024). Recent studies focusing on the synthesis of compounds that suppress CK degradation, referred to as CKX inhibitors, have demonstrated several beneficial effects in tissue cultures when a CKX inhibitor is added to the culture medium together with an isoprenoid CK (Hodai and Werbrouck 2025, Nisler *et al.,* 2021, 2022 and 2024, Sota *et al.,* 2025).

Consistent with these findings, we observed a positive effect of 5 µM iP applied in combination with the CKX inhibitor 3TFM,5Cl-2HE on lateral shoot initiation in wild service tree explants after first subculturing. During the first subculturing, the number of lateral shoots increased 1.5-fold compared with cultures grown on BAP-containing medium (Fig. 1A, 1D and Fig. 2). However, this stimulatory effect on lateral shoot formation, presumably resulting from prolonged lifetime and activity of endogenous CKs, was not observed during the second and third subculturing. One possible explanation may be that the concentrations of the two compounds used (5 µM iP and 5 µM 3TFM,5Cl-2HE) were 5- to 500-fold higher than those used in the studies investigating the physiological effects of CKX inhibitors in plant explants (Hodai and Werbrouck 2025, Nisler *et al.,* 2021, 2022 and 2024). Although Murvanidze *et al.,* (2021) tested 5 µM concentrations of two CKX inhibitors (3TFM-2HE and 3TFM,5Br-2HE) during somatic embryogenesis in *Coffea arabica*, they demonstrated that appropriate adjustment of the concentrations of both the CK and CKX inhibitors can substantially affect the balance of CK metabolism and, consequently, the efficiency of somatic embryo formation.

We therefore hypothesize that the 5 µM concentration of both the CK and CKX inhibitor used in our study has a stimulatory effect on long-term cultures maintained on BAP-containing medium mainly during the first application (Fig. 1A, 1D and Fig. 2). However, a phytotoxic effect was observed on the leaves of wild service tree explants, which remained evident following repeated application of both compounds (Fig. 1A–D). The high concentration of exogenously applied CKs adversely affected explant viability and several physiological processes, similar to those observed by Novák *et al.* (2013) in transgenic tobacco plants (*Nicotiana tabacum* L. cv. Petit Havana SR1) with enhanced CK production.

The results obtained in this study may enable the use of CKX inhibitor 3TFM,5Cl-2HE not only with other genotypes of wild service tree and potentially with other *Sorbus* species that have been maintained for long periods on BAP-containing media, but also in the National Bank of Explants of Forest Tree Species, operated by the Forestry and Game Management Research Institute (Czech Republic). This application could be particularly valuable for the conservation and propagation of genotypes with low multiplication capacity.

5. CONCLUSIONS

Our study briefly indicates the positive booster effect on shoot formation in wild service tree explants cultivated on MS medium containing 5 µM iP and 5 µM CKX inhibitor,

compound 3TFM,5Cl-2HE, compared to the *in vitro* cultures growing on MS medium supplemented with 1.76 µM BAP after first subculturing. The change of commonly used aromatic CK to the isoprenoid one in long-term subcultures increased shoot microcuttings production of wild service tree. Although this effect was not long-lasting, the disposable application of iP together with 3TFM,5Cl-2HE could be used in future subculturing to increase shoot multiplication in the other genotypes of wild service tree explants. To understand the short-term mode of action of testing compounds in plant tissue, lower concentrations of CK derivatives, use of different *Sorbus* ssp., or additional analyses such as the endogenous phytohormone profiles in explants should be investigated.

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Determining the effects of different bud sizes and culture media on anther culture in *Rosa damascena*

İlknur Eskimez, Ümit Pelin, Mehmet Polat

Isparta University of Applied Sciences, Faculty of Agriculture, Department of Horticulture, Isparta, Türkiye

ilknureskimez@isparta.edu.tr

Abstract

Rosa damascena Mill., which is considered an important raw material in essential oil production, perfumery, cosmetics, food, and pharmaceutical industries and is also widely cultivated as an ornamental plant, is a significant medicinal and aromatic plant species with high economic value. The development of tissue culture techniques for this species has the potential to contribute to breeding and propagation efforts. In this context, anther culture is an important *in vitro* approach for obtaining haploid and doubled haploid materials; however, the success of this method depends on many factors, such as genotype, the developmental stage of the explant, culture medium, and culture conditions. This study was conducted to determine the effects of different bud sizes, culture media, and culture conditions on anther culture in *Rosa damascena*. In the study, anthers isolated from flower buds approximately 1 cm and 2 cm in size were cultured on six different culture media. To determine the effects of culture conditions, some of the anthers were kept in the dark for the first four weeks and then transferred to light conditions, while the others were cultured directly under the photoperiod treatment conditions. The calli formed during the culture period were evaluated in terms of callus diameter and color. The findings are expected to provide a basis for identifying suitable bud sizes, culture media, and culture conditions for callus induction in *Rosa damascena* anther cultures and to inform future studies on *in vitro* plant regeneration.

Keywords: *explants, in vitro, rose*

1. INTRODUCTION

Oil rose (*Rosa damascena* Mill.) is a medicinal and aromatic plant species of high economic value due to its high essential oil content, characteristic fragrance, and wide range of uses. It is an important raw material for the perfumery, cosmetics, food, and pharmaceutical industries and is also valued as an ornamental plant because of its aesthetic characteristics. Widely cultivated in Türkiye, particularly in Isparta and the surrounding region, *R. damascena* is of strategic importance to both regional agriculture and the essential oil industry (Baydar, 2006). Therefore, breeding studies aimed at improving the yield, quality, and adaptation characteristics of this species are of considerable importance.

Although conventional plant breeding methods are fundamental tools for developing new and superior genotypes, they can be time-consuming, particularly in perennial and highly heterozygous plant species. In this context, plant tissue culture and other *in vitro* biotechnological techniques have emerged as valuable approaches that complement conventional breeding methods and improve the efficiency of breeding programs. *In vitro* techniques offer significant advantages, including the propagation of plant material under controlled conditions, conservation of genetic resources, acceleration of selection processes,

and generation of new plant materials for use in breeding programs (Jia *et al.*, 2014; Harmon *et al.*, 2022).

Among these techniques, anther culture is an important approach for obtaining haploid and doubled haploid materials of microspore origin. It is particularly valuable because it enables the rapid development of homozygous lines, facilitates the efficient evaluation of genetic variation, and can shorten subsequent stages of breeding programs. However, the success of anther culture is influenced by numerous factors, including genotype, physiological status of the donor plant, developmental stage of the microspore, bud size, culture medium composition, and incubation conditions (Zhou *et al.*, 2025).

The limited number of studies on anther culture in *Rosa damascena*, together with the absence of a well-established, standardized, and reproducible culture protocol for this species, highlights the need for a detailed evaluation of different culture factors. In particular, determining the effects of bud size, culture medium, and culture conditions on callus formation is important for identifying suitable conditions that may provide a basis for future haploidization, regeneration, and breeding studies for this species.

2. MATERIALS AND METHODS

2.1. Materials

The flower buds of *Rosa damascena* Mill. used in this study were of two sizes, 1 and 2 cm in length. They were collected in May from 15-year-old plants grown at an altitude of approximately 1,250 m in the Güneykent region of Isparta Province, Türkiye.

2.2. Methods

2.2.1. Preparation of Culture Media

In this study, MS (Murashige and Skoog, 1962) medium was used as the basal culture medium, and six different media were prepared according to the compositions presented in Table 1. Agar (Duchefa Biochemie) was added to the media at a concentration of 7.5 gL, and the pH was adjusted to 5.7 using 1 N NaOH or 1 N HCl. The media were sterilized by autoclaving at 121 °C and 1.2 atm for 15 min. After sterilization, the media were aseptically poured into sterile petri dishes (60 x 15 mm) at a volume of 30 mL per dish and allowed to solidify.

Table 1. Culture media used for anther culture of *Rosa damascena*

Culture media ($mg{\cdot}L^{-1}$)
Control
0.5 $mg{\cdot}L^{-1}$ BAP
2 $mg{\cdot}L^{-1}$ 2,4 D + 0.5 $mg{\cdot}L^{-1}$ BAP
4 $mg{\cdot}L^{-1}$ 2,4 D + 0.5 $mg{\cdot}L^{-1}$ BAP
2 $mg{\cdot}L^{-1}$ NAA + 0.5 $mg{\cdot}L^{-1}$ BAP
4 $mg{\cdot}L^{-1}$ NAA + 0.5 $mg{\cdot}L^{-1}$ BAP

2.2.2. Culture Conditions

Anthers transferred to the culture media were incubated in a controlled-environment growth chamber at 22 ± 2 °C. Two different culture conditions were applied to determine their effects on callus development. In the first treatment, the anthers were maintained in complete darkness for the first four weeks of culture and subsequently transferred to a 16 h light/8 h dark photoperiod. In the second treatment, the anthers were incubated under a 16 h light/8 h dark photoperiod from the beginning of the culture period. Illumination was provided by fluorescent lamps.

2.2.3. Assessment of Callus Induction Rate (%)

Anther development was monitored regularly throughout the culture period. Callus induction rate (%) was calculated by dividing the number of anthers forming callus by the total number of cultured anthers and multiplying by 100. The resulting calli were also evaluated in terms of diameter and color to assess the effects of bud size, culture medium, and culture conditions (Fig. 1).

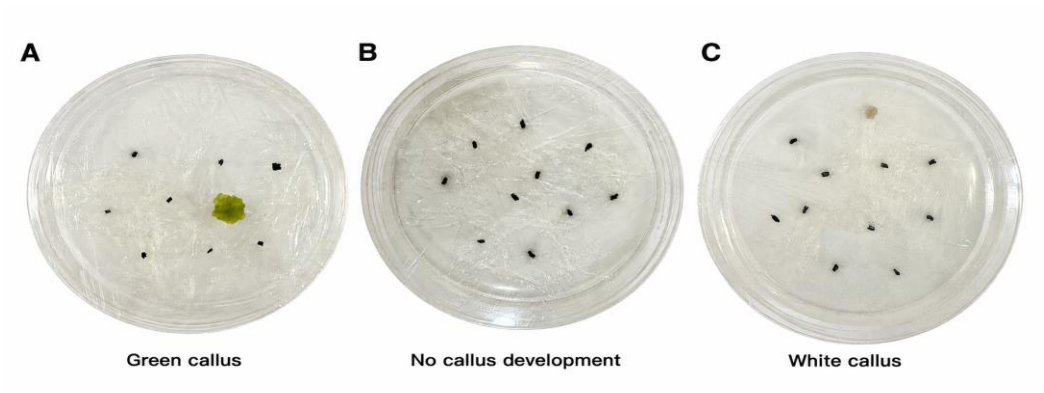


Fig. 1. Reactions observed in *Rosa damascena* anther cultures: (A) green callus formation, (B) undeveloped anthers, and (C) white callus formation

2.2.4. Statical Analyzes

The experiment included three replicate Petri dishes for each combination of culture medium, bud size, and culture conditions, with 15 anthers per dish. Each Petri dish was considered an experimental unit. Data were subjected to three-way analysis of variance (ANOVA) using Minitab 21 to assess the main effects of the three factors and their interactions. Mean differences were evaluated using Tukey's multiple comparison test at a significance level of $p < 0.05$ (Zar, 2013). The bubble plot illustrating the interaction between bud size and culture conditions was generated using the ggplot2 package (Wickham, 2016) in R version 4.3.2 (R Foundation for Statistical Computing, Vienna, Austria), with bubble areas proportional to callus induction rates (%).

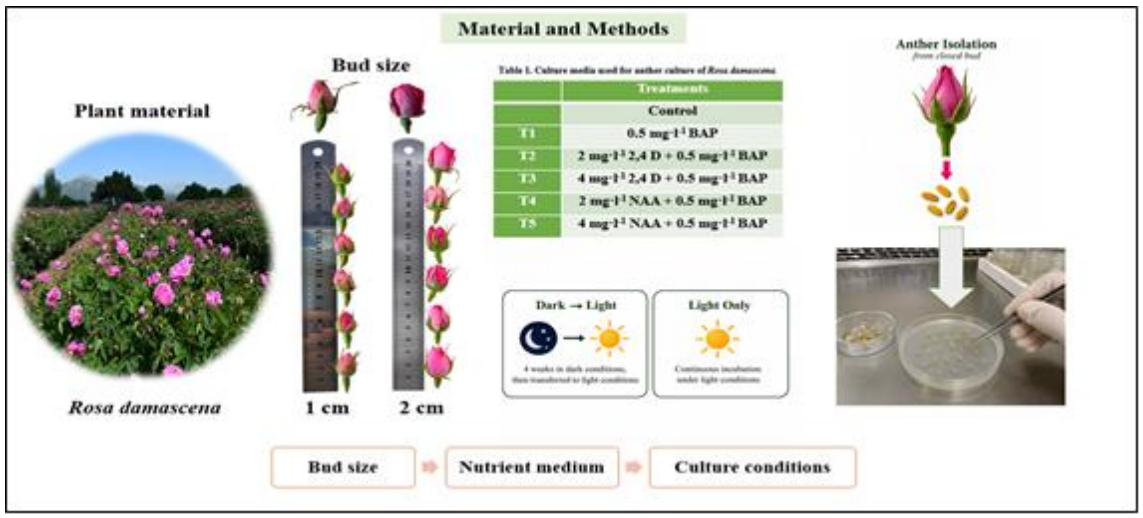


Fig. 2. Schematic representation of the experimental design for *Rosa damascena* anther culture, including plant material, bud sizes, culture media, anther isolation, and incubation conditions. (The schematic was prepared with assistance from ChatGPT (OpenAI) and reviewed by the authors).

3. RESULTS

Culture medium composition plays a key role in callus induction by regulating cellular growth and differentiation. Buds measuring 1 cm exhibited a callus induction rate of 7.65% and a mean callus diameter of 6.60 mm, whereas 2 cm buds showed a higher callus induction rate (11.77%) but a smaller mean callus diameter (5.29 mm). Cultures initially maintained in darkness exhibited a lower callus induction rate (7.94%) but a larger mean callus diameter (6.49 mm) than those maintained under light conditions (11.48% and 5.39 mm, respectively). The BS × CC interaction was significant for callus induction rate but not for callus diameter. The CM × BS and CM × BS × CC interactions were not significant for either parameter.

Table 2. Effects of culture factors on callus induction in *Rosa damascena*

Culture media (mg·L^{-1}) (CM)	**Callus induction rate (%)**	**Callus diameter (mm)**
Control	10.88 ± 1.03 B	4.13 ± 0.33 B
0.5 mg·L^{-1} BAP	9.12 ± 1.74 BC	7.94 ± 0.56 A
2 mg·L^{-1} 2,4 D + 0.5 mg·L^{-1} BAP	7.36 ± 0.80 BC	4.39 ± 0.26 B
4 mg·L^{-1} 2,4 D + 0.5 mg·L^{-1} BAP	7.36 ± 1.89 BC	9.89 ± 0.61 A
2 mg·L^{-1} NAA + 0.5 mg·L^{-1} BAP	16.77 ± 1.23 A	5.09 ± 0.40 B
4 mg·L^{-1} NAA + 0.5 mg·L^{-1} BAP	6.76 ± 1.58 C	4.21 ± 0.51 B

Bud size (cm) (BS)		
1	7.65 ± 1.08 B	6.60 ± 0.35 A
2	11.77 ± 0.63 A	5.29 ± 0.20 B
Culture conditions (CC)		
Dark	7.94 ± 1.06 B	6.49 ± 0.35 A
Light	11.48 ± 0.78 A	5.39 ± 0.25 B
ANOVA		
CM	***	***
BS	**	**
CC	*	*
CM*BS	NS	NS
BS*CC	***	NS
CM*BS*CC	NS	NS

*Note: *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; NS indicates non-significance*

Callus induction in 1-cm buds was significantly higher under light (12.35%) than under darkness (2.94%). In contrast, induction in 2-cm buds did not differ significantly between dark (12.94%) and light (10.59%) conditions. Larger buds exhibited significantly higher induction under darkness, whereas bud size had no significant effect under light (Table 3 and Fig. 3)

Table 3. Interactions effects of bud size and culture conditions on callus induction rate (%)

Bud size (cm)	**Dark**	**Light**
1	2.94 ± 0.60 B	12.35 ± 1.35 A
2	12.94 ± 0.69 A	10.59 ± 1.01 A

Note: Values are means ± SE. Different letters indicate significant differences among the four combinations (Tukey, $p < 0.05$).

Callus coloration varied depending on culture medium, bud size, and culture conditions (Table 4). Green callus was mainly observed in media containing 2,4-D or NAA, whereas white callus occurred predominantly in 2 cm buds. Consistent with Table 2, the medium containing 2 $mg{\cdot}L^{-1}$ NAA + 0.5 $mg{\cdot}L^{-1}$ BAP, which produced the highest callus induction rate, induced green callus from 1 cm buds and white callus from 2 cm buds under dark conditions.

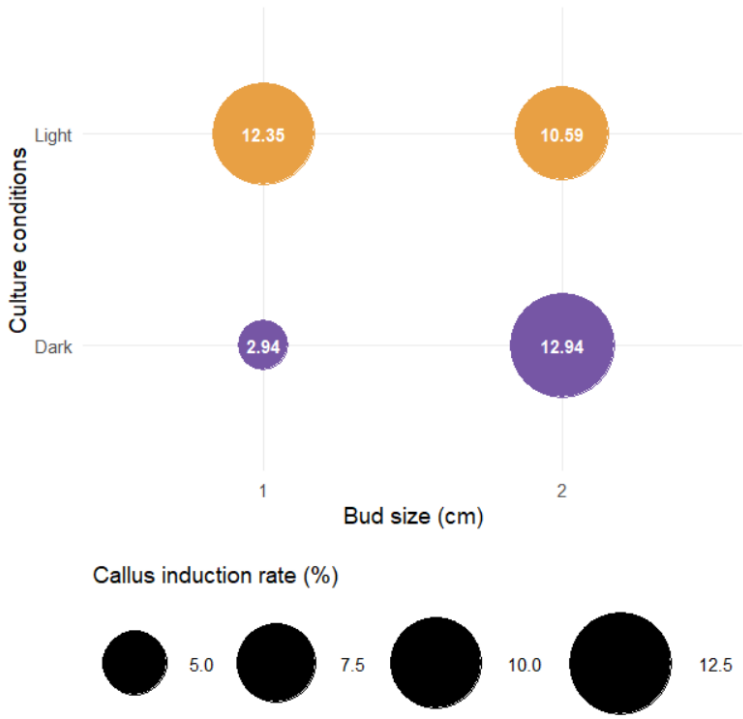


Fig. 2. Interaction effects of bud size and culture conditions on callus induction rate in *Rosa damascena* anther cultures. Bubble areas are proportional to mean callus induction rates (%), with values displayed inside the bubbles. Purple and orange represent dark and light conditions, respectively.

Table 4. Callus color under different culture conditions in *Rosa damascena*

Culture media (mg·l^{-1})	Bud size (cm)	Culture conditions (D/L)	Callus color
Control	1	Dark	-
		Light	-
	2	Dark	White
		Light	White
0.5 mg·L^{-1} BAP	1	Dark	-
		Light	-
	2	Dark	-
		Light	White

2 mg·L^{-1} 2,4D + 0.5 mg·L^{-1} BAP	1	Dark	-
		Light	Green
	2	Dark	Green
		Light	White
4 mg·L^{-1} 2,4D + 0.5 mg·L^{-1} BAP	1	Dark	-
		Light	Green
	2	Dark	-
		Light	-
2 mg·L^{-1} NAA + 0.5 mg·L^{-1} BAP	1	Dark	Green
		Light	-
	2	Dark	White
		Light	-
4 mg·L^{-1} NAA + 0.5 mg·L^{-1} BAP	1	Dark	-
		Light	-
	2	Dark	White
		Light	-

Note: (-) Callus color data were not obtained for these treatments; therefore, no color descriptions are provided. Callus color data were not obtained for these treatments; therefore, no color descriptions are provided.

4. DISCUSSION

The variation in callus formation among culture media, bud sizes, and incubation conditions suggests that both hormonal and developmental factors may influence the response of *Rosa damascena* anthers in culture. Callus formation is a process that involves the reactivation of the cell cycle and the regulation of developmental programs. Sugimoto *et al.* (2010) found that calli derived from different organs in *Arabidopsis thaliana* exhibited developmental characteristics similar to those of the root meristem. Fan *et al.* (2012) demonstrated that Lateral Organ Boundaries Domain (LBD) transcription factors, which act downstream of auxin signaling, regulate callus formation. Furthermore, Riou-Khamlichi *et al.* (1999) reported that cytokinins can activate cell division through the activation of a D-type cyclin. These findings support the notion that auxins and cytokinins jointly regulate cellular proliferation and developmental reprogramming during callus development (Ikeuchi *et al.*, 2013). However, these mechanisms were not directly examined in the present study.

Obtaining the highest callus formation rate in a medium containing 2 mg/L NAA + 0.5 mg/L BAP indicates that this combination was the most effective among the treatments tested for callus induction among the combinations tested. Olgunsoy *et al.* (2017) used the same NAA–BA combination to induce callus formation from *R. damascena* petal explants. Khosh-Khui and Sink (1982) also reported that transferring *R. manetti* leaf calli to a medium containing 2 $mg{\cdot}L^{-1}$ NAA + 0.5 $mg{\cdot}L^{-1}$ BA promoted rapid growth. In contrast, Badzhelova (2023) obtained the highest callus formation in leaf petioles and adjacent leaf segments of *R. damascena* and *R. alba* using an MS medium with reduced ammonium ions containing 1 $mg{\cdot}L^{-1}$ TDZ + 0.2 $mg{\cdot}L^{-1}$ NAA. While these results support the importance of the auxin–cytokinin combination, they also indicate that the appropriate hormone type and concentration may vary depending on the genotype, explant, and basal medium composition. Therefore, the superiority of the NAA–BAP combination in the present study should be evaluated within the context of the anther material and culture conditions examined.

When the BAP concentration remained constant while the NAA level was increased from 2 mg/L to 4 mg/L, the callus formation rate was numerically lower from 16.77% to 6.76%, indicating that under the conditions studied, an increase in NAA concentration did not promote callus formation. Recent studies conducted on Rosa species also reveal that the callus response varies depending on the composition of growth regulators and the genotype. Badzhelova (2023) reported that callus induction and proliferation in *R. damascena* and *R. alba* were influenced by genotype and the composition of the culture medium. Similarly, Zhou *et al.* (2025) obtained a callus formation rate of 18.26% in Rosa 'Pompon Veranda' anthers in a medium containing 1 $mg{\cdot}L^{-1}$ BA and 0.1 $mg{\cdot}L^{-1}$ NAA, while they determined this rate to be 45.45% in a combination of 0.6 $mg{\cdot}L^{-1}$ TDZ and 0.4 $mg{\cdot}L^{-1}$ 2,4-D. Welikala *et al.* (2024), on the other hand, reported the highest callus formation rate (72.3%) among the combinations tested on *R. centifolia* leaf discs, in a medium containing 2 $mg{\cdot}L^{-1}$ BAP and 0.2 $mg{\cdot}L^{-1}$ NAA. Although these studies do not directly compare the effects of increasing NAA concentrations, they support the notion that callus induction in rose tissues depends on the hormonal composition used. In this context, the fact that the medium containing 2 $mg{\cdot}L^{-1}$ NAA promoted higher callus formation than the medium containing 4 $mg{\cdot}L^{-1}$ NAA in the present study may be related to the different responses of anther tissue to the hormone combinations examined. However, since hormone accumulation, cell viability, or stress indicators were not measured, the decrease observed at high NAA levels cannot be directly attributed to toxicity.

The observation that callus formation rate and mean callus diameter were highest under different treatments highlights the need to assess these response variables separately. While the NAA–BAP combination stood out in terms of callus formation rate, the numerically highest mean callus diameter was obtained in a medium containing 4 $mg{\cdot}L^{-1}$ 2,4-D + 0.5 $mg{\cdot}L^{-1}$ BAP; however, this treatment was classified in the same statistical group as the medium containing only BAP. The variation in callus response in Rosa species depending on genotype and hormonal composition (Badzhelova, 2023; Welikala *et al.*, 2024; Zhou *et al.*, 2025) is consistent with the findings of the present study. However, a larger callus diameter does not directly indicate a high regeneration capacity. Indeed, Bansal *et al.* (2023) were unable to achieve plant regeneration despite high callus formation and biomass in *Catharanthus roseus* anther cultures.

Callus formation in a medium lacking growth regulators may be related to the endogenous hormone content of anthers and the wound response. Ikeuchi *et al.* (2017) reported that wounding promotes callus formation by inducing cytokinin accumulation and activating genes associated with the cell cycle. The fact that the callus formation rate is higher in buds measuring 2 cm in length, while the callus diameter is larger in 1-cm buds, suggests that the developmental stage may affect induction and growth in different ways. Although the importance of the bud development stage in *R. damascena* anther culture has been noted previously (Tabaeezadeh and Khosh-Khui, 1981), linking size differences to specific microspore stages requires cytological verification (Testillano, 2019).

The higher rate of callus formation under the photoperiod treatment, and conversely, the larger diameter in cultures initially kept in darkness, suggests that light requirements may vary depending on the culture stage. It has been reported that darkness can accelerate callus initiation in rose explants, but that continuous darkness may adversely affect subsequent development (Khosh-Khui and Sink, 1982). The green and white colors of calli may be related to pigment accumulation and plastid development (Cortleven *et al.*, 2016; Olgunsoy *et al.*, 2017); although the medium containing 2 $mg{\cdot}L^{-1}$ NAA and 0.5 $mg{\cdot}L^{-1}$ BAP was promising for callus induction, further studies are needed to determine whether the calli originated from microspores and whether they can regenerate into haploid plants. Callus coloration may provide a useful visual indicator of tissue physiological status and help identify treatments with potential for subsequent regeneration; however, color alone is insufficient to determine regenerative capacity. Future studies should therefore evaluate regeneration response, histological characteristics, and ploidy level. Overall, optimizing plant growth regulator combinations, bud size, and culture conditions is essential for developing an efficient anther culture protocol for *Rosa damascena*.

5. CONCLUSIONS

In this study, the most successful medium for callus induction in Rosa damascena anther cultures was MS medium containing 2 $mg{\cdot}L^{-1}$ NAA + 0.5 $mg{\cdot}L^{-1}$ BAP, with a formation rate of 16.77%. A higher callus formation rate was observed in buds measuring 2 cm in length, while larger callus diameters were obtained in cultures initially incubated in the dark. These findings indicate that callus induction and growth should be evaluated together when developing the protocol. In future studies, it is recommended to optimize the selected medium with different hormone levels and to determine the cellular origin of the calli, their ploidy level, and their plant regeneration capacity.

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Effect of cytokinin oxidase/dehydrogenase inhibitors on *in vitro* regeneration in *Daphne cneorum* L.

Jana Hanzal Šedivá[1], Zuzana Rottová[1], Michaela Pekařová[1], Daniel Zahradník[1], Jaroslav Nisler [2]

[1]*Landscape Research Institute v. v. i. (VÚK), Department of Plant Biotechnology, Květnové nám. 391, 252 43 Průhonice, Czech Republic; jana.sediva@vuk.gov.cz*

[2]*Institute of Experimental Botany, Isotope Laboratory, The Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czech Republic*

jana.sediva@vuk.gov.cz

Abstract

Daphne cneorum L. (fragrant daphne) is a small, covering shrub of the Thymelaceae family, which occurs naturally in many European countries, including the Czech Republic, where it is a critically endangered species. A significant reduction in the number of localities occurred in the second half of the twentieth century. The reason was changes in landscape management and the plundering of natural localities by rock gardeners. It is a recalcitrant plant species, propagation in culture is problematic. Micropropagation offers the advantages of endangered species in that it is based on a minimum amount of plant material. One of the possibilities for improving *in vitro* shoot production in recalcitrant plant species is a new group of chemical compounds (CKX inhibitors) that protect cytokinins in *in vitro* culture from degradation. The aim of our study was to evaluate the effect of cytokinin oxidase/dehydrogenase (CKX) inhibitors (diphenylurea derivatives) on multiplication in *D. cneorum*. We tested two CKX inhibitors, compounds 52 and 77 alone or in combination with isoprenoid cytokinin 2iP. CKX inhibitors had a significant effect on shoot formation compared to the control. The highest multiplicative effect was observed with the CKX-77 inhibitor compared with the CKX-52 inhibitor and cytokinin 2iP, both alone and in combination with cytokinin.

Keywords*: Daphne, cytokinin oxidase/dehydrogenase inhibitors, shoot production, in vitro*

1. INTRODUCTION

Habitat loss and reduced reproductive success can threaten the long-term survival of endangered plant populations. In *Daphne cneorum*, sexual reproduction in natural habitats is limited by low fruit production and poor seed germination (Jakubková, 1997; Žlebčík & Šedivá, 2001). Vegetative propagation therefore represents an important approach to producing plant material for conservation purposes. Vegetative propagation of *D. cneorum* has been achieved using conventional cuttings (Bärtels, 1988; Šedivá & Žlebčík, 2010) and micropropagation (Žlebčík & Šedivá, 2001; Malá & Bylinský, 2004; Noshad *et al.*, 2009). Despite the availability of established protocols, micropropagation of *D. cneorum* remains challenging due to its recalcitrance under *in vitro* conditions, particularly its limited regeneration capacity (Grant, 2000; Marks, 1996). Further optimization of micropropagation protocols is therefore needed.

Cytokinins regulate cell division (Riou-Khamlichi *et al.*, 1999) and axillary bud outgrowth (Dun *et al.*, 2012) and are widely used to promote shoot multiplication *in vitro* (Gentile *et*

al., 2014; Nisler *et al.,* 2022). However, their effects depend on plant species, concentration, and culture conditions, and higher cytokinin concentrations do not necessarily improve shoot production or quality (Capaci *et al.,* 2026). An alternative approach involves the inhibition of CKX, an enzyme that catalyses irreversible cytokinin degradation of cytokinins. CKX inhibitors can increase the availability of endogenous cytokinins and prolong the activity of exogenously applied cytokinins, potentially enhancing shoot regeneration without the need to increase cytokinin concentrations (Nisler *et al.,* 2021, 2022, 2024).

CKX inhibitors have been investigated in several *in vitro* systems, including genera such as *Populus* (Nisler *et al.,* 2021), *Coffea* (Murvanidze *et al.,* 2021), *Vaccinium* (Capaci *et al.,* 2026), and *Lavandula* (Nisler *et al.,* 2024), with contrasting responses among species. Evaluating these inhibitors in *D. cneorum*, a recalcitrant species classified as critically endangered in the Czech Republic, could provide new opportunities to improve propagation efficiency, particularly in genotypes with limited multiplication capacity. Such improvements could facilitate the production of plant material for *ex situ* collections, conservation efforts, and further research.

This study investigates the effects of two CKX inhibitors, applied individually or in combination with a cytokinin, on shoot regeneration in a single genotype of *Daphne cneorum*.

2. MATERIALS AND METHODS

2.1. Plant material

For the experiments, a genotype of *Daphne cneorum* originating from Central Bohemia, near the town of Mělník in the Czech Republic, was used (Fig. 1). This genotype belongs to the *in vitro* collection of *D. cneorum* maintained at VÚK for approximately 25 years. Stock cultures were multiplied on McCown's Woody Plant medium (WPM; Lloyd & McCown, 1980; Duchefa) containing 0.33 mg L^{-1} zeatin. The shoot explants were excised from the stock cultures and cultivated on WPM medium, supplemented with 20 g L^{-1} sucrose and solidified with 2.5 g L^{-1} Phytagel™ (basal medium). This basal medium was used in experiments evaluating two cytokinin oxidase/dehydrogenase (CKX) inhibitors, CKX-52 (Nisler *et al.,* 2022) and CKX-77 (Nisler *et al.,* 2024), each tested at a concentration of 1 µM, either alone or in combination with 1 µM of the isoprenoid cytokinin 2iP. Stock solutions of 2iP and the tested compounds were prepared in 100% dimethyl sulfoxide (DMSO) and then added to the autoclaved medium. The control was with DMSO. Explants were cultured in test tubes containing 5 mL of a WPM medium. The cultures were maintained in a growth room under a 16 h photoperiod with a photosynthetic photon flux density of 60 µmol·m^{-2}·s provided by cool-white fluorescent tubes (Tungsram, General Electric Company, Boston, MA, USA) at 22 ± 1 °C. Each treatment contained 15 tubes with one shoot (≥ 2.0 cm long). The evaluation was performed in three subcultures, each after five weeks of cultivation.



Fig. 1. The original locality of *Daphne cneorum* situated in central Bohemia (Holý vrch) from which an *in vitro* culture was derived in the past

2.2. Statistical analysis

A two-factor analysis of variance with interactions was used to evaluate the data. The dependent variables were the number of shoots per plant and the total of shoot length per plant. The explanatory variables were the treatment variant, the subculture number, and their interaction. Since the number of shoots follows a Poisson distribution, a square root transformation was applied to it (Anděl, 1998). Post-hoc analysis was performed using Tukey's multiple comparison test. The results of the post-hoc analysis are presented as homogeneous groups at a significance level of 0.05.

3. RESULTS

CKX inhibitors had a significant effect on shoot formation compared with the control (Fig. 2). A significantly higher multiplication rate was achieved with CKX-77 (3.88) compared with CKX-52 (2.74) and the cytokinin 2iP (2.40). The highest number of shoots was observed with CKX-77 in combination with 2iP (4.53), whereas the lowest was recorded in the control (1.80) in Fig. 3. The combination of a CKX inhibitor with a cytokinin had no significant effect on shoot multiplication. The effect of subculture on shoot formation was not statistically significant ($p > 0.06$). Statistically significant differences were observed among individual treatments as a whole ($p < 0.003$; Fig. 4, Table 1), however, due to the smaller number of measurements per individual treatment, no statistically significant differences were detected between pairs of individual treatments.

A positive effect on shoot elongation was observed in some treatments with CKX inhibitors compared with the control (Fig.5). The sum of shoot lengths was significantly higher with CKX-52 (80.98 mm) than with CKX-77 (68.16 mm). The combination of a CKX inhibitor with a cytokinin had no significant effect on shoot elongation. The total of shoot length was almost identical in the control, CKX-77, and 2iP treatments (67.08, 68.16, and 68.38 mm, respectively).

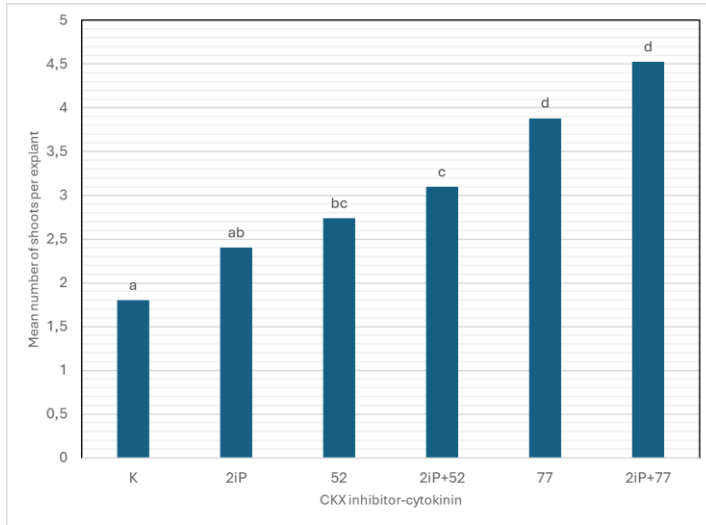


Fig. 2. Shoot production in *Daphne cneorum* cultured with CKX inhibitors alone or in combination with cytokinin

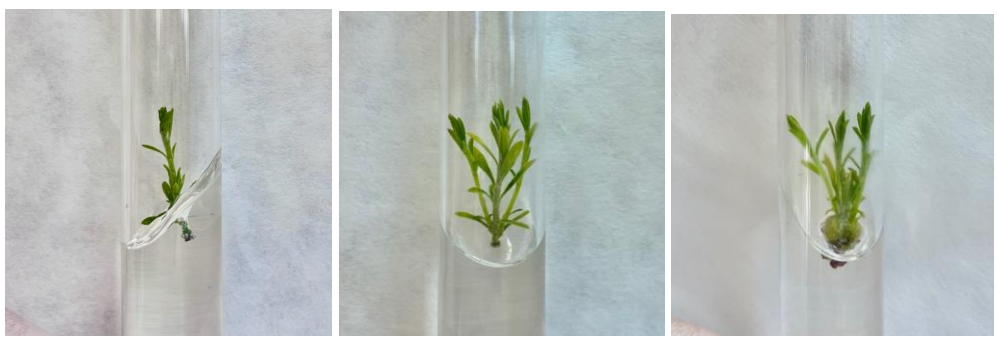


Fig. 3. The effect of CKX-77 inhibitor alone or in combination with cytokinin on shoot multiplication in *Daphne cneorum*. Left to right – control, CKX-77 and CKX-77+2iP

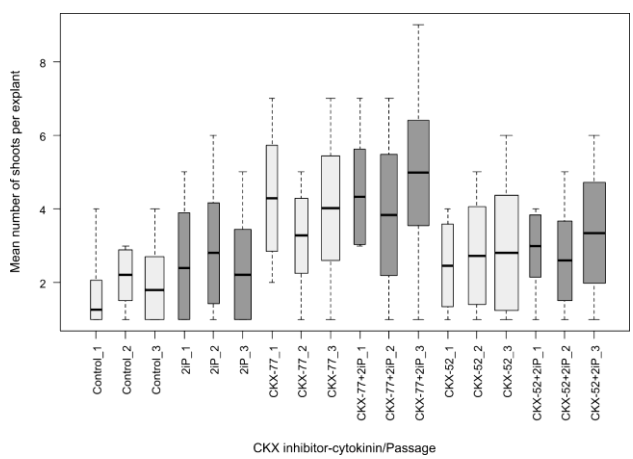


Fig. 4. Shoot production in *Daphne cneorum* over three subcultured with CKX inhibitors alone or in combination with cytokinin

Table 1. Effect of different factors (CKX inhibitor-cytokinin treatment and subculture) on shoot production in *Daphne cneorum*

	df	Mean Squares	F	
Treatment	5	6.97	47.48	***
Subculture	2	0.40	2.77	ns
Treatment × subculture	10	8.39	2.71	**
Residuals	490	0.14	12.33	

*Note: Significance codes: ** – p-value < 0,01; *** – p-value < 0,001; ns – not significant; df means degrees of freedom, and F is the F-statistic*

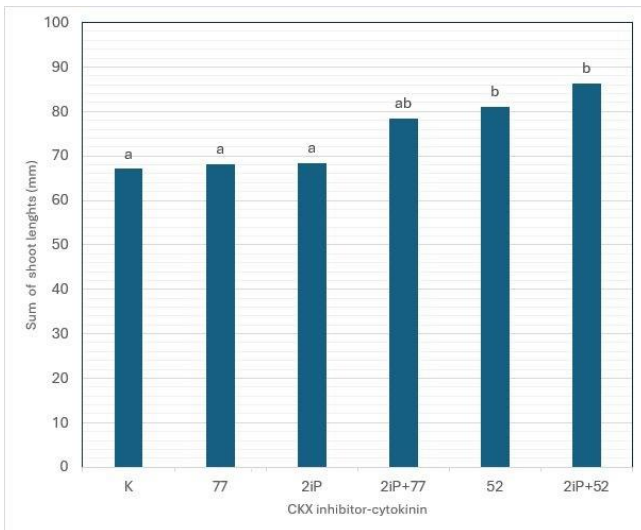


Fig. 5. Effect of CKX inhibitors alone or in combination with cytokinin on shoot elongation in Daphne cneorum

4. DISCUSSION

Plant growth regulators play a key role in adventitious shoot regeneration in woody species of the temperate zone (Durkovič & Mišalová, 2008). Cytokinins, in particular, play an important role in the induction and formation of adventitious and axillary shoots (Pierik, 1987). The irreversible degradation of cytokinins in plants is mediated by cytokinin oxidase/dehydrogenase (CKX) enzymes, thereby playing an important role in regulating endogenous cytokinin levels. CKX inhibitors suppress this degradation, thereby increasing cytokinin availability and signaling, and thus represent promising compounds for applications in agriculture and plant biotechnology (Nisler *et al.*, 2022).

Nisler *et al.* (2022) investigated shoot regeneration from leaf explants of *Nicotiana* and *Lobelia* using the CKX-52. In tobacco, two concentrations of CKX-52 and the cytokinin 2iP, 0.01 and 0.1 µM, were used. At lower concentrations of the inhibitor and cytokinin, shoot formation was low, and regeneration occurred only with the 2iP + CKX-52

combination. At the higher concentration, the addition of CKX-52 resulted in a more than twofold increase in the number of shoots compared with 2iP alone. In *Lobelia*, a concentration of 1 µM was used, and treatments without 2iP were also included. Shoot formation showed a trend similar to that observed in our results for *Daphne cneorum*: the lowest shoot production was recorded in the control, whereas the highest was observed with the combination of 2iP + CKX-52 and with CKX-52 alone.

Nisler *et al.* (2024) tested the effect of CKX-77 in ecologically and taxonomically diverse plant genera (*Lobelia, Drosera, Plectranthus, Lavandula,* and *Sempervivum*). Shoot production was species dependent. All tested plants except *Sempervivum* responded positively to the application of CKX-77 in combination with 2iP. In *Lobelia, Drosera,* and *Plectranthus,* the combination of CKX-77 + 2iP resulted in significantly higher shoot production. *Lavandula* regenerated only when CKX-77 or 2iP was applied separately. A different response was observed in *Sempervivum*, where the highest regeneration was recorded in the control, whereas no regeneration occurred with the combination of CKX-77 + 2iP. In *Daphne cneorum*, the response was similar to that observed with CKX-52: the lowest shoot production was recorded in the control, whereas the highest was observed with the combination of 2iP + CKX-77 and with CKX-77 alone.

In a study focused on CKX inhibitors, five of the most promising inhibitors were selected from more than 100 candidate compounds for potential commercial application and further testing, based on their biological activity, toxicity, and production costs. CKX-77 belongs to this selected group (Nisler, 2024).

Our results for *Daphne cneorum* demonstrated a greater multiplication-promoting effect of CKX-77 than that of CKX-52, which was not included in this group of promising inhibitors (Nisler, 2024). However, CKX-52 showed a more pronounced positive effect on shoot elongation. Although endogenous cytokinin levels were not analyzed, our results suggest that, in the presence of CKX inhibitors alone, endogenous cytokinins in *in vitro* cultures of *Daphne cneorum* were better protected against degradation than in the control treatment.

5. CONCLUSIONS

CKX inhibitors had a positive effect on shoot formation in *Daphne cneorum* and were more effective than cytokinin. CKX inhibitors were found to be effective both when applied alone and in combination with cytokinin. CKX-77 had a stronger effect on shoot formation than CKX-55.

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In vitro shoot regeneration and callus induction of *Amaranthus caudatus* L. cv. Karmin

Olha Yaroshko

Institute of Cell Biology and Genetic Engineering, National Academy of Sciences of Ukraine, Kyiv, Ukraine

90tigeryaroshko90@gmail.com

Abstract

In this study the effects of zeatin (Zea) and indole-3-acetic acid (IAA) were evaluated on callus formation and shoot regeneration in *Amaranthus caudatus* L. cv. Karmin. Explants were cultured on Murashige and Skoog (MS) basal medium without growth regulators or supplemented with 1–3 mg/L Zea, alone or in combination with 0.1 or 0.5 mg/L IAA. Callus formation was observed in all media containing growth regulators, whereas the hormone-free control did not produce callus. Callus-formation frequency ranged from 92.30% to 98.57%. The highest value, 98.57%, was recorded on MS medium containing 2 mg/L Zea and 0.5 mg/L IAA. Shoot regeneration was more dependent on the specific cytokinin–auxin balance than on the callus formation. The highest shoot-regeneration frequency, 20.23%, was obtained on MS medium supplemented with 3 mg/L Zea and 0.1 mg/L IAA. The same zeatin concentration combined with 0.5 mg/L IAA resulted in 17.18% shoot regeneration. The maximum recorded number of shoots was three per explant on MS medium containing 2 mg/L Zea. Comparison with previously published studies on callus formation in *Amaranthus* species indicates that the callus response of cv. Karmin is comparable with reported values of approximately 99–100%, whereas its shoot-regeneration response falls within the range reported for other genotypes. These results indicate that the optimal medium differed according to the regeneration stage and evaluation criterion. MS medium with 2 mg/L Zea and 0.5 mg/L IAA is proposed for initial callus induction, while MS medium with 3 mg/L Zea and 0.1 mg/L IAA is more suitable for shoot regeneration.

Keywords: *Amaranthus caudatus; callus induction; shoot regeneration.*

1. INTRODUCTION

Amaranthus caudatus L. is a nutritionally valuable pseudocereal and leafy crop with potential importance for food production, genetic improvement, and plant biotechnology (Caselato-Sousa & Amaya-Farfán, 2012; Jamrząk *et al.*, 2023). *In vitro* culture provides a useful platform for rapid multiplication, regeneration of selected genotypes, and development of transformation and genome-editing procedures (Bennici *et al.*, 1997; Montemurro *et al.*, 2020). The cytokinin–auxin balance can determine whether cultured tissue proliferates as callus or proceeds toward organogenic shoot formation (Ikeuchi *et al.*, 2020; Méndez-Hernández *et al.*, 2024).

There is limited quantity of experimental articles where was shown successful results of shoot regeneration of *Amaranthus* species (Yaroshko, 2021). *Amaranthus* species are generally considered highly recalcitrant to *in vitro* regeneration, particularly when regeneration is attempted from callus or after transformation (Bennici *et al.*, 1997; Montemurro *et al.*, 2020; Yaroshko, 2021). And there is a limited quantity of experimental

articles dedicated to shoot regeneration of *Amaranthus* species. This recalcitrance is expressed as a strong capacity for callus formation combined with a comparatively low and unstable capacity for shoot organogenesis (Gajdošová *et al.*, 2017). It may be associated with genotype-dependent endogenous hormone balance, persistent apical dominance, loss of morphogenic competence during subculture, and strong sensitivity to the type and concentration of exogenous growth regulators (Bennici *et al.*, 1997; Ikeuchi *et al.*, 2020). The results reported for *A. caudatus* cv. Helios and other *Amaranthus* species support this interpretation: some explants produced abundant callus, but callus frequently ceased growing, darkened, or died, and only a limited proportion of calli tissues regenerated shoots (Bennici *et al.*, 1997; Yaroshko, 2021).

Callus cultures may also provide material for the production and study of biologically active compounds (Montemurro *et al.*, 2020; Jamrząk *et al.*, 2023). Previous investigations of *Amaranthus* callus have focused on pigments, phenylpropanoids, and other secondary metabolites, whereas other studies have aimed to obtain regenerated plants for micropropagation or genetic transformation. The development of an efficient regeneration protocol is therefore important for both cultivar propagation and future biotechnological applications (Montemurro *et al.*, 2020; Jamrząk *et al.*, 2023).

The present study evaluated the effects of zeatin, applied alone or together with indole-3-acetic acid, on callus formation and shoot regeneration of *Amaranthus caudatus* L. cv. Karmin. The objectives were to identify the most effective medium for callus induction, determine the treatment producing the highest shoot-regeneration frequency, and evaluate the number of shoots formed per explant.

2. MATERIALS AND METHODS

The object of investigation was *Amaranthus caudatus* cv. Karmin, seeds of which were kindly provided by the staff of the Department of New Crops at the M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine.

For introduction into *in vitro culture*, seeds were surface-sterilized in a 1% solution of the commercial bleach product "Bilyzna" for 12 min, rinsed three times with distilled water, and then immersed in a 3% hydrogen peroxide solution for 12 min. Seeds were germinated under sterile conditions in glass Petry dishes on standard nutrient agar-based Murashige and Skoog (MS_{30}) medium (Murashige, Skoog, 1962) supplemented with 30 g/L sucrose and 7 mg/L agar at pH 5.7–5.9. Seedlings were grown in Petry dishes at a temperature of 22–25°C, under illumination of 3000–4500 lx, with a 16-hour light photoperiod.

The 10-14-day old cotyledon explants of *Amaranthus caudatus* L. cv. Karmin were used in experiments. The whole explants (0.4-0.7 cm long) were cultured on MS_{30} medium with the same concentration of agar, sucrose and growth conditions as mentioned above) in glass Petry dishes. The MS_{30} medium was autoclaved during 20 min, the growth stimulants were dissolved in DMSO previously and then were added to autoclaved MS_{30} medium. The MS_{30} medium was used without growth regulators as a control and other variants of MS_{30} medium were supplemented with Zea at 1, 2, or 3 mg/L, either alone or in combination with IAA at 0.1 or 0.5 mg/L. Per each Petry plate, 30 ml of MS30 mediumm was used.

Each two weeks explants were transferred to fresh MS_{30} medium with the same combinations and concentrations of growth stimulants used in the beginning for each variant of experiment. Efficiency of callus formation and shoot regeneration were evaluated 5 weeks after beginning of the experiment. Callus formation was expressed as the percentage of explants producing callus, and shoot regeneration was expressed as the percentage of explants producing shoots. The number of shoots per explant was recorded for treatments for which these observations were available.

Callus formation (%) = (number of explants producing callus / total number of cultured explants) × 100

Shoot regeneration (%) = (number of explants producing shoots / total number of cultured explants) × 100

2.1. Statistics

For each variant of experiment with different concentrations of growth stimulants, 20 explants were used (in one Petry plate), and the experiments were carried out in three replicates. Totally 60 explants were used per each variant of experiment. Statistical analysis of the results was performed using the Statistica software package (calculation of Student's t-test). Significance levels (P) of differences or ratios between mean values were determined using tables for small sample sizes. Significant differences were defined as a significance level of 1% (p-value < 0.01) or 5% (p-value < 0.05).

3. RESULTS

After three weeks of explants cultivation on different variants of media, the transfer of differentiated tissues to callus tissue was detected. Primary callus appeared as creamy-yellow, compact, dense, slightly nodular tissue emerging from basal cut ends of the explants. After two subcultures, the callus in all variants of experiment except only control, remained compact and dense in the interior, whereas the surface layer remained friable, with a smooth to weakly tuberculate surface and occasional small meristematic nodules (Fig. 1 A, B, D, F). The callus was compact in the interior, whereas the surface layer remained friable. The majority of calli exhibited pink–red pigmentation, which may indicate that these calli were synthesizing secondary metabolites.

No callus formation or shoot regeneration was recorded on hormone-free MS_{30} medium. All media containing growth regulators induced callus formation, with values from 92.30% to 98.57% (Table 1, Fig. 1, Fig. 2 A). The highest callus-formation frequency was observed on MS_{30} medium containing 2 mg/L Zea and 0.5 mg/L IAA. The second-highest value was recorded on MS_{30} medium containing 3 mg/L Zea and 0.5 mg/L IAA.

After five weeks of cultivation of explants on media supplemented with different concentrations of Zea and IAA, Small yellowish-pink shoot primordia were observed as dot-like protrusions on the callus surface. Later those structures transformed into tiny shoots (Fig. 1 A, C). Formation of all shoots were observed from callus tissue, no direct regeneration of explant tissue was detected. Shoot regeneration varied more widely than callus formation. The highest regeneration frequency, 20.23%, was obtained on MS_{30} medium supplemented with 3 mg/L Zea and 0.1 mg/L IAA (Fig.1). The corresponding treatment containing 0.5 mg/L IAA resulted in 17.18% regeneration (Table 1, Fig.2 B).

Other growth-regulator treatments resulted in 2.70% and 7.69% shoot regeneration (Table 1, Fig. 2). We found significant differences in efficiency of shoot formation according to different variant of media which were used.

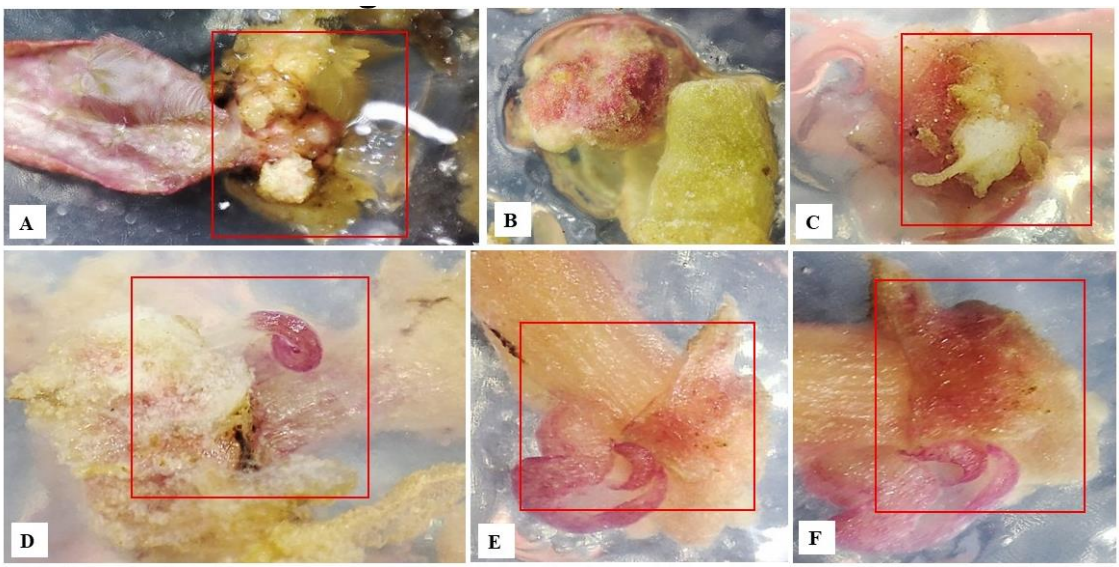


Fig. 1. Callus initiation and shoot regeneration (*de novo* organogenesis, in red frame) from cotyledon explants of *A. caudatus* cv. Karmin on MS medium supplemented with growth stimulants. A, C, E, F – MS + 3 mg/L Zea + 0.1 mg/L IAA, B and D – MS + 3 mg/L Zea + 0.5 mg/L IAA.

The maximum recorded shoot number was three shoots per explant on MS medium containing 2 mg/L Zea. MS medium supplemented with 3 mg/L Zea in combination with either 0.1 or 0.5 mg/L IAA resulted in two shoots per explant (Table 1). Thus, the treatment producing the highest regeneration percentage was not identical to the treatment producing the highest recorded shoot number per explant.

Table 1. Effects of Zea and IAA on callus formation and shoot regeneration in Amaranthus caudatus L. cv. Karmin.

Medium	**Callus formation (%)**	**Shoot regeneration (%)**	**Shoots per explant**
Control	0.00	0.00	—
1 mg/L Zea	97.87	5.50	1
2 mg/L Zea	92.30	7.69	3.00
3 mg/L Zea	94.73	6.14	1.2
1 mg/L Zea + 0.1 mg/L IAA	96.39	2.70	1.6
1 mg/L Zea + 0.5 mg/L IAA	97.46	5.06	1.8
2 mg/L Zea + 0.1 mg/L IAA	96.59	6.82	2

2 mg/L Zea + 0.5 mg/L IAA	98.57	2.85	2
3 mg/L Zea + 0.1 mg/L IAA	96.42	20.23	2.00
3 mg/L Zea + 0.5 mg/L IAA	98.43	17.18	2.00

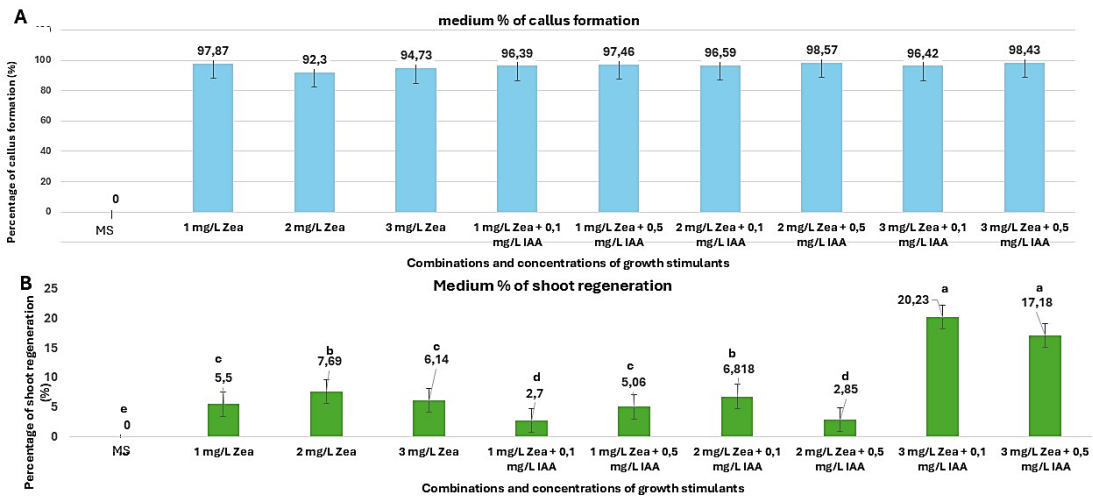


Fig. 2. Effect of growth regulators on efficiency of callus formation (A) and shoot regeneration (B) from cotyledon segments of *A. caudatus* cv. Karmin in MS_{30} medium after five weeks of cultivation. Data are the mean ±SE (standard error); n = 20. Letters indicate significant differences between samples (B, p-value < 0.01 (a,c) or p-value < 0.05 (b,d)).

4. DISCUSSION

The present study demonstrated that *Amaranthus caudatus* L. cv. Karmin has a high capacity for callus formation *in vitro*. Callus formation exceeded 92% on all media containing growth regulators, and the highest value, 98.57%, was recorded on MS medium supplemented with 2 mg/L Zea and 0.5 mg/L IAA. However, shoot regeneration was considerably lower, with the highest value of 20.23% obtained on MS medium containing 3 mg/L Zea and 0.1 mg/L IAA. These results confirm that efficient callus induction and efficient shoot regeneration are distinct processes and require different hormonal conditions.

The high callus-forming ability observed in cv. Karmin agrees with results reported for other *Amaranthus* species and their genotypes. Amin et al. (2015) obtained callus formation in 99.7 ± 0.2% of *Amaranthus gangeticus* explants derived from stems on MS medium containing 2 mg/L NAA (1-naphthaleneacetic acid) and 1 mg/L BA (6-benzylaminopurine). Similarly, Bennici *et al.* (1997) reported almost complete callus formation in most tested lines of *A. caudatus*, *A. cruentus*, *A. hybridus*, and *A. hypochondriacus*. In their experiments, callus was induced on media containing 2,4-D (2,4-dichlorophenoxyacetic acid) with kinetin or combinations of NAA and BA. Thus, the 98.57% callus-formation frequency recorded in the present study is comparable with the highest values reported for other *Amaranthus* genotypes.

Nevertheless, the results of Bennici *et al.* (1997) also demonstrate that callus induction is strongly genotype-dependent. Although many lines formed callus at frequencies close to 100%, some *A. caudatus* lines responded poorly. This variability is important when comparing the present results with those of other researchers

The results obtained for cv. Karmin also agree with the earlier study callus formation of cv. Helios. In that study, stable callus cultures were obtained on MS_{30} medium supplemented with 1 mg/L 2,4-D and 1 mg/L kinetin or with 2 mg/L 2,4-D and 10% coconut milk (Yaroshko, 2017). Callus was induced from hypocotyl, leaf, and root explants, although hypocotyl explants showed the strongest response. In the experiments with Helios cultivar, the efficiency of callus formation on media supplemented with 2,4-D alone was lower than the efficiency of callus formation on medium supplemented with a combination of 2,4-D and kinetin, whereas kinetin alone did not induce visible changes. For cv. Karmin, the highest callus formation was obtained using Zea and IAA rather than 2,4-D and kinetin. This difference suggests that the optimum auxin–cytokinin combination is cultivar-specific.

A comparison of callus induction efficiency of *A. tricolor* with obtained callus formation efficiency in our experiments with cv. Karmin further supports the importance of hormonal balance. Biswas *et al.* (2013) established a stable callus line for the production of betalain pigments using stem explants on MS medium containing 0.25 mg/L NAA and 2 mg/L BA. The high callus-formation frequency observed in cv. Karmin suggests that this cultivar may also be suitable for further studies of callus biomass accumulation and secondary-metabolite production, but biochemical analyses are required before this potential can be evaluated.

Other studies have shown that callus formation may be highly efficient while regeneration remains limited. Flores *et al.* (1982) reported rapid callus growth and abnormal root formation in *A. hypochondriacus* and *A. cruentus* leaf discs on MS medium containing 0.1–1.0 mg/L 2,4-D. At higher concentrations of 2,4-D, embryo-like structures developed, but regeneration depended on the subsequent transfer of tissue to a medium containing NAA and Zea. In the present study, the highest callus-formation frequency was obtained on MS + 2 mg/L Zea + 0.5 mg/L IAA, but shoot regeneration on the same variant of medium reached only 2.85%. This result similarly indicates that a medium favorable for callus proliferation may not be suitable for shoot organogenesis.

The shoot-regeneration frequency obtained for cv. Karmin can be compared with the low regeneration frequencies reported for other *Amaranthus* genotypes. Bennici *et al.* (1997) obtained regenerants from *A. hybridus* line 1047 and *A. hypochondriacus* line 674, but regeneration frequencies were only 8.5% and 14.3%, respectively. Bennici *et al.* (1992) reported regeneration of *A. caudatus* on MS_{30} medium containing 3 mg/L kinetin and 0.3 mg/L IAA, with an overall regeneration frequency of approximately 26%. The highest value in the present study, 20.23%, is therefore within the range reported for other *Amaranthus* species.

The present regeneration response is lower than that reported in some studies based on direct regeneration from young epicotyls. Gajdošová *et al.* (2007, 2013) reported direct regeneration of *A. cruentus* cv. Ficha and *A. hybridus* K-433 on media containing high cytokinin concentrations, including 5 mg/L BA + 0.01 mg/L NAA, 1 mg/L TDZ (thidiazuron), or 3 mg/L TDZ + 0.01 mg/L NAA. However, regeneration was strongly affected by genotype, plant age, and explant type. Their results indicated that epicotyls were

more suitable for regeneration than roots or leaves. The present experiment should therefore be extended by comparing hypocotyl, cotyledon, epicotyl, leaf, and root explants of cv. Karmin for shoot regeneration induction.

High cytokinin concentrations have frequently been associated with shoot regeneration in *Amaranthus*. Flores *et al.* (1982) obtained shoots from hypocotyl-derived callus on B5 medium containing 0.1 mg/L NAA and 0.1–1.0 mg/L Zea. Gajdošová *et al.* (2007) obtained direct regeneration on media containing BA or TDZ, while the experiments conducted with *A. caudatus* cv. Helios demonstrated that 1 mg/L TDZ was effective for microclonal propagation and adventitious-bud formation (Yaroshko, 2017). In the present study, the best shoot-regeneration response was obtained on MS media with 3 mg/L Zea and 0.1 mg/L IAA. The high cytokinin-to-auxin ratio in this treatment is consistent with the general requirement for increased cytokinin influence during shoot induction.

The present results also show that increasing the auxin concentration may reduce regeneration. At 3 mg/L Zea, shoot regeneration decreased from 20.23% with 0.1 mg/L IAA to 17.18% with 0.5 mg/L IAA. A stronger reduction was observed at 2 mg/L Zea and 1 mg/L IAA, where regeneration declined from 6.82% to 2.85% (2 mg/L Zea + 0.5 mg/L IAA). According to these results, we can suggest that excessive auxin may maintain tissues in a callogenic state or suppress shoot differentiation.

The present results suggest a strategy for induction of shoots of cv. Karmin. Callus could initially be induced on MS + 2 mg/L Zea + 0.5 mg/L IAA, and responsive calli could then be transferred to MS + 3 mg/L Zea + 0.1 mg/L IAA for shoot induction. Separate media should subsequently be tested for shoot elongation and rooting.

The results are also relevant to the development of genetic transformation systems. Yaroshko (2021) emphasized that regeneration is one of the principal limitations for genetic engineering of *Amaranthus*. Although transformed tissues and plants have been obtained in several *Amaranthus* species, transformation success depends on the availability of a reliable regeneration system. Methods suitable for callus initiation but not efficient for shoot regeneration cannot support the recovery of stable transformed plants. Therefore, the 20.23% shoot-regeneration frequency recorded in cv. Karmin represents a useful starting point for future transformation experiments, but it must be improved and validated for media with selective agents used during *Agrobacterium*-mediated transformation.

Genotype dependence is particularly important for transformation work. Regeneration ability may vary among species, cultivars, lines, explant types, and seedling ages. Regeneration conditions developed for *A. cruentus*, *A. hybridus*, or *A. hypochondriacus* cannot be directly transferred to *A. caudatus* cv. Karmin without optimization. The present study provides cultivar-specific information that may be useful for future *Agrobacterium*-mediated transformation, but the effects of infection time, bacterial density, co-cultivation period, selection agent, and regeneration under selection still need to be determined.

Overall, the results obtained for cv. Karmin are consistent with the general pattern reported for *Amaranthus*: callus formation is usually high, whereas shoot regeneration is more limited and strongly genotype-dependent. The 98.57% callus-formation frequency obtained on MS + 2 mg/L Zea + 0.5 mg/L IAA is comparable with the approximately 99–100% values reported for *A. gangeticus* and several *Amaranthus* lines. The maximum shoot-

regeneration frequency of 20.23% is within the range reported for other genotypes and is higher than the regeneration frequencies recorded for several lines of *A. hybridus* and *A. hypochondriacus*. Further optimization should focus on explant type, seedling age, subculture interval, callus quality, shoot elongation, rooting, and regeneration under selection conditions.

5. CONCLUSIONS

Zea and IAA influenced on callus induction and shoot regeneration of *Amaranthus caudatus* L. cv. Karmin in a concentration-dependent and stage-specific manner. The highest callus formation, 98.57%, was obtained on MS medium with 2 mg/L Zea and 0.5 mg/L IAA. The highest shoot-regeneration frequency, 20.23%, was recorded on MS medium with 3 mg/L Zea and 0.1 mg/L IAA. The maximum recorded number of shoots per explant was 3 on MS medium with 2 mg/L Zea. The callus response was comparable with the highest values reported for other *Amaranthus* species and lines, while the shoot-regeneration response was within the range reported for this genus. These findings provide a basis for developing a two-stage regeneration system and may support future micropropagation and genetic-transformation studies. Further replicated experiments with complete statistical analysis are required before the protocol can be considered fully optimized.

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Comparison of inoculation protocols for transient Agrobacterium-mediated transformation in different ornamental sunflower (*Helianthus annuus* L.) explant types

Şükran Yıldız[1], Ashkan Hodaei[2], Sara Yasemin[3], Dilek Tekdal[1], Stefaan P. O. Werbrouck[2]

1Department of Biotechnology, Faculty of Science, Mersin University, Mersin, Türkiye

2Department of Plants & Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

3Department of Horticulture, Faculty of Agriculture, Siirt University, Siirt, Türkiye

yldzskran@gmail.com

Abstract

Efficient and reproducible genetic transformation remains a major limitation for applying genome-editing technologies in sunflower (*Helianthus annuus* L.). This study compared transient Agrobacterium-mediated transformation in three ornamental sunflower explant types-root, leaf, and cotyledon-using two different inoculation regimes: a vacuum-assisted $MgCl_2$ infiltration protocol (10 min) versus a non-vacuum ½ MS liquid immersion protocol (30 min). After inoculation, explants were co-cultured for 16 h, and transient T-DNA expression was evaluated by luminescence imaging using the luciferase reporter gene. No luciferase signal was detected in root and cotyledon explants under either method. Leaf explants, however, showed clear luciferase expression in both treatments, with vacuum infiltration producing stronger luminescence than non-vacuum infiltration despite the shorter inoculation time. These results indicate that leaf tissue is the most responsive explant type for transient *Agrobacterium*-mediated transformation in ornamental sunflower, and that the vacuum-assisted $MgCl_2$ protocol substantially enhances transient reporter-gene delivery compared to conventional immersion under the tested conditions.

Keywords: *vacuum infiltration, luciferase reporter gene, ornamental plants, transient expression*

1. INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an economically valuable crop prized for its vibrant yellow flowers and seeds rich in high-quality oil (Puttha *et al.*, 2023). In ornamental sunflower breeding, flower color, flowering type, vase life, and resistance to pathogens are areas of interest (Cvejić *et al.*, 2016; Gavrilova *et al.*, 2025). Recent advances in CRISPR/Cas genome editing technology promise to accelerate plant breeding by enabling efficient and targeted modifications in most plants (Chen *et al.*, 2019).

In dicotyledonous plant species, the preferred method for both stable and transient gene transfer is transformation mediated by the soil bacteria *Agrobacterium tumefaciens* (Hiei *et al.*, 2014). However, sunflower is among the species for which regeneration in tissue culture is difficult and highly genotype-dependent (Lewi *et al.*, 2006). This problem manifests as low yield, poor reproducibility and excessive sensitivity to numerous variables-from the explant to the culture medium-in both organogenesis and somatic embryogenesis-based systems (Knittel *et al.*, 1991). These regeneration barriers also affect stable sunflower transformation protocols. Furthermore, they make screening of genome-editing vectors via stable transformation a highly costly and time-consuming process.

Transient expression systems offer the ability to rapidly analyze the functionality of genetic constructs at the cellular level, providing time and labor efficiency compared to stable transformation. This system can be successfully applied in a wide range of plant species and tissues, supporting its use as a common tool in functional genomics studies (Zhang *et al.*, 2020). Gene transformation studies are typically conducted using various techniques, one of which is a vacuum-assisted gene transformation technique. The application of vacuum and subsequent repressurization forces the bacterial suspension into the interior of the leaf tissue, increases its entry into intercellular air spaces, and in most systems, yields higher transient or stable transformation efficiency compared to standard inoculation methods such as syringe injection or dipping (Tague and Mantis, 2006).

Luciferase (LUC) from fireflies is highly significant in transient expression analyses in plants because, compared to classical reporters such as GFP and GUS, it offers important advantages such as the absence of endogenous background light production in cells and much more sensitive detection. Luciferase-based bioluminescence imaging (BLI) systems provide low background signal and high resolution because they do not require external light stimulation. Compared to fluorescent and colorimetric methods, luciferase systems offer superior sensitivity, a wide dynamic range and low background interference (Li *et al.*, 2021; Xi and Yeh, 2026). Therefore, in this study, the susceptibility of three different explant types (root, leaf, and cotyledon) from ornamental sunflower (*Helianthus annuus* L.) to *Agrobacterium*-mediated transient gene transfer was compared using a luciferase reporter system. Furthermore, we compared the performance of two widely practiced inoculation regimes-a rapid vacuum-assisted $MgCl_2$ infiltration protocol (10 min) versus a standard non-vacuum ½ MS immersion protocol (30 min)-to establish an optimized transient expression platform for functional genomics and vector screening in sunflower.

2. MATERIALS AND METHODS

2.1. Vector and Agrobacterium-mediated transformation

The pMKV057 plasmid carrying the AtUBQ10:LUC:pNOS cassette as reporter gene, kindly provided by Dr. Daniel Voytas, was propagated in *Escherichia coli* and isolated using the Thermo Fisher Plasmid Miniprep Kit. The purified plasmid was subsequently introduced into electrocompetent *Agrobacterium tumefaciens* GV3101 cells. Briefly, 50 µL of electrocompetent *Agrobacterium* cells were mixed with 1 µL of plasmid DNA (100 ng/µL) on ice and transferred to a pre-chilled 2-mm electroporation cuvette. Electroporation was performed at 2.5 kV, 25 µF capacitance, and 400 Ω resistance. Immediately after electroporation, 950 µL of YEB medium was added, and the cells were recovered at 28 °C for 2 h with shaking. The transformed cells were plated on selective YEB medium and incubated at 28 °C for colony formation.

For LUC activity detection, the NightSHADE imaging system (Berthold Technologies GmbH & Co.KG) located at Vlaams Instituut voor Biotechnologie (VIB) was used. Explants were sprayed with 1 mM D-luciferin (GoldBio™) and subsequently imaged using IndiGo software (version 2.0.3.0; Berthold Technologies). Luminescence was recorded using 1-min image acquisition intervals, with imaging continued at regular intervals to monitor LUC activity over time.

2.2. Preparation of the Bacterial Culture and Infiltration Suspension

The *A. tumefaciens* GV3101 strain was cultured overnight at 28°C with shaking at 220 rpm in LB medium (containing 10 g Bacto-peptone, 10 g Bacto-yeast extract, and 7 g Bacto-tryptone in 1 L of distilled water; pH 7.2). After the 1.0 optical density (OD600) of the culture was determined spectrophotometrically, the bacterial suspension was centrifuged to form a pellet. The resulting pellet was divided into two equal portions. The first portion was resuspended in liquid 1/2 MS (Murashige & Skoog) medium for non-vacuum inoculation. The second group was suspended in an infiltration buffer containing 10 mM $MgCl_2$ for vacuum-assisted infiltration. 100 µM Acetosyringone was added to both suspensions, and they were incubated at room temperature for 1 hour prior to inoculation.

2.3. Plant Material, Explant Inoculation and Co-Culture

Under sterile conditions, sections of approximately equal size were prepared from root, leaf, and cotyledon tissues of *Helianthus annuus* cv. 'Music box' (ornamental sunflower). Two different protocols were applied to compare inoculation methods:

Protocol 1 (Vacuum-Assisted Infiltration): Ten explants each from root, leaf, and cotyledon tissues were placed in petri dishes containing a bacterial suspension in the infiltration buffer and exposed to negative vacuum pressure in a desiccator for 10 minutes.

Protocol 2 (Non-Vacuum Infiltration): Ten explants from each tissue type (root, leaf, cotyledon) were added to a bacterial suspension in 1/2 MS medium and incubated at atmospheric pressure for 30 minutes.

At the end of the inoculation periods, the explants from both groups were transferred to sterile filter paper to remove excess bacterial suspension from their surfaces. The explants were then transferred to a solid co-culture medium containing full-strength MS salts and 0.7% (w/v) agar and left for co-culture in the dark for 16 hours.

2.4. Quantitative Image Analysis of Sunflower Leaves

Transient luciferase (LUC) reporter gene expression was evaluated using a CCD camera-based luminescence imaging system following a 16-hour co-culture period. The photon signals emitted by the explants were recorded under standard exposure conditions, and luminescence intensity was quantified using Fiji (ImageJ-based) software. For leaf explants in both inoculation methods (vacuum-assisted and non-vacuum), the boundaries were individually defined as regions of interest (ROI) using the "Polygon/Freehand Selection Tool" and saved in the ROI Manager. To correct for optical and instrument background noise, three independent reference areas were selected from the non-luminescent empty backgrounds within the petri dish, and the average background signal (B_mean") was calculated. To determine the net luminescence output independent of variations in tissue size, the background average was subtracted from the raw signal of each explant to obtain the Corrected Mean Luminescence Intensity. These experiments were designed as a preliminary, exploratory screening study to determine whether transient T-DNA expression could be detected in different tissue samples; therefore, the results are presented as qualitative and semi-quantitative observations. The experiment was conducted once (a single independent replicate) for each tissue sample and protocol, using ten tissue samples per sample and protocol.

3. RESULTS

The tissue specificity of *Agrobacterium*-mediated transient gene expression in ornamental sunflower (*Helianthus annuus* L.) was determined by testing root, cotyledon, and leaf explants using both vacuum-assisted (10 min) and non-vacuum immersion (30 min) techniques. During bioluminescence imaging following a 16-hour co-culture period, no luciferase (LUC) emission could be detected in any of the root or cotyledon explants, no matter which inoculation method was used (Figure 1A). The fact that the control groups (consisting of bacterial suspension alone and untransformed explants) showed no background signal proved that the luminescence measured was completely due to T-DNA-mediated expression. Unlike the root and cotyledon tissues, the leaf explants showed a clear luminescence signal with both inoculation methods, which shows that leaves are the tissue most sensitive to transient transformation in ornamental sunflowers.

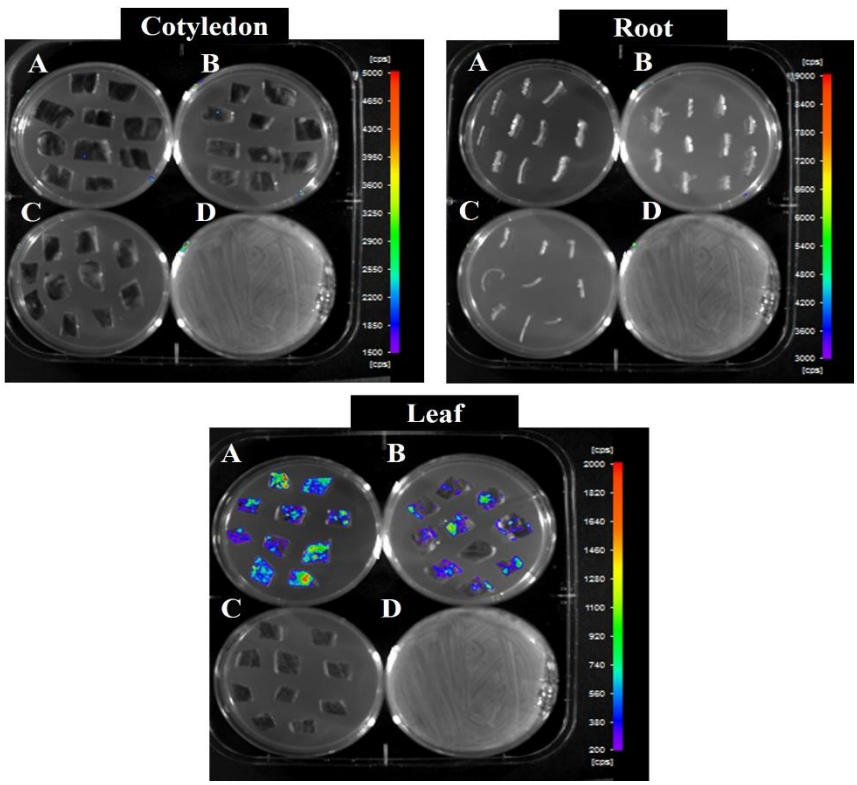


Fig 1. Transient luciferase (LUC) expression in sunflower explants. (A) Explants subjected to vacuum-assisted infiltration (Group A). (B) Explants into which plasmid transfer was performed without vacuum (Group B). (C) Untransformed explants (negative control). (D) *Agrobacterium* suspension control. The color scale on the right shows luciferase activity in counts per second.

Luciferase activity in leaf explants was measured by selecting pixel-based regions of interest (ROIs) in Fiji (ImageJ) and adjusting for background noise. The corrected mean luminescence intensity indicated that vacuum treatment was essential for efficient gene transfer. Even though the inoculation time was only 10 minutes, the vacuum-assisted

protocol led to a much higher level of transient reporter gene expression than the standard 30-minute method.

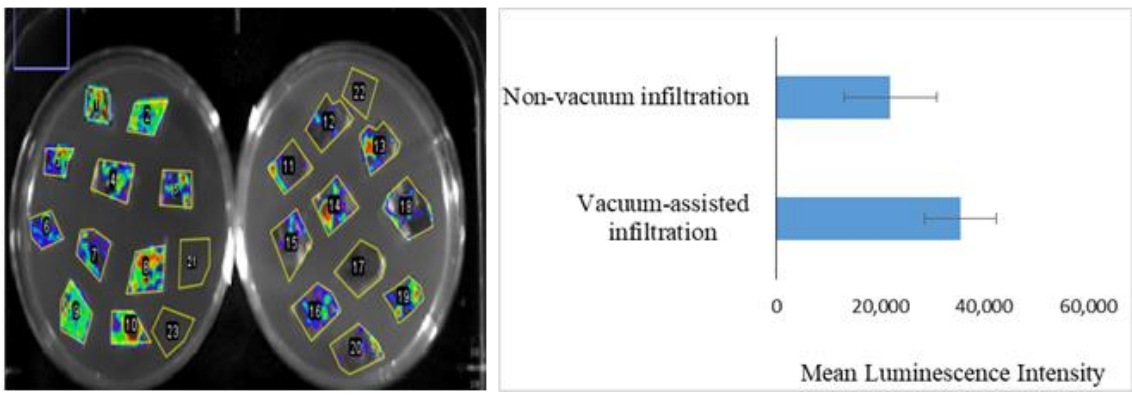


Fig. 2. This figure shows a quantitative image analysis of transient luciferase (LUC) reporter gene expression in ornamental sunflower (*Helianthus annuus* L.) leaf explants after vacuum-assisted and non-vacuum *Agrobacterium* inoculation. (A) Luminescence intensity maps display leaf explants in two different Petri dishes following 16 hours of co-culture after inoculation (left Petri dish: vacuum-assisted inoculation; right Petri dish: non-vacuum inoculation). We defined the boundaries of each explant as regions of interest (ROIs) using the Polygon/Freehand Selection tool in Fiji (ImageJ) for quantification. (B) The graph compares corrected mean luminescence intensities for explants treated with non-vacuum and vacuum-assisted inoculation. Columns show group means, and error bars represent the standard error of the mean (± SE). The values were obtained from a single experiment (10 explants per protocol, with each protocol conducted in a single Petri dish); the error bars reflect the variation among explants within the same dish rather than the variation among independent biological replicates.

4. DISCUSSION

Sunflower (*Helianthus annuus* L.) is considered recalcitrant for *in vitro* culture and transformation (Radonic *et al.*, 2014). Low regeneration efficiency prevents the direct development of transgenic sunflowers and, in particular, delays the integration of genome editing techniques such as CRISPR/Cas9 via stable transformation (Mabuza *et al.*, 2023). Testing transient expression systems designed to overcome such limitations offers a rapid screening approach for genomic studies. In this study, we investigated the susceptibility of different explant types (root, cotyledon, and leaf) in ornamental sunflowers to *Agrobacterium*-mediated transient gene transfer and demonstrated the effect of vacuum-assisted infiltration on transfection efficiency using the luciferase (LUC) reporter gene. This finding is consistent with previous studies reporting that sunflower is a species highly dependent on genotype and explant type in both organogenesis and somatic embryogenesis-based regeneration systems (Ionas *et al.*, 2026; Bayraktaroglu and Dağüstü, 2010). Although the absence of any T-DNA-derived fluorescence signal in root and cotyledon explants does not imply complete resistance to transformation, the short-duration (10-30 minutes) inoculation protocol and the 16-hour co-culture period may not be sufficient for effective T-DNA transfer in these tissue types. Studies in which sunflower cotyledon explants were successfully transformed highlight strategies such as optimizing genotype

and explant maturity (Sujatha *et al.*, 2012), using wounding pretreatments, or extending co-culture periods with low inoculum levels (Zhang and Finer, 2016). This suggests that the *Agrobacterium* susceptibility of cotyledon tissue depends largely on the physiological age used and the tissue preparation protocol. The low light-response of root tissue, on the other hand, is attributed to the root epidermis possessing apoplastic barriers such as a thicker cuticle and suberin accumulation (Nawrath *et al.*, 2013; Barberon, 2017), the limited availability of competent cell areas exhibiting active cell division necessary for T-DNA integration (Château *et al.*, 2000), and the presence of wound-induced phenolic compounds which trigger *vir* gene induction, in a different profile or concentration compared to leaf tissue (Stachel *et al.*, 1985).

5. CONCLUSION

This study investigated the susceptibility of various explant types from ornamental sunflower (*Helianthus annuus* L.), a species known for its resistance to genetic transformation, to *Agrobacterium*-mediated transient gene transfer using a luciferase (LUC) reporter and a vacuum-assisted agroinfiltration method. Results indicate that transient gene expression is influenced by explant type. Among the tissues tested, leaf explants exhibited the highest efficiency, as evidenced by a strong bioluminescence signal following short-term inoculation and 16-hour co-culture, while root and cotyledon tissues showed no detectable signal. These findings suggest that vacuum-assisted agroinfiltration of leaf explants offers a practical, reliable, and highly efficient approach for rapid screening of gene functions, vector constructs, and CRISPR/Cas9-based genome editing components in sunflowers, eliminating the need for prolonged and labor-intensive tissue culture required for stable transformation.

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WORKING GROUP 2

Diagnosis, sanitation, and conservation

Preliminary phytosanitary assessment of the autochthonous Albanian grapevine cultivars 'Shesh i bardhë' and 'Shesh i zi'

Adriatik Çakalli[1], Doriana Xhulaj (Bode)[2], Magdalena Cara[3,4], Klevis Hoxhallari[3,4], Jordan Merkuri[4]

[1]Institute of Plant Genetic Resources, Agricultural University of Tirana

[2]Faculty of Agriculture and Environment, Agricultural University of Tirana

[3]Department of Plant Protection, Agricultural University of Tirana

[4]NanoAlb/NanoBalkan, Academy of Sciences of Albania

acakalli@ubt.edu.al

Abstract

'Shesh i Bardhë' and 'Shesh i Zi' are autochthonous Albanian grapevine cultivars with important value for local viticulture and for the conservation of national grapevine diversity. The sanitary quality of their propagation material is relevant for maintaining productive vineyards and for future selection of healthy mother vines. A preliminary phytosanitary survey was conducted during 2024–2025 in vineyards representing the two cultivars in several Albanian viticultural areas. A total of 131 plant samples (58 'Shesh i Bardhë' and 73 'Shesh i Zi') were examined following field inspection for virus-like symptoms and assessment of varietal conformity. Serological screening was performed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for Grapevine leafroll-associated virus 3 (GLRaV-3), Grapevine fanleaf virus (GFLV), Grapevine virus A (GVA), Grapevine fleck virus (GFkV) and Arabis mosaic virus (ArMV). GLRaV-3 was the most frequently detected virus, occurring in 16 of 58 'Shesh i Bardhë' samples (28%) and 23 of 73 'Shesh i Zi' samples (32%). GFLV occurred in 7 (12%) and 10 (14%) samples, respectively, whereas GVA was detected in 2 (3%) and 4 (5%) samples and GFkV in 1 (2%) and 1 (1%) sample. ArMV was not detected in either cultivar. The results provide a preliminary picture of the sanitary status of these two traditional cultivars and identify GLRaV-3 and GFLV as the main targets for continued monitoring. The study also illustrates the usefulness of combining field inspection with serological screening when selecting candidate mother vines. Because ELISA may not detect low-titre or unevenly distributed infections, the present results should be regarded as a preliminary screening step rather than as a definitive certification test.

Keywords: *Vitis vinifera; 'Shesh i Bardhë'; 'Shesh i Zi'; grapevine viruses; DAS-ELISA; GLRaV-3; GFLV; phytosanitary selection*

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an important perennial crop in Albania, where traditional cultivars remain part of the country's viticultural heritage. Among them, 'Shesh i Bardhë' and 'Shesh i Zi' have a long history of cultivation in central Albania and are traditionally used for wine production. Their conservation involves not only maintaining cultivar identity and agronomic characteristics, but also preserving healthy and traceable propagation material. In perennial crops, viruses can be transmitted from infected mother plants to their progeny through vegetative propagation, contributing to the spread and long-term persistence of viral infections in vineyards.

Viral diseases therefore represent a persistent phytosanitary challenge in grapevine cultivation (Martelli, 2017). Several economically important viral diseases affect grapevine, including fanleaf degeneration, leafroll disease, and the rugose wood complex. Grapevine fanleaf virus (GFLV) is associated with fanleaf degeneration and is transmitted by the soil-borne nematode *Xiphinema index*. Grapevine leafroll-associated viruses are associated with leafroll disease and can be spread through infected propagation material and, for some viruses, by mealybugs. GVA is associated with the rugose wood complex, whereas GFkV may remain largely symptomless but is relevant when the sanitary status of propagation material is assessed. These characteristics make visual inspection alone insufficient for reliable sanitary selection.

Serological methods remain useful for screening large numbers of grapevine samples. DAS-ELISA is relatively simple, comparatively inexpensive and suitable for processing many samples in parallel. The current EPPO standard PM 7/125 (2) describes ELISA as a method for detection and identification of plant viruses and recognises its usefulness for large-scale screening (EPPO, 2025). The EPPO certification scheme for pathogen-tested grapevine material also recommends ELISA for GFLV and other European nepoviruses, GLRaVs, GVA/GVB and GFkV when suitable antisera are available (EPPO, 2008).

Previous work has demonstrated the relevance of grapevine viruses in Albania. Merkuri *et al.* (1994) reported grapevine fanleaf, fleck, virus A and leafroll-associated viruses in several Albanian viticultural districts. Sadikaj *et al.* (2016) subsequently assessed four native cultivars, including ‘Shesh i Bardhë’ and ‘Shesh i Zi’, using ELISA. Their results confirmed the occurrence of several grapevine viruses in Albanian vineyards and highlighted the importance of sanitary control of propagation material.

The present study was designed as a focused preliminary assessment rather than a national epidemiological survey. The objectives were to assess the sanitary status of ‘Shesh i Bardhë’ and ‘Shesh i Zi’ using field inspection and DAS-ELISA, identify the viruses most frequently detected in the material examined, and provide a practical basis for continued selection of candidate mother vines. Compared with previous Albanian surveys, the contribution of the present work is the updated 2024–2025 assessment of the two traditional cultivars in selected vineyards from Tirana, Durrës and Lezhë, combined with traceable sample codes linking field observations, varietal conformity and laboratory results.

2. MATERIALS AND METHODS

2.1. Study design and plant material

The survey was conducted during the 2024–2025 vegetative seasons. Sampling focused on vineyards containing the traditional cultivars ‘Shesh i Bardhë’ and ‘Shesh i Zi’. The working dataset comprised 131 plant samples (58 ‘Shesh i Bardhë’ and 73 ‘Shesh i Zi’) collected from representative vineyards in Tirana, Durrës and Lezhë, according to the field survey material (Table 1). The study was intended as a preliminary screening and was not designed to estimate virus prevalence at national scale.

For the purposes of this proceedings paper, the working dataset was treated at vine level: each sample code corresponded to one individually labeled vine and was counted once in the incidence summary. Field symptoms were recorded during inspection and used to guide

sample selection, but the present short proceedings contribution reports the main descriptive laboratory findings rather than a separate quantitative symptom-incidence analysis.

Plants were inspected for general vigour, varietal conformity and symptoms potentially associated with viral diseases. Particular attention was paid to leaf discoloration, interveinal reddening or yellowing, downward rolling of leaf margins, short internodes, abnormal leaf shape, double nodes and fanleaf-like symptoms. Vines with good agronomic performance and vines showing virus-like symptoms were labeled in September and sampled, because grapevine virus infections can remain latent or show weak and variable symptom expression.

2.2. Sampling and traceability

Samples were collected from individual vines and labeled with a unique sample code. The cultivar, sampling site and field observations were recorded at collection. Plant material was transported to the laboratory under cool conditions and processed for serological testing. Traceable sample codes linked laboratory results to the corresponding field observations and candidate-vine selection process.

The unique sample-code system was used to maintain traceability between each vine, its cultivar, sampling site, field observations and laboratory result, and to avoid duplicate counting in the descriptive incidence summary.

2.3. Ampelographic assessment

Varietal conformity was assessed using visible ampelographic characters of the shoot and mature leaf, together with bunch and berry characteristics where available. The assessment followed the principles of the OIV descriptor system for *Vitis* varieties. The current OIV framework emphasises harmonised descriptors for variety identification and recommends combining phenotypic information with genetic profiling when formal identification is required. In this preliminary study, ampelography was used as a field selection criterion and not as a substitute for formal genetic identification (OIV, 2023).

2.4. DAS-ELISA

Serological testing was performed by DAS-ELISA (Clark & Adams, 1977) for five target viruses: GLRaV-3, GFLV, GVA, GFkV and ArMV. The assays followed the instructions of the corresponding commercial reagent systems and the general principles described in EPPO PM 7/125(2) (EPPO, 2025). Positive and negative controls were included, and samples were analyzed in duplicate. ELISA was selected because it permits simultaneous screening of a relatively large number of grapevine samples and is an established tool in phytosanitary testing of propagation material. EPPO guidance emphasises the importance of appropriate controls and validated interpretation criteria when establishing positive and negative results.

All 131 samples were screened for the same five target viruses. DAS-ELISA was performed using commercial diagnostic kits supplied by Agritest S.r.l. Leaf material from mature leaves was used for testing; when visible symptoms were present, symptomatic leaves were preferentially sampled, whereas apparently healthy vines were sampled from representative mature leaves. Samples were classified as positive according to the positivity threshold recommended in the Agritest kit instructions and by comparison with the negative and positive controls included in each assay.

2.5. Data presentation

Virus incidence was expressed as the percentage of ELISA-positive samples within the examined material for each cultivar, rounded to the nearest whole number. The proceedings paper reports the main descriptive findings. No attempt was made to infer national prevalence, cultivar resistance or epidemiological relationships from the limited preliminary dataset.

The present proceedings paper reports target-specific incidence for each cultivar. Vine-level sanitary categories, including vines positive for at least one target virus, vines with mixed infections and vines negative for all five targets, require the complete sample-by-virus matrix and should be reported in a subsequent extended analysis. In the present selection framework, vines with appropriate field characteristics and negative ELISA results for the five tested targets were retained for further evaluation, whereas vines positive for one or more target viruses were excluded from direct use as propagation sources at this preliminary screening stage.

Table 1. Main characteristics of the preliminary survey.

Parameter	Description
Study period	2024–2025
Target cultivars	‘Shesh i Bardhë’ and ‘Shesh i Zi’
Number of samples	131 (58 ‘Shesh i Bardhë’; 73 ‘Shesh i Zi’)
Main sampling areas	Tirana, Durrës and Lezhë
Primary diagnostic method	DAS-ELISA
Target viruses	GLRaV-3, GFLV, GVA, GFkV and ArMV
Purpose	Preliminary phytosanitary screening and candidate mother-vine selection

3. RESULTS

3.1. Field observations

Field inspection showed that virus-like symptoms varied among vineyards and between individual vines. In ‘Shesh i Bardhë’, plants were frequently asymptomatic or showed mild interveinal discoloration, whereas ‘Shesh i Zi’ more often showed reddening and downward rolling of leaves compatible with leafroll-like symptoms. Fanleaf-type deformations, short internodes and abnormal shoot or leaf development were observed sporadically, particularly in older plantings. These observations were not interpreted as diagnostic for a particular virus because similar symptoms may result from different viral complexes or non-viral causes.

The occurrence of apparently healthy vines among laboratory-positive material was also relevant. This supports the use of laboratory screening during selection of mother plants rather than relying exclusively on visual appearance. Uneven virus distribution and symptom variability are recognised limitations of field observation and can also affect serological detection in grapevine (Rowhani *et al.*, 1997).

Some vines that appeared visually healthy during field inspection tested positive by ELISA. This observation confirms that visual assessment alone is insufficient for mother-vine selection and supports the combined use of field inspection, traceable sampling and laboratory screening. The exact proportion of asymptomatic ELISA-positive vines should be quantified in a future vine-level analysis using the original field-record matrix.

3.2. Virus detection by DAS-ELISA

GLRaV-3 was the most frequently detected virus in both cultivars. It was detected in 16 of 58 'Shesh i Bardhë' samples (28%) and in 23 of 73 'Shesh i Zi' samples (32%). GFLV was the second most frequently detected target, detected in 7 (12%) and 10 (14%) samples, respectively. GVA was detected at lower levels (2 'Shesh i Bardhë' samples, 3%; 4 'Shesh i Zi' samples, 5%), while GFkV occurred in 1 sample of each cultivar (2% and 1%). ArMV was not detected in either cultivar in the material examined. Thus, the same general order of virus detection was observed in both cultivars, with GLRaV-3 clearly predominating (Table 2).

The difference between the two cultivars was modest for most targets. 'Shesh i Zi' showed somewhat higher recorded incidence of GLRaV-3, GFLV and GVA, whereas GFkV was slightly higher in 'Shesh i Bardhë'. These descriptive differences should not be interpreted as evidence of cultivar-specific resistance or susceptibility because the present dataset was not designed or powered for such a comparison.

Table 2. Incidence (%) of five grapevine viruses by DAS-ELISA in 'Shesh i Bardhë' and 'Shesh i Zi'.

Virus	'Shesh i Bardhë', % (n = 58)	'Shesh i Zi', % (n = 73)
GLRaV-3	28	32
GFLV	12	14
GVA	3	5
GFkV	2	1
ArMV	0	0

3.3. Implications for candidate mother-vine selection

The serological results were incorporated into the preliminary selection process. Vines with favorable agronomic and varietal characteristics were considered potential mother vines only after their sanitary status had been assessed. Plants that tested positive for one or more target viruses were not considered suitable for direct use as candidate propagation sources within this screening stage. Vines with appropriate field characteristics and negative ELISA results were retained for further evaluation. This provides a practical first filter before confirmatory testing or establishment of a protected mother block.

4. DISCUSSION

The predominance of GLRaV-3 in both 'Shesh i Bardhë' and 'Shesh i Zi' is consistent with the importance of leafroll-associated viruses in grapevine sanitary programs. Previous Albanian surveys also identified GLRaV-3 among the principal viruses detected in local

cultivars. Sadikaj *et al.* (2016) reported GLRaV-3 in 'Shesh i Bardhë' and 'Shesh i Zi' within a broader survey of Albanian native varieties, while earlier work by Merkuri *et al.* (1994) documented leafroll-associated viruses together with GFLV, GFkV and GVA in several viticultural districts. The present findings complement earlier Albanian surveys by providing updated, cultivar-specific serological data from selected vineyards surveyed during 2024–2025. The occurrence of GFLV in both cultivars is also relevant because GFLV causes grapevine fanleaf degeneration and is associated with the nematode vector *Xiphinema index*. GFLV is considered one of the major virus diseases of grapevine, and management depends strongly on healthy propagation material and prevention of spread (Andret-Link *et al.*, 2004).

The detection of GVA and GFkV at lower frequencies remains important for sanitary selection. GVA is associated with the rugose wood complex, while GFkV may be symptomless in many cultivated situations. Consequently, absence of conspicuous symptoms does not necessarily indicate that a vine is suitable as a propagation source.

The slightly higher incidence recorded in 'Shesh i Zi' for GLRaV-3, GFLV and GVA should be interpreted cautiously. The study was designed as a preliminary assessment and not to test genetic differences in virus susceptibility. Differences may reflect vineyard history, source material, age of vines, vector pressure, sampling distribution or other environmental factors. Similar caution is required when interpreting symptom expression because leafroll symptoms vary with cultivar and season.

The role of vectors is particularly relevant to GLRaV-3. Field experiments in Spain demonstrated transmission of GLRaV-3 by *Planococcus citri* and showed that incidence can increase substantially in established vineyards. These observations support sanitation together with vector management when infected vineyards are used as sources of propagation material (Cabaleiro & Segura, 1997).

The use of DAS-ELISA in the present study is appropriate for preliminary screening because it allows many samples to be processed in parallel and is recognised in EPPO diagnostic and grapevine certification guidance. The current EPPO PM 7/125(2) standard provides general requirements for ELISA testing, including appropriate positive and negative controls and validated interpretation criteria. The EPPO grapevine certification standard also lists ELISA for GFLV and other European nepoviruses, GLRaVs, GVA/GVB and GFkV when suitable antisera are available (EPPO, 2008).

At the same time, ELISA alone is not sufficient for final certification. Serological detection can be affected by virus titre, tissue type, season and uneven virus distribution. Rowhani *et al.* (1997) showed that ELISA-positive tissues can be unevenly distributed within chronically infected grapevines. The present results should therefore be considered a screening assessment. Candidate mother vines intended for formal certification should subsequently undergo the testing required by the applicable certification scheme.

The findings have practical relevance for conservation of Albanian grapevine germplasm. A conservation program should not rely only on preserving cultivar identity or phenotype. The sanitary status of vines used to establish collections and propagation blocks is equally important. Field selection, traceability and targeted ELISA screening provide an accessible first-stage procedure that can be applied to traditional vineyards before more intensive confirmatory testing.

5. CONCLUSIONS

This preliminary assessment showed that the traditional Albanian cultivars 'Shesh i Bardhë' and 'Shesh i Zi' carry several grapevine viruses of phytosanitary relevance. GLRaV-3 was the most frequently detected virus in both cultivars, followed by GFLV, whereas GVA and GFkV occurred at lower frequencies and ArMV was not detected in the material examined. The results reinforce the value of sanitary screening when selecting propagation sources and show that visual inspection alone cannot provide sufficient confidence for the selection of mother vines. DAS-ELISA represents a practical first-line screening method, particularly when a relatively large number of samples must be processed. However, the findings are preliminary and should not be interpreted as a substitute for the complete testing required for formal certification. Further work should focus on repeated testing of selected negative vines, confirmation according to the applicable certification scheme, and establishment of traceable healthy mother material for the conservation and propagation of 'Shesh i Bardhë' and 'Shesh i Zi'.

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Cryopreservation as a tool for the long-term conservation of edited embryogenic cultures in European chestnut

Mª Teresa Martínez, Cristina Buceta, Elena Corredoira

Grupo de Biotecnología y Mejora Forestal, Misión Biológica de Galicia (MBG-CSIC), Avd Vigo s/n, 15705, Santiago de Compostela, La Coruña, Spain

elenac@mbg.csic.es

Abstract

Cryopreservation is a highly valuable biotechnological tool for long-term conservation and has been successfully applied to a wide range of plant materials. To overcome the limitations of maintaining somatic embryogenic lines through repetitive embryogenesis - the emergence of somaclonal variation, reduced capacity to produce new embryos, losses due to contamination, and high labor and material costs, cryopreservation has emerged as a key tool. The aim of this study was to validate a previously developed cryopreservation protocol for somatic embryos of *Castanea sativa* and to assess its effectiveness for storing gene-edited embryogenic lines while their modified traits are being evaluated. Somatic embryos in which the susceptibility genes *PMR4* (Powdery Mildew Resistant 4) and *DMR6* (Downy Mildew Resistant 6) had been edited using the CRISPR/Cas9 system were cryopreserved using a vitrification-based procedure. Groups of 2 to 3 edited somatic embryos at the globular and early cotyledonary developmental stages (from 5 *DMR6* and 3 *PMR4* lines) were treated with PVS2 solution (60 min at 0 °C) prior to storage in liquid nitrogen. After cryopreservation, embryo recovery rates ranged from 46% to 83% varying by line. Thus, these results show that the vitrification-based cryopreservation procedure developed for European chestnut is an effective strategy for the long-term conservation of genome-edited embryogenic lines.

Keywords: *Castanea sativa, CRISPR/Cas9, vitrification-based procedure, somatic embryogenesis*

1. INTRODUCTION

The conservation of forest biodiversity should not be limited to the preservation of strategically important populations for conservation or elite specimens but should also encompass the maintenance of novel genotypes generated through breeding programs and plant biotechnology methods. In this context, the development of plant biotechnology, particularly advances *in vitro* culture, has provided valuable tools for the management, breeding and conservation of wide range of species and plant materials. Among these techniques, cryopreservation, based on the ultra-low temperature (−196 °C) storage of biological material in liquid nitrogen (LN), represents an effective strategy for long-term conservation (Engelmann, 2004). This approach can be applied to a wide range of plant materials, including seeds, meristematic tissues, cells, calli, pollen, and somatic embryos (Corredoira *et al.*, 2017). A recent state-of-the-art review on somatic embryo cryopreservation in broadleaf forest species shows that cryopreservation is a well-established approach and plays a relevant role for the long-term conservation of materials derived from plant biotechnology programs (Ballesteros *et al.*, 2024).

Cryopreservation is a highly recommended tool for the conservation of multiple embryogenic lines generated through transformation and genome editing while their

characteristics are evaluated (Lambardi *et al.,* 2008). Cryopreservation overcomes key limitations in the long-term maintenance of somatic embryos through repeated subculturing and cycles of secondary embryogenesis, among other advantages, avoids somaclonal variation, loss of embryogenic capacity, contamination, and high material and labor costs (Corredoira *et al.,* 2017). Thus, cryopreservation provides an efficient strategy for the stable long-term storage of a large number of lines, which can subsequently be recovered when required for further characterization, evaluation and other purposes.

Our research group has developed efficient regeneration systems based on somatic embryogenesis for several forest tree species of interest, including the European chestnut (*Castanea sativa* Mill.) (Fernandes *et al.,* 2026). The availability of these systems has enabled the incorporation of new biotechnological approaches, such as genome editing, aimed at the targeted modification of candidate genes involved in plant defense mechanisms against pathogens. Specifically, edited European chestnut lines have been obtained targeting the *PMR4* (Powdery mildew resistance 4) and *DMR6* (Downy mildew resistance 6) susceptibility genes, which are involved in processes associated with the plant's response to pathogens. These genes encode a callose synthase and a 2-oxoglutarate/Fe(II)-dependent dioxygenase, respectively.

The chestnut lines obtained through genome editing require further characterization to determine the effects of the introduced modifications and, in particular, to assess their potential contribution to increased tolerance to *Phytophthora cinnamomi* Rands. This process may require relatively long periods and involves the maintenance of a large number of independent lines. In this context, cryopreservation represents a particularly valuable strategy, as it allows edited lines to be preserved without the need for continuous maintenance through *in vitro* subculturing.

Therefore, this study aimed to evaluate the efficacy of a vitrification-based cryopreservation protocol, previously established and originally designed for unedited European chestnut lines, for the long-term storage and subsequent recovery of genome-edited lines.

2. MATERIALS AND METHODS

2.1. Plant Material

Somatic embryos carrying CRISPR/Cas9-mediated edits in *PMR4* and *DMR6* genes were used to validate the previously defined protocol. Edited embryogenic lines, three *PMR4* and five *DMR6*, were maintained on proliferation medium consisting of MS (Murashige and Skoog, 1962) basal medium containing half-strength macronutrients and supplemented with 438 mg L^{-1} glutamine, 3% (w/v) sucrose, and 0.7% (w/v) Sigma agar.

All plant media were adjusted to pH 5.6–5.7 prior to autoclaving at 115 °C for 20 min. After sterilization, the medium was poured into 90-mm disposable Petri dishes, with 10 groups of somatic embryos placed in each dish. Unless otherwise indicated, cultures were incubated under a 16-h photoperiod (50–60 μmol m^{-2} s^{-1} from cool white fluorescent lamps) at 25 °C in the light and 20 °C in the dark (standard conditions).

2.2. Cryopreservation procedure

Cryopreservation was performed using a vitrification-based procedure adapted from Corredoira *et al.* (2004). Inocula were collected from four-week-old embryogenic cultures and consisted of small clumps of 2–3 somatic embryos at the globular or early torpedo stage. The embryo clumps were precultured for 3 days on proliferation medium supplemented with 0.3 M sucrose. Subsequently, they were treated with Plant Vitrification Solution 2 (PVS2 Sakai *et al.*, 1990) at 0 °C for 60 min to promote dehydration. Following PVS2 treatment, the embryo clumps were rapidly immersed in LN and cryostored for 1 month. For recovery, cryopreserved embryo clumps were rapidly thawed at 40–42 °C and immediately transferred to a 1.2 M sucrose solution for two successive washing steps of 10 min each. The somatic embryos were then transferred to proliferation medium under standard culture conditions. Post-cryopreservation recovery was assessed by embryo survival and the capacity to develop new embryos.

2.3. Data analysis

In each edited line, 3 Petri dishes with 10 embryo clumps per dish were used (n=30). After 6 weeks of culture the following parameters were evaluated: (i) survival defined as the percentage of explants exhibiting any sign of growth, including callus formation and (ii) embryo recovery rate defined as the percentage of explants that showed resumption of somatic embryogenesis.

3. RESULTS

The vitrification method was successfully applied to the cryopreservation of gene-edited chestnut lines, demonstrating its potential for the long-term conservation of these valuable genetic resources. All three lines carrying the edited *PMR4* gene exhibited survival rates exceeding 60%, whereas embryo recovery rates ranged from 46.7% to 53.3% (Fig. 1).

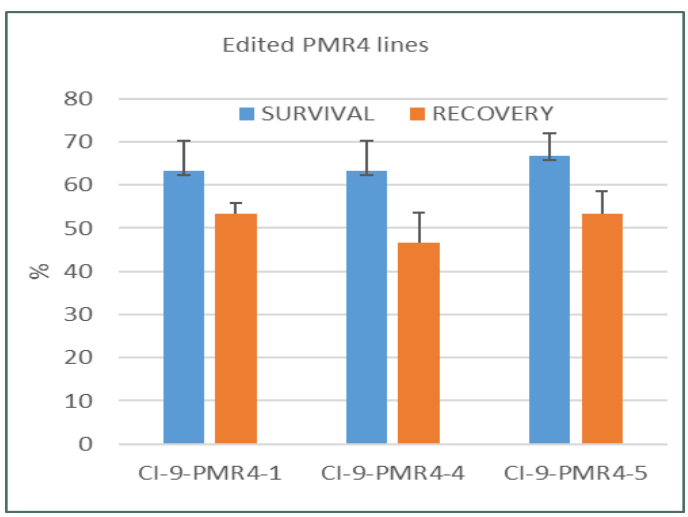


Fig. 1. Survival and embryo recovery of *PMR4* gene-edited lines following preculture on sucrose medium, 60 min exposure to PVS2, and subsequent immersion in LN. Survival and embryo recovery were assessed 6 weeks after treatment (n = 30).

Five *DMR6* gene-edited lines were evaluated for their response to cryopreservation. All lines exhibited high survival rates following storage in LN, ranging from 66.7% to 86.7%. Embryo recovery rates were also consistently above 55% across all evaluated lines (Fig. 2). In both *PMR4* and *DMR6* gene-edited lines, the recovered somatic embryos displayed a normal morphological appearance and resumed development following cryopreservation with no evident morphological abnormalities observed (Fig. 3). These results indicate that the vitrification procedure preserved the morphological integrity and developmental capacity of the somatic embryos in both gene-edited lines. Importantly, the efficient recovery and normal development of embryos from lines exhibiting different degrees of gene editing suggest that the level of editing did not adversely affect their ability to withstand the cryopreservation procedure. Currently, we have 17 edited lines stored in LN, comprising ten *DMR6*-edited lines and seven *PMR4*-edited lines, whose tolerance to *P. cinnamomi* is being evaluated.

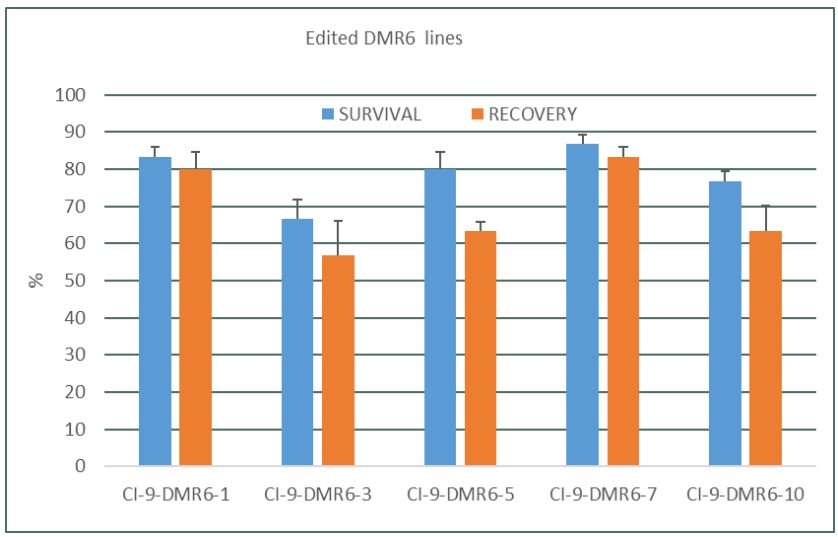


Fig. 2. Survival and embryo recovery of *DMR6* gene-edited lines following preculture on sucrose medium, 60 min exposure to PVS2, and subsequent immersion in LN. Survival and embryo recovery were assessed 6 weeks after treatment (n = 30).

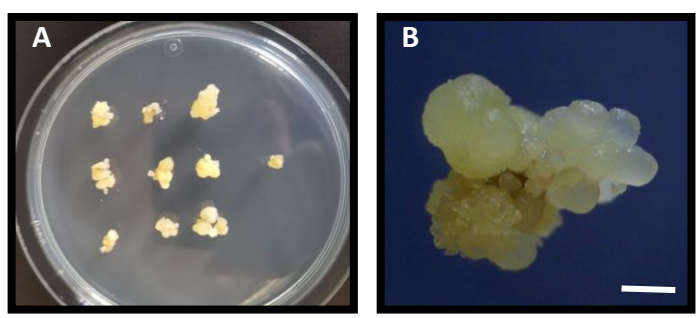


Fig. 3. A. Regrowth of somatic embryos from cryopreserved embryo clumps of an edited line following exposure to PVS2 solution for 60 min, subsequent immersion in LN and after 6 weeks of regrowth. Dish diameter = 90 mm. B. Detailed view of an embryo clump after cryopreservation and regrowth. Scale bar = 1 mm.

4. DISCUSSION

The successful cryopreservation of both *PMR4* and *DMR6* gene-edited chestnut lines demonstrates the applicability of the vitrification procedure for the long-term conservation of genetically edited material. Vitrification was used to minimize intracellular ice formation while preserving the viability (Sakai, 2004) and developmental potential of CRISPR/Cas9-edited somatic embryos during long-term storage. Although differences in cryotolerance were observed between the two groups of lines, all lines maintained relatively high survival and embryo recovery rates following PVS2 treatment and exposure to LN. The *DMR*6 lines exhibited the highest overall survival rates (66.7–86.7%) and consistently high embryo recovery rates (>55%), whereas the *PMR4* lines showed survival rates above 60%, with embryo recovery ranging from 46.7% to 53.3%. Notably, lines with different levels of gene editing displayed comparable responses to cryopreservation, suggesting that the extent of gene editing did not substantially affect their tolerance to dehydration and cryogenic stress or their capacity to recover following cryopreservation. Overall, these results validate the vitrification protocol previously established for European chestnut [7] and demonstrate its robustness and applicability to genetically edited lines, confirming its suitability for the long-term conservation of CRISPR/Cas9-derived germplasm.

Cryopreservation has previously been successfully applied to genetically transformed plant material, including embryogenic lines of European chestnut. Corredoira *et al.* (2007) demonstrated the successful vitrification and long-term storage of transgenic *C. sativa* embryogenic lines, with stable transgene integration confirmed in plants regenerated following cryopreservation. Similar approaches were subsequently applied to transgenic holm oak and cork oak lines using PVS2-based vitrification protocols (Cano *et al.,* 2020; Cano *et al.,* 2021). In other woody species, such as silver birch (Ryynänen *et al.,* 2002) and the hybrid *Populus tremula* L. × *Populus tremuloides* Michx. (Jokipii-Lukkari *et al.,* 2004), cryopreservation of transgenic material has likewise been shown to maintain the viability and regenerative capacity of the material, as well as the genetic characteristics of the recovered plants. However, to the best of our knowledge, reports on the cryopreservation of CRISPR/Cas9-edited embryogenic lines remains scarce.

5. CONCLUSIONS

Overall, these findings confirm that cryopreservation is an effective long-term storage strategy for genetically modified chestnut lines. The protocol can be broadly applied across multiple lines yealding high survival and embryo recovery rates. This is particularly relevant for the conservation of the generated gene-edited material, as it provides a reliable means of safeguarding these lines while their phenotypic characteristics and potential contribution to tolerance or resistance to the major diseases affecting chestnut are being evaluated. Consequently, successful cryopreservation represents a vital step towar maintaining these lines for future functional validation studies and breeding programs.

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Establishing a protocol for a high throughput screening test of hybrid larch by inducing osmotic stress *in vitro* during germination

Emma Ehmke, Marieluise Hohloch, Madlen Walther, Andrea Rupps

Dept. of Plant Evolution and Biodiversity, Späth-Arboretum, Institute of Biology, Humboldt-Universität zu Berlin, Späthstr. 80/81, 12437 Berlin, Germany

emma.ehmke@hu-berlin.de

Abstract

Somatic embryogenesis (SE) allows for the propagation of plants with known traits. However, characterizing sE larch *in vivo* is time and space intensive. In an effort to establish an early screening method for drought stress tolerance of hybrid larch, multiplied via SE, we applied mild and sublethal osmotic stress during germination using sorbitol as osmoticum. The earliest trials date back to 2018. To optimize the protocol, we lowered sorbitol concentration from 10% to 8% to induce osmotic stress bordering the permanent wilting point. Additionally, we added mild stress (4%). We used 21-day old plantlets and applied stress for 21 days. These changes improved efficiency, handling of roots and the ability to add regeneration capacity to the recorded parameters. We tested and ranked 45 genotypes of hybrid and European larch based on SSI of root and early leaf growth, quality score and regeneration capacity. All genotypes reacted by stunted growth. Sublethal stress led to more wilting and lower regeneration capacity than mild stress. Furthermore, we showed that several genotypes behaved differently to mild stress than to sublethal stress. Another optimization to the protocol included taking photos for analysis via Fiji, potentially enabling further parameters to be extracted. Our future aim is linking these findings to drought stress tolerance *in vivo*.

Keywords: *somatic embryogenesis, hybrid larch, early screening, osmotic stress, woody tree germination*

1. INTRODUCTION

Forestry needs to prepare for increased frequency and intensity of drought events due to the climate crisis. Trends in climate change since 1970 have shown higher temperatures, anomalies in temperature and precipitation as well as changes in relative and absolute humidity, with Europe exceeding many of the global averages (Bilgili & Tokmakci, 2025). Native range of European Larch (*Larix decidua* MILL.) has been documented with an average annual temperature of 4°C, while temperature in cultivation areas averaged to up to 9°C (Falk *et al.*, 2012). Fossil studies of *Larix decidua* in the Central European Alps have shown changes in distribution as response to periods of rising temperatures (Wagner *et al.*, 2015). Thus, rising temperatures might deem *L. decidua* unsuitable for formally established areas of cultivation in the future.

Temperature, rather than vapor pressure deficit, was found to impact mortality of European Larch (Sagar *et al.*, 2025). The same study found Japanese Larch (*L. kaempferi* (LAMB.) CARR.) to be more sensitive to high vapor pressure deficit and summer drought. The F1 generation of hybrid larch (*L. x eurolepis* HENRY), the hybrid of European and Japanese larch, has been shown to exhibit heterosis effects for example in regard to greater biomass, wood quality and improved stem length and form (Matyssek & Schulze, 1987; Rieckmann *et al.*, 2020). Crucially, these effects were shown to decrease with subsequent generations

and might not affect all crossings and individual hybrid trees (Pâques *et al.*, 2026). Beyond traits regarding growth, the heterosis effect may extend to tolerance of certain hybrid larch genotypes to drought events (Malm & Stener, 2002; Wolf *et al.*, 2021). One solution to ensure plant material with favorable traits can be planting of genotypes, multiplied via somatic embryogenesis (SE) (Park *et al.*, 2016). However, characterizing and testing conifer genotypes *in vivo* is very time and space intensive. Field testing for breeding programs of larch can take between 10-15 years (Aronen *et al.*, 2025). Testing younger trees (ie one to two years old) for drought stress tolerance under controlled conditions requires greenhouse space and resources for data collection for over two months, limiting the amount of testable genotypes (Wolf *et al.*, 2021). Furthermore, concerns for biodiversity of clonally propagated trees require an extensive clonal archive with regular addition of newly initiated genotypes. To alleviate these intensive resources needed for characterization of genotypes, a high throughput early screening test for drought tolerance is needed.

An early screening of potato for osmotic stress tolerance *in vitro* found the stress susceptibility index in combination with the ratio of root to shoot dry mass allowed for identification of the most tolerant and most sensitive genotypes to drought stress *in vivo* (Bündig *et al.*, 2017). Early trials on hybrid larch using sorbitol as osmoticum linked plant vitality to sorbitol concentration in media (Johst & Rüdinger, 2018). Further trials on European and hybrid larch found that root length, cotyledon length and a quality score for vitality serve as significant and reproducible parameters to distinguish genotype-specific reactions to osmotic stress *in vitro* (Benneckenstein & Tietje, 2018). Efforts to link results of this early screening to experiments in the greenhouse have not been successful yet, one stated reason was considerable effort required for testing, resulting in 8 genotypes available for validation (Wolf *et al.*, 2021). To test a larger number of genotypes at an early stage of plant development, we optimized the protocol and applied mild and sublethal osmotic stress in germination to potentially link the reaction *in vitro* to drought stress tolerance *ex vitro*. We lowered sorbitol concentration from 10% to 8% to induce osmotic stress bordering the permanent wilting point (Ford *et al.*, 2005; Johst & Rüdinger, 2018). We started treatment on 21-day old plantlets, without direct tracking of individual plants and lowered treatment time to 21 days. This allowed for improved handling of roots, more efficient experiments and the ability to add regeneration capacity to the recorded parameters. In summary, we modified the following parameters compared to earlier trials: shortened exposure to osmoticum, testing on younger plantlets without direct tracking of individual plants, lowering of sublethal stress concentration from 10% to 8%, addition of mild stress at 4% sorbitol and addition of recovery as tested parameter. With these optimizations, we were able to test and rank 45 genotypes regarding osmotic stress tolerance. Furthermore, the last optimization included taking photos for analysis via Fiji, which allowed a larger number of genotypes to be probed at once (Schindelin *et al.*, 2012).

2. MATERIALS AND METHODS

2.1. Materials

Plant material consists of 45 SE cell cultures (genotypes) initiated between the years 2013 to 2024. We tested two genotypes of *L. decidua* and 43 genotypes of *L. x eurolepis*. Hybrid larch genotypes either originate from direct crosses or open pollinated mother tree from a

seed orchard. 32 genotypes can be sorted into five established progenies. 11 genotypes belong to unnamed progenies with known parent trees. Genotypes tested more than once at different times are indicated by an underscore.

Basic culture media ingredients for proliferation and maturation of larch are reported in Kraft & Kadolsky, 2018. Standard media for germination found in Kraft & Kadolsky, 2018 was modified for potassium nitrate content according to Gupta & Holmstrom, 2005.

To induce osmotic stress, sorbitol was added to the standard germination media. Control media contained 0% sorbitol, mild stress inducing media contained 4% (40 g/L) sorbitol and sublethal stress inducing media contained 8% (80 g/L) sorbitol.

2.2. Methods

Proliferation and maturation of embryogenic cultures were performed according to standard procedures (Kraft & Kadolsky, 2018).

Approximately 125 normally developed somatic embryos per genotype were germinated on standard media (25 embryos per culture vessel), kept in the dark for 3-6 days and subsequently placed under LED growth lights. Growing conditions consisted of temperatures of 22 ± 1°C and photo period of 16 h light at an intensity of 120 µmol*s^{-1}*m^{-2}. After 14 days on germination media, plantlets were checked for sufficient growth and placed in an upright position with root submerged. After 20-22 days on standard media, approximately 13 vital plants per treatment group were chosen for testing. Plants were placed on a sterile dish with graph paper for scale and pictures were taken. Plants were subsequently transferred to treatment media, either containing 0% sorbitol, 4% sorbitol or 8% sorbitol. 21+-1 days later, treated plants were photographed again and placed on standard germination media for regeneration. Photos were analyzed using Fiji/ImageJ and measurements of root length and cotyledon/leaf length were taken (Schindelin *et al.*, 2012). Furthermore, each plant was given a quality score between 0 (fully vital) and 4 (mostly wilted) (table 1.) after stress treatment. In trials taking place before 2025 each plant was measured by hand and ruler under sterile conditions at the start and end of stress treatment. This data is included in this study. If plants showed new growth and/or no visual decline 21+-1 days after stress treatment, regeneration was positive. Several genotypes had to be excluded from regeneration analysis due to missing data.

Plants were not individually tracked to improve efficiency compared to earlier trials. To analyze stress tolerance, root and leaf growth was calculated as difference of organ length at end and start of stress treatment. To limit variance due to mismatched individual measurements, plants were sorted by root length before calculations. For each combination of organ and stress intensity, the stress susceptibility index (SSI) based on Fischer & Maurer, 1978 was calculated as follows:

$$SSI_{(Genotype)} = \frac{1 - \left(\frac{P_s}{P_c}\right)}{1 - \left(\frac{mean\ P_s}{mean\ P_c}\right)}$$

With P_s and P_c as parameters of one genotype under stress and control conditions and mean P_s and mean P_c as mean of parameters of the tested population. Two genotypes with negative mean root growth under control conditions have been excluded from SSI

calculations. Figure 1 was created with the viridis color palette, all graphs were created in RStudio (v. 4.5.1) (Garnier *et al.*, 2021; RStudio Team, 2015).

Table 1.: Overview of the quality score of vitality for plantlets of SE larch including characteristics and exemplary photos. Quality score was given to each plantlet after stress treatment to assess the effects of osmotic stress on plantlet phenotype.

Quality score	**0**	**1**	**2**	**3**	**4**
characteristic	Plant is fully vital	Plant shows minor yellow discoloration of leaf tips	Plant shows major yellow discoloration of leaves, but no wilting	Plant shows wilting, as well as dark and yellow discoloration	Plant is mostly wilted
example					

3. RESULTS

SSI reveals diverging tolerance to stress intensities. In an effort to rank all genotypes regarding stress tolerance *in vitro*, SSI based on growth of different organs under stress conditions were calculated (figure 1.).

Both European larch genotypes (7 and 96) showed SSI > 1 for growth of leaves indicating that these genotypes are more sensitive than average under both stress conditions. The root growth SSI of genotype 96 under sublethal stress corresponds to less pronounced stress response, however mean root growth of genotype 96 under control conditions was low (3.6 mm).

Four genotypes had negative SSI indicating higher growth of organs under mild stress conditions than under control conditions. Genotypes tested more than once showed differences in median growth (data not shown) but tended to show similar tendency regarding tolerance based on SSI. Neither year of initiation nor progeny can be found as a clear indicator for good (< 1) SSI under both stress intensities. Under mild stress conditions, 15 genotypes were found with SSI < 1 for both measured organs. Based on SSI for root growth, 24 genotypes appeared to be more tolerant to mild stress. 26 genotypes appeared more tolerant to mild stress in regard to leaf growth. For sublethal stress, 12 genotypes exhibited good SSI for both organs, 20 genotypes for root growth and 23 for leaf growth respectively. Interestingly, overlap of most tolerant genotypes under both stress conditions is 42%, indicating diverging tolerance of genotypes to mild and sublethal stress.

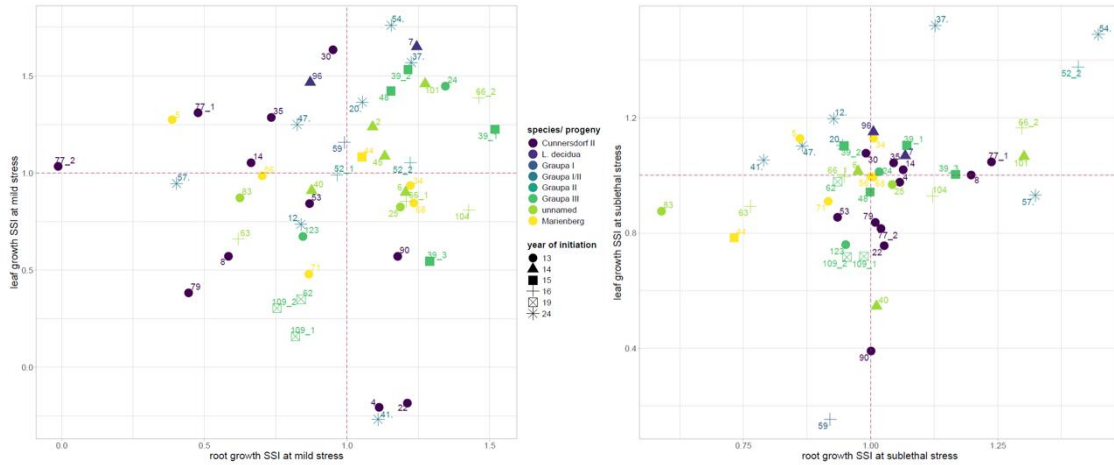


Fig. 1.: SSI of root growth (x) and leaf growth (y) under mild (left) or sublethal (right) stress conditions. Color corresponds to progeny or species; different shapes indicate the year of initiation; genotypes tested more than once are indicated by underscore. SSI < 1 is categorized as more tolerant than average to stress conditions, SSI > 1 is considered more sensitive than average to stress conditions. Lower left quadrant contains the most tolerant genotypes. Upper right quadrant contains the least tolerant genotypes. For sublethal stress, four genotypes (2, 42, 45 and 52_1) had to be excluded due to missing data.

All genotypes react to osmotic stress.

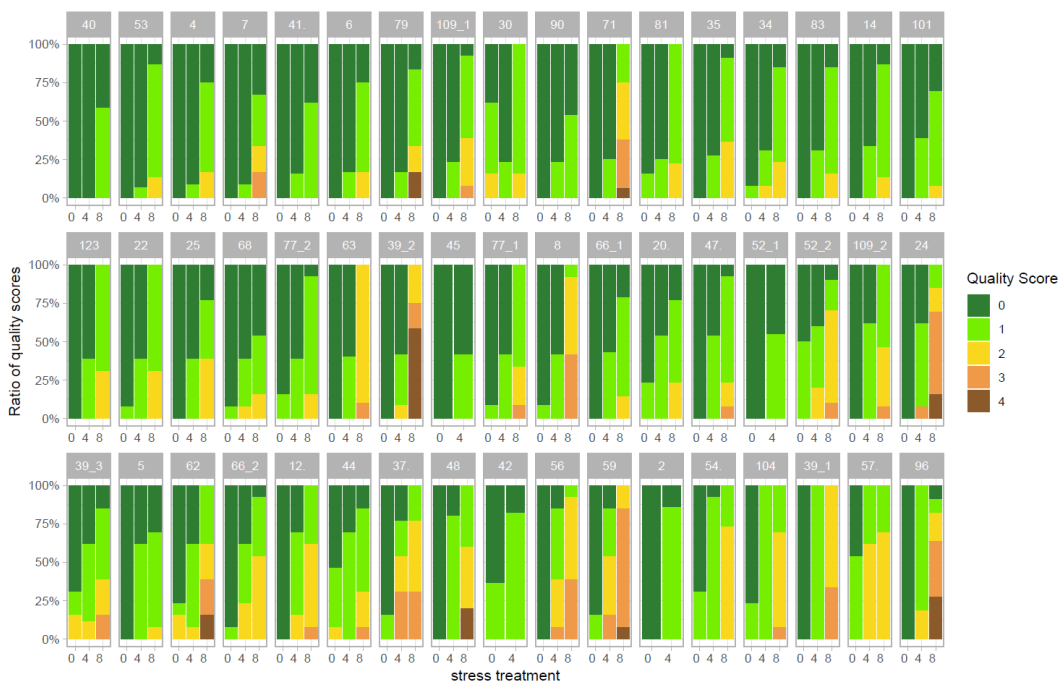


Fig. 2. Ratio of quality scores for each genotype per treatment. 0 = control, 4 = mild stress and 8 = sublethal stress. Genotypes are sorted by decreased ratio of fully vital plants after mild stress. Genotypes tested more than once are indicated by underscore.

After stress treatment, each plant was given a quality score of vitality. To visualize the effects of stress treatment, ratios of quality score occurrence were calculated and genotypes sorted by decreased ratio of fully vital plants under mild stress (figure 2.). Ratio of quality scores showed that all genotypes react to stress treatment. As expected, the control treatment contained the highest ratio of vital plants for almost all genotypes. However, not all genotypes exhibited 100% vital plants under control conditions, hinting at additional factors of culture conditions contributing to plant quality besides sorbitol content. Quality scores 3 and 4 were rare under mild stress but could be found at sublethal stress. 14 out of 17 genotypes found in the upper row also exhibited a higher tolerance for stress based on mild stress SSI, except for genotype 101, genotype 7 (European larch) and genotype 81 (omitted from SSI analysis). 11 out of 17 genotypes overlapped with leaf growth SSI and seven out of 17 overlapped with root growth SSI. Severe wilting under sublethal stress was observed in all rows. Once again it does not appear, that reaction to mild and sublethal stress are necessarily linked.

Some genotypes show improved regeneration capacity compared to quality scores. Ratio of regeneration capacity was calculated to visualize the lasting effects of stress treatment. Regeneration capacity was measured 21 days after stress treatment by visual inspection of plantlets. Over 50% of genotypes fully regenerated after mild stress (figure 3.). Sublethal stress led to loss of regeneration capacity in most genotypes. Nine genotypes showed less than 100% regeneration capacity under control conditions, indicating that culture conditions or increased handling of plantlets during the experiments could contribute to loss of regeneration capacity. For many genotypes ratio of regeneration mirrored the ratio of quality scores for example 52_2 and 56. However a few genotypes (for example 109_1 & 2, 30 and 8) showed improved regeneration capacity compared to quality scores. This difference might be informative, when exploring these results in the context of drought experiments *ex vitro* containing a rewatering phase.

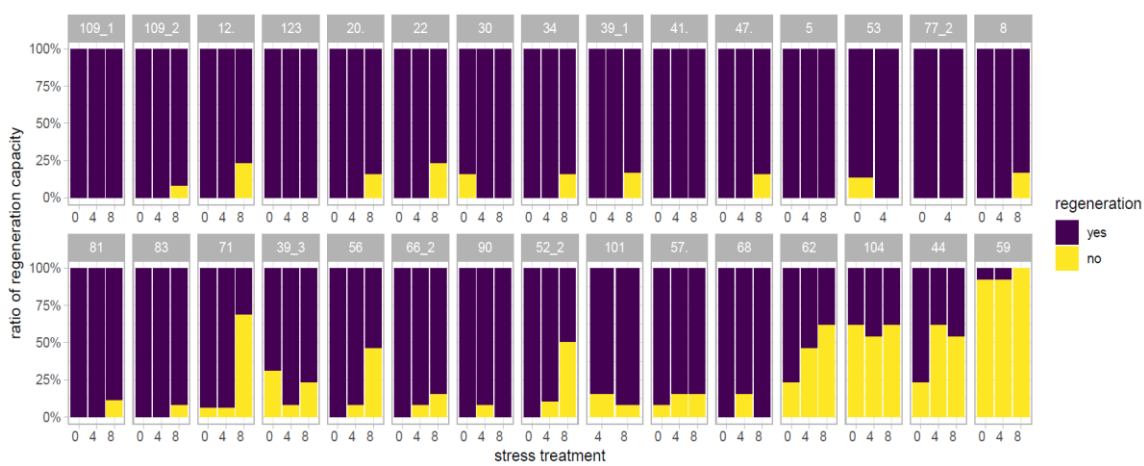


Fig. 3. Ratio of regeneration capacity for each genotype per treatment. 0 = control, 4 = mild stress and 8 = sublethal stress. Genotypes are sorted by decreased ratio of regeneration under mild stress. Many genotypes were excluded due to missing data. Genotypes tested more than once are indicated by underscore.

4. DISCUSSION

In an effort to establish a high throughput early screening test for larch genotypes multiplied via SE, we induced mild and sublethal osmotic stress during germination and ranked genotypes using SSI, quality score and regeneration capacity. Based on SSI, 15 genotypes were found more tolerant than average under mild stress conditions and 12 genotypes under sublethal stress. Overlap of these groups were 42%. We found that reaction of several genotypes to mild or sublethal stress diverged, indicating different physiological responses to the two stress intensities.

Japanese larch has been shown to reduce transpiration by reducing leaf surface area while hybrid larch might utilize sensitivity of stomata under drought stress conditions (Sasani *et al.*, 2021). As Japanese larch was shown as the most sensitive towards general drought conditions, SSI based on leaf size might be useful to indicate an improved reaction of tested genotypes compared to this parent species (Sagar *et al.*, 2025). In potatoes more tolerant genotypes increased root development in relation to shoot under osmotic stress (Bündig *et al.*, 2017). The SSI of root growth is a relative measure and does not take shoots into account, nonetheless root growth is a common response mechanism to drought and might also be beneficial regardless of overall tree size (Sagar *et al.*, 2025). The usage of SSI comes with limitations: SSI reflects only relative stress tolerance which can change depending on the group of tested genotypes. Genotypes exhibiting very low growth under control conditions are easily ranked more tolerant. When linking SSI *in vitro* to drought stress tolerance *ex vitro*, special attention should be given to genotypes with low growth under control conditions.

The quality score of vitality ensures the inclusion of a metric of stress reaction unrelated to growth parameters. Combining *in vitro* quality scores of 8 genotypes tested in earlier trials and *ex vitro* drought stress experiments has not been successful, but results are inconclusive due to the small sample size (Wolf *et al.*, 2021). However due to the low variability of quality scores for mild stress, one might question the informative value extracted after 21 days. Quality score is mainly based on appearance of leaves, which might explain the large overlap (11/17) with genotypes deemed more tolerant based on leaf growth SSI under mild stress. Anecdotally, leaves of different genotypes are varied in hue; nonetheless further work could focus on extracting quality scores bioinformatically based on color for example via Fiji. In comparison to earlier trials using 10% sorbitol for 35 days, the number of partially vital plants (0-2) under sublethal stress allows for regeneration to be added as a parameter (Benneckenstein & Tietje, 2018). Larch has been found to reduce growth for several years after drought events, thus it is beneficial to find genotypes with high regeneration capacity (Du *et al.*, 2024). Especially genotypes with improved regeneration capacity compared to quality scores should be considered when linking *in vitro* to *ex vitro* data. We found that physiological responses of several genotypes to sublethal and mild stress diverged, mirroring findings on larch tree ring data and different genotypes of almond (Du *et al.*, 2024; Yadollahi *et al.*, 2011).

Drought stress is comprised of several parameters and is often accompanied by other stressors. Temperature, humidity, duration, soil mixture and many other factors can contribute to drought stress reaction *in vivo*. This protocol only accounts for the reduction of water availability in the media. In the future, our findings need to be linked to drought stress *ex vitro* to confirm its applicability as a high throughput early screening for drought tolerance. Altogether, we were able to optimize the high throughput screening protocol for larch in several ways. We added two different stress intensities, lowered sublethal stress from 10% (higher than permanent wilting

point according to Johst & Rüdinger, 2018) to 8% sorbitol concentration and added regeneration to the measured parameters. We streamlined the process by lowering treatment time and adding analysis via Fiji. This last optimization might allow the extraction of further parameters (i.e. number of leaves, length of hypocotyl etc.) long after experiments have been completed. We found different physiological reactions of several genotypes to the two osmotic stress intensities. Furthermore, we identified genotypes with improved regeneration capacity compared to quality scores. A complete picture requires testing of genotypes for drought stress in a greenhouse setting. Nonetheless, we improved efficiency of testing *in vitro* and were able to rank the 45 tested genotypes under mild and sublethal osmotic stress conditions.

5. CONCLUSION

In this study, we present an optimized *in vitro* osmotic-stress screening procedure for hybrid larch germination. We aimed to rank the 45 tested genotypes based on stress reaction under two stress intensities. Our optimized protocol has proven promising for future approaches requiring a high efficiency screening of larger sample sizes. By including a digital step and quantifying parameters in Fiji, we could increase the number of genotypes tested *in vitro*. Furthermore, the digital archive enables the quantification of further parameters if necessary. However, our results are preliminary and need to be considered as such, especially in the context of drought stress *ex vitro*. In a next step, relevant genotypes should be tested repeatedly to confirm reproducibility and representative genotypes should be tested in a greenhouse context, to explore if the detected differences in osmotic stress tolerance can be linked to drought stress *in vivo*.

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Tiny allies and hidden threats: bacteria in woody plant micropropagation

Stefaan P. O. Werbrouck

Laboratory for Applied In vitro Plant Biotechnology, Ghent University, Ghent, Belgium

Stefaan.werbrouck@ugent.be

Abstract

Reliable micropropagation of woody plants depends not only on optimized culture media and regeneration protocols but also on the effective management of bacteria that accompany plant material into culture. Bacteria in plant tissue culture are often regarded solely as contaminants, yet their roles are more complex. Some cause visible losses, tissue necrosis, and failed propagation, whereas others persist as latent endophytes that may contribute to plant vigor, stress tolerance, and developmental stability. Particular attention is given to endogenous bacteria, including intracellular cytobacts, which challenge the assumption that fully axenic plant material is always attainable. In woody plant cloning, where recalcitrance, long production cycles, clean-stock maintenance, and commercial scalability are major concerns, understanding this hidden microbiome is essential. A shift is therefore needed from indiscriminate sterilization toward evidence-based microbial governance: detecting harmful taxa early, preventing outbreaks, applying targeted sanitation where required, and preserving plant physiological quality. Integrating molecular diagnostics, mother-plant indexing, improved asepsis, antibiotics, and Plant Preservative Mixture (PPM™) can strengthen the reliability, safety, and sustainability of micropropagation pipelines for elite woody genotypes.

Keywords: *bacterial endophytes; cytobacts; microbial contamination; clean stocks; microbial governance; sanitation; tissue culture*

1. INTRODUCTION

Micropropagation is an important tool for the rapid multiplication, conservation, sanitation, and distribution of elite plant genotypes. Its application to woody plants, however, can be particularly demanding because cultures may be recalcitrant, production cycles can be long, and valuable clones may remain *in vitro* through many successive subcultures. Under these circumstances, microbial contamination can cause direct losses and can also compromise the reproducibility and reliability of propagation systems (Herman, 2017; Leifert *et al.*, 1991).

Historically, plant tissue culture has been built around the principle of asepsis. Bacteria appearing in a culture vessel are therefore commonly interpreted as contaminants that should be eliminated. This interpretation remains justified for microorganisms introduced during handling, media preparation, equipment failure, or other breaches of aseptic procedure. However, it becomes less satisfactory when bacteria are already present inside the source plant. Plants naturally harbor diverse bacterial communities. Endophytic bacteria may occur in intercellular spaces, vascular tissues, and even within living plant cells (Hardoim *et al.*, 2015; Thomas & Franco, 2021). Their ecological roles range from beneficial symbiosis through apparently neutral associations to latent pathogenicity. Consequently, the presence of a bacterium in an *in vitro* plant does not by itself establish that the organism is harmful.

Recent syntheses likewise argue that microorganisms in tissue culture should be interpreted according to microbial identity, host context, and culture conditions rather than through a purely sterile-versus-contaminated binary (Permadi *et al.*, 2026).

This distinction is particularly relevant to woody-plant micropropagation. Valuable genotypes are often maintained for long periods, giving latent microbial populations considerable opportunity to persist unnoticed. At the same time, aggressive sanitation can injure physiologically sensitive or difficult-to-establish material. Microbial management in woody plants should therefore be approached as a problem of risk assessment and microbial governance, rather than simply as an attempt to eradicate every microorganism detected (Aasim *et al.*, 2026).

2. EXOGENOUS CONTAMINANTS AND ENDOGENOUS BACTERIA

A useful first distinction is between exogenous microbial contamination and endogenous bacterial colonization. Exogenous contaminants originate outside the explant and enter cultures through laboratory processes. Common sources include operators, inadequately sterilized instruments or media, contaminated work surfaces, defective filtration systems, and environmental air. The identity of the contaminant can sometimes provide information about its source. Skin-associated microorganisms, for example, can indicate shortcomings in operator technique, whereas environmental organisms may point toward failures of laboratory hygiene or air handling (Herman, 2017; Leifert *et al.*, 1991). These contaminants should be considered preventable. Their repeated appearance calls for investigation of aseptic procedures, sterilization processes, personnel training, equipment maintenance, and laboratory design rather than increasingly aggressive treatment of the plant material.

Endogenous bacteria are fundamentally different. They are present inside the mother plant before explants enter the tissue-culture laboratory. Endophytes may be acquired from the environment through roots, stomata, lenticels, wounds, or vectors, and some bacterial associations may also be vertically transmitted (Lengrand *et al.*, 2024; Thomas *et al.*, 2022). Once bacteria have colonized vascular or internal tissues, conventional surface disinfection cannot be expected to remove them.

This distinction has important diagnostic consequences. Repeated recovery of the same endogenous bacterium from a line maintained under otherwise satisfactory aseptic conditions does not necessarily indicate repeated failures by laboratory personnel. Conversely, recurring exogenous organisms should be regarded as indicators of deficiencies within the production process. Microbial quality assurance should therefore ask not simply, "Is a bacterium present?" but rather, "Where did it come from, where is it located, and what does its presence mean for this particular plant genotype and propagation system?"

3. ENDOPHYTES: HIDDEN TENANTS OF *IN VITRO* PLANTS

Endophytic bacteria form a continuum of ecological interactions. Some benefit their hosts, some appear largely neutral, and others can behave as opportunistic or latent pathogens depending on environmental and physiological conditions (Hardoim *et al.*, 2015). This helps explain a familiar problem in plant micropropagation: cultures may remain apparently healthy through numerous subcultures before bacterial growth suddenly appears in the

medium. Such an event does not necessarily mean that contamination occurred during the most recent transfer. Instead, bacteria already established within plant tissues may have persisted at low or spatially confined populations before a change in host physiology or culture conditions permitted their proliferation. The concept of balanced antagonism is useful for interpreting these associations. Schulz and Boyle (2005) proposed that asymptomatic endophytic colonization can reflect a dynamic equilibrium between microbial virulence and host defense. As long as neither partner gains sufficient advantage, the association remains symptomless. When the physiological balance changes, however, a formerly inconspicuous endophyte may proliferate.

In vitro plants already experience artificial conditions, including high carbohydrate availability, restricted gas exchange, altered irradiance, repeated wounding, and periodic nutrient depletion. Additional stress associated with prolonged culture, cutting, transport, temperature fluctuations, or poor physiological condition can further destabilize the plant–microbe equilibrium. Under such conditions, latent bacteria may multiply, leave internal plant tissues, colonize the nutrient medium, and eventually interfere with shoot development or cause tissue necrosis (Schulz & Boyle, 2005). This phenomenon has particular relevance for commercial woody-plant propagation. A bacterial population may remain undetected while a clone is multiplied through many generations, only becoming visible after the material has been distributed across a large number of vessels. Preventing physiological deterioration is therefore not merely a matter of improving plant growth; it may also reduce the likelihood that latent endophytes develop into destructive microbial outbreaks. A direct woody-plant example is provided by *Staphylea pinnata*, in which latent endophytic bacteria became problematic only after prolonged *in vitro* maintenance, with bacterial manifestation reported after about two years of culture (Szewczyk-Taranek *et al.*, 2020).

4. CYTOBACTS AND THE LIMITS OF AXENICITY

The conventional definition of an axenic plant becomes still more complicated in light of evidence for bacteria residing inside living plant cells. Microscopy and molecular investigations have demonstrated diverse bacterial populations within the cytoplasm of apparently healthy plant cells. Thomas and Franco (2021) described abundant intracellular bacteria in several plant systems, while Thomas *et al*. (2022) termed these intracellular bacteria cytobacts and provided evidence that such organisms can occur in embryonic tissues and may be vertically transmitted. Earlier observations had already revealed intracellular bacterial populations in *in vitro* cultures considered to be axenic. Esposito-Polesi *et al.* (2017), for example, detected bacteria including *Bacillus*, *Acinetobacter*, and *Methylobacterium* in supposedly axenic cultures of pineapple and orchids.

Intracellular localization has major implications for sanitation. A bacterium present inside a living plant cell cannot be reached by a surface disinfectant without crossing the same biological barriers that protect the plant cell. Similarly, the efficacy of antibiotics or physical treatments against free-living bacteria cannot automatically be extrapolated to bacteria residing within plant tissues or cells.

The occurrence of cytobacts therefore challenges the assumption that increasingly severe treatment will inevitably produce a completely bacteria-free plant. In some plant systems, absolute axenicity may be biologically unrealistic (Thomas & Franco, 2021; Thomas *et al.*,

2022). For woody plants maintained for prolonged periods *in vitro*, the more useful operational distinction may therefore be between a culture that is microbiologically stable and phytosanitarily safe and one containing bacteria capable of proliferating, spreading, causing disease, or compromising certification.

5. BACTERIA AS POTENTIAL ALLIES

The persistence of bacteria within healthy plant tissues raises another important question: are all endogenous bacteria undesirable? Endophytes can influence nutrient acquisition, plant growth, stress tolerance, and physiological homeostasis (Cantabella *et al.,* 2026; Compant *et al.,* 2019; Hardoim *et al.,* 2015). Their effects are therefore potentially relevant in tissue culture, where plants develop in highly artificial environments and depend on carefully regulated nutrient, hormonal, and environmental conditions. Esposito-Polesi *et al.* (2017) found abundant bacterial communities in supposedly axenic *in vitro* plants that had remained healthy over extended periods. Such findings suggest that the simple coexistence of an endophyte and an *in vitro* plant is not necessarily detrimental. Recent experimental evidence from an axenic *in vitro* model provides direct support for this view: inoculation of *Origanum heracleoticum* plantlets with the endophyte *Bacillus safensis* OHL2 increased photosynthetic rate, leaf dry biomass, average leaf area, and chlorophyll content (Semenzato *et al.,* 2026).

Repeated micropropagation could even act as an unintended selection process. Plant–microbe combinations that remain compatible under *in vitro* conditions may persist through repeated subcultures, while less stable combinations disappear when either the plant or the associated microorganism fails to survive. This raises the possibility that some differences in vigor among tissue-culture lines may be influenced not only by genotype and culture conditions but also by differences in their associated microbiota (Compant *et al.,* 2019; Hardoim *et al.,* 2015).

Indiscriminate elimination of endogenous microorganisms may therefore have unintended consequences. Removal of functionally useful bacteria could theoretically reduce host resilience or alter physiological stability. This does not mean that endophytes should automatically be retained. Some bacteria can shift toward pathogenic behavior, and propagation systems must remain compliant with phytosanitary and certification requirements. Rather, the evidence argues against treating every bacterium as biologically equivalent. The objective should be to discriminate between harmful, potentially harmful, neutral, and potentially beneficial organisms whenever the available diagnostic information permits such a distinction.

6. DETECT BEFORE TREATING

Visual observation remains the simplest form of contamination monitoring, but it cannot reliably identify latent internal bacteria. Conventional plating extends detection capacity but is limited to microorganisms capable of growing under the chosen laboratory conditions. Indexing of mother plants and *in vitro* lines therefore provides an important preventive strategy. A sequential approach combining visual examination, medium indexing, and tissue indexing can reveal covert bacteria before they become obvious in routine propagation.

Using bacteriological media differing in nutrient concentration and other growth conditions can further increase the range of cultivable organisms recovered (Hamill & Rames, 2018). This limitation also applies after sanitation: visually clean material should be indexed before it is considered microbiologically clean, as demonstrated for PPM™-treated walnut shoots (Kushnarenko *et al.,* 2022). Molecular techniques substantially expand this diagnostic capacity. Bacterial identification using 16S rRNA sequencing can provide taxonomic information on recovered isolates, whereas PCR and quantitative PCR can be used for targeted detection. Next-generation sequencing can reveal broader microbial communities, including low-abundance and difficult-to-culture organisms that conventional plating may miss (Nafea *et al.,* 2024).

For commercial woody-plant production, these methods need not all be applied routinely to every vessel. A hierarchical system is more realistic. Routine visual inspection and indexing can be used for large-scale screening, while molecular identification can be reserved for valuable stock plants, recurrent contamination, unexplained decline, certification-critical material, or bacterial populations that repeatedly survive sanitation. Most importantly, diagnosis should precede antimicrobial treatment whenever practicable. The use of a broad-spectrum antimicrobial against an unidentified organism may suppress visible growth without solving the underlying problem and may expose valuable plant material to unnecessary phytotoxicity. In *Bambusa balcooa*, antibiotic surface treatment failed to prevent endophytic contamination during multiplication, whereas subsequent isolation, 16S rDNA identification, and susceptibility testing enabled a more targeted treatment strategy (Sinha Ray *et al.,* 2017).

7. PREVENTION AS THE FIRST LINE OF DEFENSE

Recognition of endogenous microbiomes does not diminish the importance of aseptic technique. Exogenous contamination remains avoidable and can rapidly destroy cultures because nutrient-rich plant media also support microbial proliferation. Laboratory design should therefore support separation of clean and less-clean operations, while laminar airflow equipment, appropriate filtration, validated sterilization procedures, and regular surface sanitation remain basic components of contamination prevention (George *et al.,* 2008). Personnel training is equally important because each transfer introduces opportunities for microorganisms to enter the culture. In addition to mastering good aseptic technique, personnel should be trained to recognize subtle indicators of contamination or equipment malfunction before and during routine operations. These may include unusual turbidity, discoloration, condensation patterns, microbial growth, or other visual changes in cultures, as well as unexpected odors that may indicate microbial activity. Operators should also develop the habit of checking critical equipment before starting work, including the laminar-flow cabinet, airflow, sterilization equipment, and the cleanliness and integrity of tools and work surfaces. Appropriate protective clothing, controlled movement, sterile instruments, and properly maintained workspaces further reduce the risk of introducing contaminants (George *et al.,* 2008; Herman, 2017). Such attention to detail should be regarded as part of good microbiological practice rather than merely as compliance with an aseptic protocol. In woody plants, mother-stock management deserves particular emphasis. Field-grown woody plants are continually exposed to soil microorganisms, aerosols, insects, wounds, and other potential entry routes for endophytes. Establishing protected and indexed

mother stocks can therefore reduce the microbial burden introduced into the laboratory. Once clean, vigorous material has been established, maintaining high-quality mother plants under controlled conditions is generally preferable to repeatedly initiating cultures from uncontrolled field material. Effective prevention is consequently multilayered: it includes mother-plant health, explant preparation, facility design, equipment maintenance, personnel behavior, vessel integrity, and routine monitoring.

8. ANTIBIOTICS: PRECISION TOOLS RATHER THAN UNIVERSAL REMEDIES

Antibiotics remain valuable when problematic bacteria must be removed from irreplaceable germplasm. Their use, however, illustrates the fundamental compromise between antimicrobial efficacy and plant phytotoxicity. Identifying the bacterial organism before treatment allows antimicrobial selection to be based on susceptibility rather than trial and error. In *Prunus yedoensis*, *Pantoea* and *Curtobacterium* isolates were controlled using cefotaxime and tetracycline, although the effects of the compounds on shoot development differed (Cheong *et al.*, 2020). The therapeutic window can be particularly narrow. Liang *et al.* (2019) found that low tetracycline concentrations were effective for bacterial control in cherry rootstock 'Gisela 6', whereas substantially higher concentrations induced chlorosis and inhibited differentiation. These results demonstrate why antimicrobial efficacy measured against an isolated bacterium cannot be considered independently of the response of the plant genotype.

Comparable problems occur in other woody plants. In *Eucalyptus cloeziana*, Timentin reduced bacterial migration into the culture medium and was associated with increased plant dry mass and altered nutrient uptake (Leone *et al.*, 2019). Antibiotics can also directly influence plant morphogenesis. Although studied in the context of *Agrobacterium*-mediated transformation, Tambarussi et al. (2015) showed that the response of teak (*Tectona grandis*) depended strongly on antibiotic type, concentration, and explant type, with Timentin and cefotaxime at 100–300 mg L^{-1} better tolerated than higher concentrations or carbenicillin. Comparable genotype- and treatment-dependent responses were observed in mature citrus tissues, where cefotaxime could support shoot regeneration while helping to suppress bacterial growth, although none of the tested beta-lactams completely eliminated contamination (Oliveira *et al.*, 2010). The response may also change between stages of propagation. In loblolly pine (*Pinus taeda*), ampicillin, carbenicillin, and cefotaxime enhanced callus formation and shoot regeneration but reduced subsequent rooting (Tang *et al.*, 2003). Moreover, increased regeneration does not necessarily indicate improved plantlet quality. In hybrid aspens (*Populus* spp.), cefotaxime promoted shoot development but also increased hyperhydricity (Bosela, 2009).

Together, these studies show that antibiotics in woody-plant cultures are not biologically neutral. Their effects depend on the antimicrobial compound and dose, plant species or genotype, explant type, culture stage, and the developmental trait being evaluated. Similar variability is reflected in hazelnut, where no single antibiotic removed all internal bacterial contaminants and combinations were required for acceptable control (Reed *et al.*, 1998), and in broader woody shoot cultures, where susceptibility varied among bacterial isolates and plant genotypes (Young *et al.*, 1984). Antibiotic selection should therefore consider not

only bacterial suppression and shoot multiplication but also plantlet quality, rooting capacity, and subsequent recovery.

There is therefore no universal "best antibiotic" for woody-plant tissue culture. Treatment should ideally involve identification of the bacterium, antimicrobial-susceptibility testing, determination of the lowest effective dose, assessment of plant toxicity and morphogenic effects, and confirmation that the organism does not reappear after treatment. Short or pulse-style treatments followed by transfer to antibiotic-free medium may also be preferable to prolonged exposure where the plant system permits this approach. Antibiotics should therefore be regarded primarily as **targeted rescue tools for valuable germplasm**, not as routine substitutes for good microbiological management. This diagnostic logic has also been demonstrated in *Staphylea pinnata*, where identification and susceptibility testing preceded in-culture antibiotic treatment; gentamicin at 100 mg L^{-1} provided the best balance between bacterial suppression and shoot survival, while higher concentrations increased phytotoxicity (Szewczyk-Taranek *et al.,* 2020).

9. PLANT PRESERVATIVE MIXTURE (PPM™)

Plant Preservative Mixture (PPM™) is widely used as a broad-spectrum antimicrobial alternative or supplement to conventional antibiotics in plant tissue culture. Its practical advantages include activity against bacteria and fungi and the possibility of incorporating it directly into culture media. Evidence from woody plants demonstrates both its usefulness and the need for careful optimization. Romadanova *et al.* (2022) reported control of endophytic bacteria in *in vitro* apple shoots. In walnut, a short PPM™ pretreatment followed by culture on PPM™-containing initiation medium substantially reduced contamination and increased the recovery of viable shoots, whereas prolonged exposure caused severe tissue injury (Kushnarenko *et al.,* 2022).

These findings also show why treatment success should not be judged by appearance alone. In walnut, 12.5% of shoots considered visually clean after PPM™ treatment still tested positive for bacteria when indexed on 523 detection medium (Kushnarenko *et al.*, 2022). More recent studies in blackberry showed that three subcultures on medium containing 0.2% PPM™ followed by transfer to PPM™-free medium produced shoots in which bacterial contamination was no longer detected during extended follow-up (Tolegen *et al.,* 2025). The authors nevertheless cautioned that efficacy may depend on genotype and on the nature of the contamination. As with antibiotics, PPM™ should therefore be treated as a biologically active management tool rather than a universal sterilant: concentration and exposure time should be optimized for each plant system, and treatment success should preferably be confirmed by indexing after the biocide has been withdrawn (George & Tripepi, 2001; Kushnarenko *et al.,* 2022; Tolegen *et al.,* 2025).

10. FROM STERILIZATION TO MICROBIAL GOVERNANCE

The evidence reviewed above supports a conceptual change in the management of bacteria in woody plant micropropagation. The traditional model is essentially binary: a culture is either sterile and acceptable or contaminated and unacceptable. This framework works reasonably well for obvious exogenous contamination but is insufficient for complex

endogenous microbiomes and intracellular bacteria. A microbial-governance model asks a more informative series of questions. Is the organism exogenous or endogenous? Is it increasing in abundance? Is it associated with plant decline? Is it a known pathogen? Can it be detected repeatedly in healthy material? Does treatment improve plant performance or merely remove a detectable organism? What is the phytotoxic cost of treatment? Microbial risk is also context dependent: strain identity, inoculum density, explant type and physiological state, medium composition, and culture stage can all influence whether a microorganism behaves as a compatible partner or a disruptive contaminant (Permadi *et al.*, 2026).

On this basis, at least four operational categories can be distinguished:

1. **Preventable exogenous contaminants** should trigger correction of aseptic procedures, equipment, laboratory hygiene, or operator practices (Herman, 2017; Leifert *et al.*, 1991).
2. **Known pathogens or aggressive endogenous bacteria** require exclusion, sanitation, or destruction of affected material according to the relevant phytosanitary requirements.
3. **Latent bacteria associated with declining cultures** should be identified and selectively controlled while minimizing damage to valuable plant material.
4. **Stable asymptomatic endophytes without demonstrated phytosanitary risk** warrant characterization and monitoring rather than automatic use of increasingly aggressive eradication treatments.

This approach is particularly appropriate for elite woody genotypes because the plant material itself may be irreplaceable. Destruction or phytotoxic sanitation treatment of a unique clone can represent a greater loss than the presence of a stable, nonpathogenic bacterial population. Microbial governance therefore does not mean relaxing hygiene. It means combining rigorous exclusion of preventable contaminants with better discrimination among endogenous microorganisms.

11. IMPLICATIONS FOR COMMERCIAL WOODY-PLANT PROPAGATION

Microbial governance has direct practical consequences for commercial laboratories.

First, systematic indexing can reduce the risk of multiplying problematic mother plants into large production populations. Once an internal bacterial problem has been propagated through thousands of shoots, remediation becomes considerably more expensive than early detection. Second, molecular diagnosis can reduce empirical antimicrobial use. Identifying the bacterial population enables a laboratory to decide whether treatment is required and, when it is, to choose a more rational intervention. Third, maintaining plant physiological quality should be considered part of contamination management. If the expression of latent bacteria depends partly on host stress and deterioration, then culture conditions that maintain vigorous plant growth also contribute indirectly to microbiological stability (Hardoim *et al.*, 2015; Schulz & Boyle, 2005). Fourth, targeted sanitation can preserve genotypes that would otherwise be discarded. This is particularly important for woody plants derived from breeding programs, mature elite selections, conservation collections, or unique germplasm. This approach also changes how culture quality is interpreted. A

visually clean culture at a single observation point is not necessarily microbiologically stable, while detection of bacterial DNA does not automatically demonstrate that a culture is unsuitable and has to be destroyed.

12. FUTURE PERSPECTIVES

One of the most interesting future directions is the transition from defensive microbial control toward deliberate management of beneficial plant-associated microorganisms. Plant microbiome research increasingly recognizes that microbial communities contribute to plant growth and resilience (Compant *et al.*, 2019; Hardoim *et al.*, 2015). If specific bacterial strains can reproducibly improve rooting, stress tolerance, acclimatization, nutrient acquisition, or developmental stability in woody micropropagation, controlled inoculation could eventually become an additional component of propagation technology. The feasibility of such targeted inoculation is supported by axenic *in vitro* systems in which defined endophytes can be introduced, tracked across host tissues, and evaluated for physiological effects (Semenzato *et al.*, 2026).

Such an approach will require stringent safeguards. Candidate bacteria must be identified accurately, their effects must be reproducible, and their pathogenic and phytosanitary risks must be assessed. Their behavior during long-term subculture and after transfer to *ex vitro* conditions will also require investigation. Improved sequencing and quantitative diagnostics will facilitate this transition. Instead of merely asking whether bacteria are present, laboratories will increasingly be able to characterize microbial composition, abundance, localization, and dynamics through successive propagation stages (Nafea *et al.*, 2024). Accordingly, deliberate inoculation should be preceded by baseline microbiome assessment and controlled for strain, inoculum density, and timing, because beneficial behavior may not remain stable under *in vitro* conditions (Permadi *et al.*, 2026).

The occurrence of cytobacts adds another dimension. If intracellular bacterial populations are common and vertically transmitted, the plant microbiome may represent a more persistent biological component of elite genotypes than previously recognized (Thomas & Franco, 2021; Thomas *et al.*, 2022). For long-lived woody plants, understanding this relationship could help explain why apparently similar tissue-culture lines may show large variation in vigor, stability, rooting, acclimatization, and susceptibility to microbial outbreaks.

13. CONCLUSION

Bacteria in woody plant micropropagation are both hidden threats and potential allies. Exogenous bacteria remain important indicators of failures in aseptic practice and should be rigorously prevented. Endogenous bacteria require a more nuanced interpretation because they may persist latently in vascular, intercellular, or intracellular locations and may escape conventional surface sterilization (Hardoim *et al.*, 2015; Thomas & Franco, 2021). Latent endophytes can become problematic when disruption of the host–microbe equilibrium allows bacterial proliferation. At the same time, some endophytes may contribute to plant vigor, physiological stability, and stress tolerance (Compant *et al.*, 2019; Schulz & Boyle, 2005). The recognition of intracellular cytobacts further challenges the assumption that

absolute axenicity is always attainable (Thomas *et al.,* 2022). Consequently, successful microbial management should not be defined exclusively as the complete elimination of all detectable bacteria. For elite woody genotypes, a more resilient strategy combines healthy and indexed mother stocks, rigorous asepsis, early microbial detection, molecular identification, maintenance of plant physiological quality, and targeted use of antibiotics, PPM™, or other antimicrobial treatments when justified. The future of reliable woody-plant micropropagation therefore lies in evidence-based microbial governance: preventing avoidable contamination, identifying microbial risks accurately, intervening selectively, and preserving the physiological quality of the plant material while meeting phytosanitary requirements.

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From virome to clean planting stock, cost-effective ngs-guided sanitation for *in vitro* propagation of heritage grapevine

Sebastián Gómez Talquenca[1], Humberto Debat[2], Raul Cuello[3], Diego Zavallo[3], Rocio Torres[1], Sebastian Asurmendi[3]

1Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (EEA-Mendoza-INTA), Mendoza, Argentina

2Instituto de Patología Vegetal, Centro de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria (IPAVE-CIAP-INTA), Córdoba, Argentina

3Instituto de Agrobiotecnología y Biología Molecular (IABIMO), CICVyA. Instituto Nacional de Tecnología Agropecuaria (INTA), Consejo Nacional de investigaciones Científicas y Técnicas (CONICET), Los Reseros y Nicolas Repetto, Hurlingham, Buenos Aires, Argentina

gomez.talquenca@inta.gob.ar

Abstract

We characterized, by total RNA sequencing, the virome of grapevine germplasm conserved at INTA Mendoza, Argentina (five individual plants and two pools of accessions), quantified each agent against a curated reference that combined public genomes, locally assembled genomes and a grapevine genome decoy, and applied explicit detection criteria. One sample carried no virus, two carried one and the others three to nine, with abundances from 15 to about 51,000 reads per million (RPM). Viroids were present in every sample. Divergent local isolates of grapevine rupestris stem pitting-associated virus (GRSPaV) and grapevine leafroll-associated virus 2 were quantified through their own genomes. Of 720 meristem tips, 394 regenerated into lines. Lines were screened by RT-qPCR until at least two per sample tested negative for every virus of the mother plant; 74 lines were screened and 15 passed. The proportion of screened lines negative for a given virus ranged from over 90% for leafroll-associated viruses to 8% for grapevine virus A, and screening effort increased with the number of viruses and with the presence of hard-to-eliminate ones. GRSPaV, which resisted meristem culture, is not regulated in Argentina and has been proposed for exclusion from regulation internationally. In a cost model based on a commercial quotation, pooling ten plants at equal depth per plant would lower library and sequencing cost from USD 236 to 56 per plant. A curated virome inventory before sanitation identifies the viruses likely to limit it and allows effort to be anticipated.

Keywords: *grapevine virome, high-throughput sequencing, meristem culture, sanitation, clean stock certification, Criolla varieties*

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is propagated vegetatively, so any virus present in a mother plant is carried, and often accumulated, through every cycle of cuttings and grafts. More than 100 viral species have now been reported in grapevine (Fuchs, 2025). Sanitary selection first relied on biological indexing on woody indicators, which takes three to five years of field observation to release a single plant and, for many diseases, was carried out without an identified causal agent. High-throughput sequencing (HTS) has since moved, in the United States, from research into quarantine and certification diagnostics at clean plant

centers (Soltani *et al.,* 2021; Al Rwahnih *et al.,* 2025), and the interpretive and regulatory gaps that remain have recently been reviewed (Debat *et al.,* 2026).

The shift from diagnosing diseases to diagnosing pathogens has also changed what is considered worth regulating. Virologists have asked that grapevine viruses and viroids without demonstrated economic impact be left out of regulatory oversight (Fuchs *et al.,* 2025), and that agents named in regulations but never characterized be removed from them (Tzanetakis *et al.,* 2024). Argentina certifies against viruses that have economic impact and known natural spread. In practice, this turns the certification panel into a closed list of seven viruses (grapevine fanleaf virus, GFLV; grapevine virus A, GVA; grapevine leafroll-associated viruses 1, 2, 3 and 4, GLRaV-1 to -4; and grapevine fleck virus, GFkV; INASE, 2018), while the testing methods are set by a separate instrument (INASE, 2023).

Local heritage germplasm, including Criolla varieties and old clones of Torrontés Riojano, adds a further constraint: sanitation of a clone selected for its phenotype must not alter that phenotype. For this reason, meristem culture without heat therapy was chosen as the sanitation route in the project that frames this work.

Our premise is that sanitation should be preceded by a complete characterization of what each mother plant carries. Here we describe the virome of seven samples (five individual plants and two pools of accessions), follow 720 meristem tips through to virus-tested lines, and model the cost of the sequencing that supports the strategy. The study is a case series on a small, heterogeneous set of samples; it aims to show how inventory data can inform sanitation planning, not to estimate prevalence or general elimination rates.

2. MATERIALS AND METHODS

2.1. Materials

Seven samples were analyzed: five individual plants, Malbec clone 18 (M1), Criolla Chica (171), Chardonnay (CH40), the rootstock Paulsen 1103 (P1103) and Torrontés Riojano, and two pools of accessions, M50 (candidate clones of Syrah, two accessions, and Bonarda, four accessions) and M150 (five Criolla Chica accessions). Five libraries, M1, CH40, 171, P1103 and the Ribo-Zero library of Torrontés Riojano (TorrRZ), were generated in a study of host RNA depletion in which M1 is designated MB21 (Cuello *et al.,* 2026). Total RNA was extracted from cambial scrapings (RNAprep Pure Plant Plus Kit, Tiangen Biotech, China) and checked by NanoDrop, agarose gel and Agilent 2100 Bioanalyzer; degraded samples were discarded. rRNA was depleted with the Ribo-Zero Magnetic Kit (Illumina) and libraries were sequenced paired-end on an Illumina platform. M50 and M150 were processed in the same way, with RNA extracted and quality-controlled (QC) plant by plant before pooling. Libraries were sequenced in three lanes: M1, CH40, 171 and P1103 in one, M50 and M150 in a second and TorrRZ in a third. Individual plants were sequenced at 104 to 128 M read pairs (P1103, 104 M; M1, 113.5 M; CH40, 126 M; 171, 128 M), except Torrontés Riojano (30.1 M), and the pools M50 and M150 at 37 M and 56 M read pairs, respectively. About 90% of the reads of the individual libraries (Cuello *et al.,* 2026) and 82% of those of the pools mapped to the grapevine genome. Except for TorrRZ, which was analyzed completely (58.2 M reads after trimming), read pairs aligning to the grapevine genome were removed with Bowtie2 before virus analysis, leaving 13.1 to 19.9 M reads after trimming. Raw reads and local genomes will be deposited in SRA and GenBank.

2.2. Methods

2.2.1. Assembly, virus identification and curation

Reads were trimmed with Trimmomatic v0.39 (adapter removal, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:36; Bolger *et al.*, 2014) and each library was assembled de novo with SPAdes in rnaviral mode (Bankevich *et al.*, 2012; Meleshko *et al.*, 2022) in Galaxy (usegalaxy.eu; The Galaxy Community, 2024). Scaffolds were annotated by megablast against a database of grapevine virus genomes and by BLASTX against plant viral proteins (BLAST+; Camacho *et al.*, 2009; E-value ≤ 1e-5). A hit was accepted only when the alignment covered at least 30% of the scaffold; when a scaffold matched several taxa, identity was assigned to the hit with the largest coverage; and alignments of identical length and position recurring across libraries were inspected as possible host artifacts. Every local scaffold later used as a mapping reference was checked by BLASTN against the NCBI nucleotide collection, and scaffolds whose best hit was a grapevine sequence were excluded.

2.2.2. Reference-based quantification

Abundance was estimated by mapping each library with Bowtie2 v2.5.5 (--very-sensitive-local; Langmead and Salzberg, 2012) to a single reference that contained (i) the grapevine PN40024 genome assembly with its chloroplast and mitochondrion, as a competitive decoy; (ii) 36,712 public sequences of viruses reported in grapevine, after removal of 830 records that are not viral genomes or are too short to be informative (148 protein-structure entries, 417 patent sequences and 265 sequences shorter than 150 nt); (iii) public viroid genomes together with viroid scaffolds assembled from our libraries; and (iv) 22 viral scaffolds assembled from our libraries and checked as described above. Labels of local viroid scaffolds were checked by pairwise alignment against reference genomes (section 4.3). Reads per reference were counted with samtools coverage v1.22 (Danecek *et al.*, 2021), excluding unmapped, secondary, QC-failed and duplicate alignments, and summed by agent. Abundance is reported at mapping quality 0 in reads per million reads of the complete library (RPM): mapped reads, counting both mates, divided by twice the number of sequenced read pairs, which keeps host-depleted and complete libraries comparable. Reads on references covered over less than 5% of their length were not counted, and 97 public references that collected such narrow pileups in three or more of the seven libraries, or in M1, were excluded as artifact-prone. A virus was considered detected when it had at least 500 reads, at least 50% coverage of one reference and a mean depth of at least 2×; a viroid, when it had at least 100 reads and 90% coverage. Weaker signals (at least 20 reads and 10% coverage) are reported in parentheses and not counted as detections.

2.2.3. Meristem culture

Meristem culture followed the virus elimination protocol of Foundation Plant Services (FPS), University of California, Davis (Foundation Plant Services, 2018; Sim *et al.*, 2012; Golino *et al.*, 2017), with minor modifications. Shoots were forced from dormant woody cuttings stored at 4 °C for at least 30 days and sprouted as single-node cuttings at 24 °C. Actively growing shoot tips were shaken for 15 min in water with 0.1% Tween 20 and for a further 15 min in 5% (v/v) commercial bleach (55 g/L active chlorine) with 0.1% Tween 20, then rinsed two times in sterile distilled water. Meristem tips smaller than 0.5 mm (apical dome with zero or one leaf primordium) were excised under a stereomicroscope (40×) in a laminar flow hood and placed, ten per 55 mm-Petri dish, on MSB medium: Murashige and Skoog (1962) salts

and vitamins (4.43 g/L), 30 g/L sucrose, 1 mg/L 6-benzylaminopurine (BAP, 4.4 µM) and 6.1 g/L agar, pH 5.8. One hundred meristem tips were excised from each individual plant and 20 from each accession of the pools (120 for M50 and 100 for M150), 720 in total. Cultures were kept at 24 °C under a 16 h photoperiod provided by LED grow lamps and transferred to fresh MSB every 15 days; contamination was negligible. Explants that reached 1 cm were moved individually to 25 × 200 mm culture tubes with MSB, and at 3 cm to rooting medium RM: half-strength MS salts and vitamins (2.22 g/L), 15 g/L sucrose, 1 mg/L indole-3-acetic acid (IAA, 5.7 µM) and 6.1 g/L agar, pH 5.8. Media components were from PhytoTechnology Laboratories (USA).

When a plantlet reached 6 cm, at least two cuttings were taken and kept in RM tubes until testing was completed, and the remaining tissue (collar, shoot and leaves) was used for RNA extraction. A meristem tip was counted as a regenerated line when these cuttings became established; cuttings from one meristem tip were treated as a single line. Lines that tested negative were multiplied by apical subculture on RM when plantlets reached six leaves and acclimatized in blocks of coconut-fiber substrate (Cocomix) enclosed in 50 µm polyethylene bags, which were progressively opened after one week; 70 to 90% of plants survived acclimatization.

2.2.4. Screening

Regenerated lines were screened in successive batches until at least two negative lines for every virus detected in the mother plant (or pool) had been obtained for each sample; for M1, which carried no virus, two lines were taken forward. All tissue left on each plantlet after the cuttings were taken was used for RNA extraction with the Spectrum Plant Total RNA Kit (Sigma-Aldrich, USA), and 1 µg was reverse-transcribed with random hexamers and M-MLV reverse transcriptase (Thermo Fisher Scientific, USA). Lines were tested by RT-qPCR with assays designed on the genomes reconstructed from the local isolates, with 18S rRNA as internal reference and a cut-off of Ct 38; assay design and validation will be described separately. Extreme abundances (grapevine Muscat rose virus, GMRV; GRSPaV; grapevine Pinot gris virus, GPGV) were checked by independent RT-qPCR. Grapevine satellite virus (GV-Sat) and viroids were not included in screening. Lines negative for all screened viruses will be retested after one year of growth before release.

2.2.5. Cost model

Sequencing cost was based on a quotation from Macrogen (Korea; February 2026): USD 236 per sample for a Ribo-Zero Plant total RNA library sequenced on Illumina NovaSeq X (150 bp paired-end, 40 M reads), including provider QC and excluding shipping. This price was split into a fixed cost per library of USD 200 and USD 0.90 per million reads (each mate counted). At equal depth per plant, the cost per plant in a pool of n plants is $200/n + 0.9M$, where M is millions of reads per plant (base scenario, 40 M). Local QC, extraction, bioinformatics and culture are not included.

3. RESULTS

3.1. Local germplasm carries a broad and uneven virome

Viral load differed sharply among samples (Fig. 1). M1 carried no virus, 171 and P1103 carried one each (GPGV and GRSPaV, respectively), CH40 three, the pools M50 and M150

five and eight, and Torrontés Riojano nine, counting GV-Sat. Viroids were found in every sample (hop stunt viroid, HSVd, in all seven, GYSVd-1 in six, and Australian grapevine viroid (AGVd), grapevine hammerhead viroid-like RNA (GHVd) and GYSVd-2 in one each). M1 had the highest viroid load of the set (GYSVd-1, 284 RPM; 64,412 reads) although it carried no virus; its weak GMRV and GPGV signals (0.5 and 0.3 RPM) did not meet the detection criteria, and in an independent analysis of the same library the only viral signals were 53 GPGV and 3 GRSPaV reads, neither confirmed by PCR (Cuello *et al.*, 2026). Detected viruses spanned more than three orders of magnitude, from 15 RPM (GLRaV-1 in Torrontés Riojano) to about 51,000 RPM (GFLV in Torrontés Riojano), and the same virus differed widely among samples (GPGV, 27 to 2,163 RPM; GRSPaV, 28 to 5,286 RPM). Five of the 12 viruses detected here (GMRV, GRLDaV, GRVFV, GPGV and GV-Sat) were outside the nine-virus panel of a targeted survey of Mendoza vineyards (Gomez Talquenca *et al.*, 2023). For TorrRZ, abundances of GFLV, GPGV, GRLDaV, GRSPaV and GVA were within 17% of those obtained by Cuello *et al.* (2026) with a different reference and pipeline.

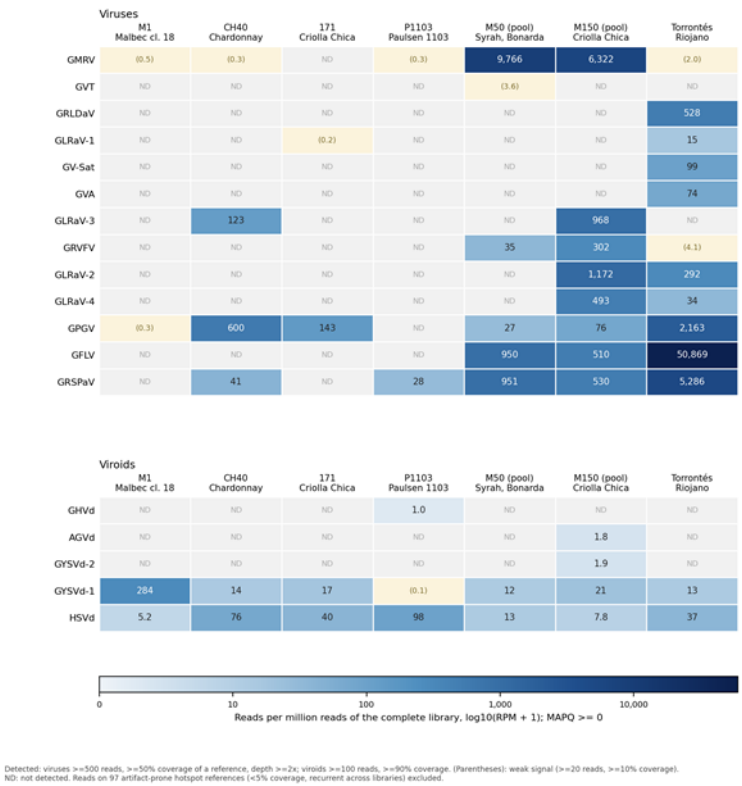


Fig. 1. Virome of seven samples. Columns are libraries; M50 and M150 are pools of accessions (M50, two Syrah and four Bonarda; M150, five Criolla Chica). Upper panel, viruses; lower panel, viroids. Values are reads per million reads of the complete library (RPM) at mapping quality 0, and color follows log10(RPM + 1). Detected: viruses with at least 500 reads, at least 50% coverage of a reference and mean depth of at least 2×; viroids with at least 100 reads and 90% coverage. Values in parentheses are weak signals (at least 20 reads and 10% coverage) not counted as detections; ND, not detected. GRLDaV, grapevine Roditis leaf discoloration-associated virus; GRVFV, grapevine rupestris vein feathering virus; other abbreviations as in the text.

Assembly recovered complete or near-complete genomes of GLRaV-3 (18.4 to 18.5 kb in CH40 and M150, 99.4% identity to the closest reference), GLRaV-2 (16.6 kb in M150, 86.0%), GPGV (about 7.2 kb in four samples, 98.2 to 98.4%) and GRSPaV (8.7 to 8.9 kb in four samples), and two partial scaffolds of GLRaV-4 of the strain 9 group (6.2 and 4.6 kb in M150, 91.5 to 94.7%). Three of the GRSPaV genomes (CH40, M50 and M150) shared only about 83% identity with the closest reference over three quarters of their length, and the P1103 genome 94.3%. These isolates were quantified mainly through their own genomes; in P1103, no public GRSPaV genome was covered over more than 34% of its length. GMRV RNA3 was assembled in M50 at more than 20,000× coverage, and four short scaffolds in M50 (0.6 to 1.2 kb) belonged to grapevine virus T (GVT; 83 to 85% nucleotide identity), a foveavirus that the initial annotation had assigned to GVA. Local genomes also differed from public ones at the primer binding sites of published assays, so the assays used for screening were designed on the local genomes (section 2.2.4).

3.2. From 720 meristem tips to virus-negative lines

The projected circuit comprises sequencing (2 months), meristem establishment (5 months), plant development and testing (3 months) and retesting before release (12 months), so tested wood would be released after 22 months. Testing by woody indexing would take at least three seasons (36 months) and up to five years, instead of the 15 months of testing and retesting planned here. The *in vitro* stages took eight months, similar to the seven months from excision to potted plant reported at FPS (Sim *et al.*, 2012).

Regeneration ranged from 50 to 58 lines per 100 meristem tips (394 of 720, 54.7%; Table 1). Because screening stopped once two virus-negative lines had been obtained, only 74 lines (19% of regenerated lines) were screened, and 15 were negative for every virus of their mother plant or pool: two per sample, except 171 (three). The proportion of lines screened is therefore a consequence of this stopping rule, not a property of the material.

3.3. Meristem culture does not treat all viruses equally

The proportion of screened lines negative for each virus (Fig. 4B) was above 90% for GLRaV-3 (19/20), GLRaV-4 (32/35), GLRaV-1 (25/26), GLRaV-2 (32/35), GFLV (44/47) and GRVFV (20/21); intermediate for GRLDaV (16/26) and GMRV (7/21), and lowest for GPGV (16/63, 25%), GRSPaV (15/67, 22%) and GVA (2/26, 8%). Wilson 95% confidence intervals were wide (GVA, 2 to 24%; GRSPaV, 14 to 34%; GPGV, 16 to 37%) and overlapped among the three hardest viruses. In the pools, the infection status of each accession is unknown, so a negative line may come from an accession that never carried the virus; for viruses found only in pools (GMRV, GRVFV) the proportion cannot be read as an elimination rate (section 4.4).

At sample level, screening effort increased with the number of viruses present (Spearman $\rho = 0.83$, $n = 7$; Table 1, Fig. 4A), but composition also mattered. Torrontés Riojano, with GVA among its eight screened viruses, needed 26 lines, whereas M150, with eight viruses but no GVA, needed nine, fewer than CH40 with three viruses (11 lines). With seven samples and a stopping rule, these data cannot separate the effect of the number of viruses from that of particular viruses.

Table 1. Viruses in the inventory, limiting virus and screening effort per sample. The limiting virus is the one with the lowest proportion of negative lines; values in parentheses are negative lines over lines screened. One hundred meristem tips were excised per sample except M50 (120). GV-Sat was not screened.

Sample	Viruses in inventory	Limiting virus (negative / screened)	Lines regenerated	Lines screened	Negative for all
M1 (Malbec clone 18)	0	none	57	2	2
171 (Criolla Chica)	1	GPGV (3/5)	57	5	3
P1103 (Paulsen 1103)	1	GRSPaV (2/9)	58	9	2
M150 (pool, Criolla Chica)	8	GRSPaV (2/9)	53	9	2
CH40 (Chardonnay)	3	GPGV (2/11)	58	11	2
M50 (pool, Syrah and Bonarda)	5	GRSPaV (3/12)	60	12	2
Torrontés Riojano	9 (8 screened)	GVA (2/26)	51	26	2
Total			394	74	15

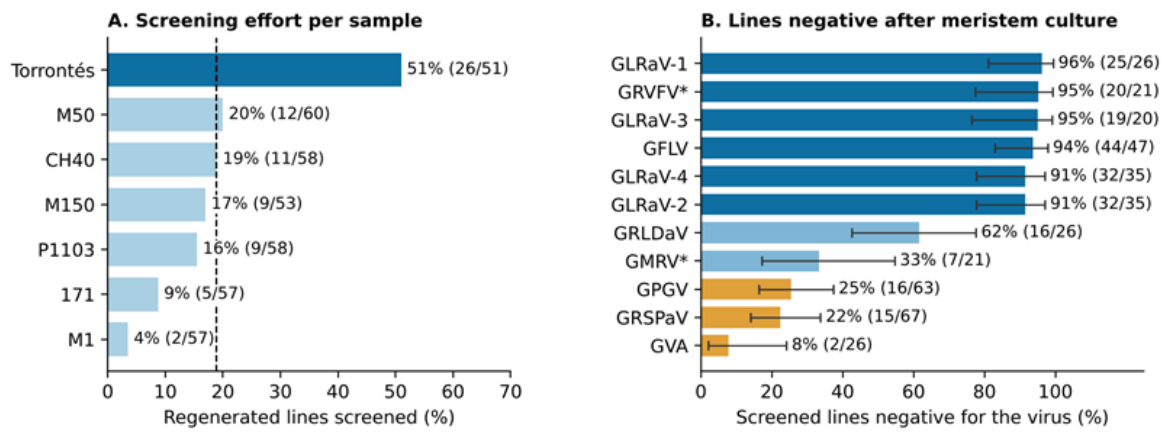


Fig. 4. (A) Lines screened as a percentage of regenerated lines per sample (screened/regenerated in parentheses); dashed line, overall 19% (74/394). Screening stopped once two lines negative for all viruses had been obtained. (B) Proportion of screened lines negative for each virus, pooled across samples (negative/screened in parentheses), with Wilson 95% confidence intervals; orange bars, below 30%. Viruses marked with an asterisk were found only in pools, so their values include lines from accessions of unknown infection status.

3.4. What is amortized is the library, not the sequencing

At the quoted depth of 40 M reads, library preparation represents 85% of the cost of a single sample (USD 200 of 236). Pooling at equal depth per plant lowers the cost to USD 76, 56 and 46 per plant for pools of 5, 10 and 20 plants, approaching an asymptote of USD 36 (sequencing alone).

The saving depends on depth per plant (Table 2). At 10 M reads per plant, below the 12 and 22 M reads per plant of the pools analyzed here, library plus sequencing falls from USD 209 for a single plant to 29 in a pool of ten. At 100 M reads per plant, sequencing becomes the main cost and the reduction is smaller (USD 290 to 110). Depth and sensitivity are linked: at 10 M reads per plant, a virus at 15 RPM, the lowest abundance detected here, it would yield about 150 reads, below the 500 reads required for detection, whereas at the quoted 40 M reads it would yield about 600.

Table 2. Sensitivity of library plus sequencing cost per plant (USD) to pool size and depth per plant. Cost = 200/n + 0.9 × M, with M in millions of reads per plant; local QC per plant is not included.

Reads per plant	1 plant	5 plants	10 plants	20 plants
10 M	209	49	29	19
40 M	236	76	56	46
100 M	290	130	110	100

4. DISCUSSION

4.1. Characterizing before sanitizing

A complete virome inventory tells which viruses a mother plant carries before the first meristem tip is cut, and therefore which of them are likely to limit sanitation. Combined with local elimination data, the inventory gives a first estimate of effort that can be refined as lines are tested. A pool of ten plants reduces the cost per plant about fourfold, which brings inventories of whole collections within reach of a public institution.

Pooling has a cost in sensitivity. A virus present in one plant of a pool of *n* is diluted about *n*-fold in relative abundance when total depth is fixed. GLRaV-1 in Torrontés Riojano (15 RPM, 931 reads in 60 M reads) would be represented by about 90 reads in a ten-plant pool sequenced at the same total depth, below the detection criterion. Keeping depth per plant constant preserves read counts at the price of a larger run. Pools also leave the status of each accession unknown, so pooled mother plants should be tested individually by RT-qPCR before sanitation; this was not done here for M50 and M150.

4.2. Regulatory status: two different questions

Two statuses should be kept apart: Some agents are *not regulated in Argentina*, meaning that they are absent from the panel of Res. 199/18 (INASE, 2018). Others have been *proposed for exclusion internationally* by a group of virologists (Fuchs *et al.*, 2025). Only the first has legal effect here. The regulatory matrix (Fig. 6) shows mismatches in both

directions: GFkV is required by Res. 199/18 but proposed for exclusion, whereas GRSPaV (five samples) and grapevine rupestris vein feathering virus (GRVFV, two samples) are not regulated in Argentina and are on the exclusion list. The signal first assigned to GVA in M50 belonged to GVT, which is not regulated, an example of how a misassignment between related viruses can create a regulatory finding where there is none. GPGV is on neither list: latent infections with no direct impact on production have been documented (Messmer *et al.,* 2024), while symptomatic infections are reported elsewhere, so its relevance depends on the variant present and not simply on detection.

Our data do not argue for leaving GRSPaV in foundation material. Where a program or its end users require lines free of it, elimination is feasible but costly: GRSPaV was the limiting virus in three samples (Table 1) and only 22% of screened lines were negative. In P1103, where GRSPaV was the only virus, not requiring its elimination would have made sanitation unnecessary. The inventory makes this cost visible and allows an explicit decision. Fewer than 15 of the more than 102 viruses recorded in grapevine are characterized well enough to support a fully informed regulatory decision (Debat *et al.,* 2026), which argues for panels built on evidence of impact and for periodic review of their content.

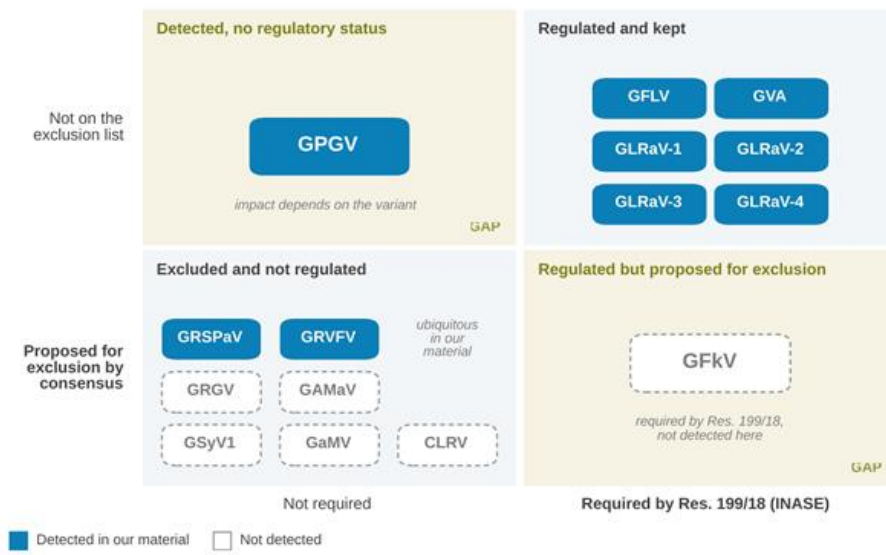


Fig. 6. Regulatory status of viruses. Columns: not required (left) or required (right) by Res. 199/18 (INASE). Rows: not on (top) or on (bottom) the list proposed for exclusion from regulation by Fuchs *et al.* (2025). Filled boxes, detected in our material; dashed boxes, not detected. GPGV is in neither list (upper left), and GFkV is required in Argentina but proposed for exclusion (lower right). GMRV, GRLDaV and GV-Sat, also detected, are not required by Res. 199/18 and are not shown. Sources: INASE (2018), Fuchs *et al.* (2025), Messmer *et al.* (2024), Debat *et al.* (2026).

4.3. Curation of HTS results

Curation changed the results substantially. First, de novo contigs built from highly expressed grapevine transcripts or plastid DNA can carry viral labels: in an earlier reference

build, 11 of 17 locally assembled contigs labeled as viral had their best BLASTN hit in grapevine, and four short scaffolds labeled as GLRaV-4 behaved the same way, collecting reads in every library including M1. Second, public records that are not viral genomes and references that gathered narrow pileups collected spurious reads and were removed (section 2.2.2). Third, labels propagate: five viroid scaffolds labeled grapevine yellow speckle viroid 2 (GYSVd-2) were closer to GYSVd-1 (87 to 92% identity, against about 80%), one labeled GYSVd-1 was GYSVd-2 (99.8%), and GVT fragments annotated as GVA would have reported a regulated virus that was not present. GLRaV-3 in CH40, negative by PCR, collected more than 30,000 reads over a complete genome, a PCR false negative. Conversely, GLRaV-3 reported in TorrRZ at 48 RPM (Cuello *et al.*, 2026) did not pass the coverage filter here: of the 462 reads assigned to it in our pipeline, all but two fell on narrow pileups covering less than 5% of a reference. Divergent local isolates were measured reliably only once their own genomes entered the reference: with public references only, P1103 and CH40 gave 1,638 and 308 GRSPaV reads (Cuello *et al.*, 2026), against 5,747 and 10,424 here. Weak GMRV signals (0.3 to 2.0 RPM) appeared in M1, CH40, P1103 and TorrRZ, sequenced in lanes other than that of the pools where GMRV was abundant; index hopping within a lane therefore does not explain them, and their origin remains unresolved.

4.4. Limitations and generalization

Seven samples cannot represent Argentine germplasm. Pools cannot be attributed to individual plants, and the infection status of each pooled accession is unknown. Per-virus proportions pool lines from different infection backgrounds, rest on 20 to 67 lines per virus with screening stopped at two negative lines, and treat lines from one mother plant as independent; they are specific to this set and protocol. No extraction blanks or healthy-plant controls were sequenced, and M1 was used both to identify artifact-prone references and as the virus-free sample, so its status is not independent of the curation. Detection thresholds are conventions chosen for these libraries; because they are set in reads, the limit of detection in RPM differs about fourfold between the deepest and the shallowest library. Lines were screened for viruses but not for viroids, which were present in every sample and are not reliably eliminated by meristem culture; their status and the result of the one-year retest must be established before release. Cultivar and phenological stage affect regeneration and virus elimination (Marković *et al.*, 2021); explant sources and somatic embryogenesis are being compared, together with epigenome stability.

5. CONCLUSIONS

A curated virome inventory identifies the viruses likely to limit meristem culture sanitation; in this set, GVA, GPGV and GRSPaV were the hardest to eliminate. Pooling makes such inventories affordable, because the library is the main cost, provided that depth per plant is kept and accessions within a pool are tested individually before sanitation. Some of the hardest viruses, such as GRSPaV, are not regulated in Argentina; where programs or end users nevertheless require their elimination, the inventory makes the cost visible before sanitation starts. Lines negative at first screening still require viroid testing and a one-year retest before they can enter certification schemes.

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Cadmium effects on ion balance, leaf structure, and antioxidant regulation in micropropagated plantlets of *Paulownia* clone Pao Tong Z07

Viktor Husak[1], Volodymyr Kotsyubynsky[2], Ulyana Kilchytska[1], Viktoriia Nykolyshyn[1], Yuliia Savchuk[1], Viktoriia Zadarko[1], Volodymyr I. Lushchak[1,3]

1 Department of Biochemistry and Biotechnology, Vasyl Stefanyk Carpathian National University, 57 Shevchenko Str, Ivano-Frankivsk, 76018, Ukraine

2 Department of Applied Physics and Materials Science, Vasyl Stefanyk Carpathian National University, 57 Shevchenko Str, Ivano-Frankivsk, 76018, Ukraine

3 Research and Development University, 13a Shota Rustaveli Str, Ivano-Frankivsk, 76018, Ukraine

volodymyr.lushchak@cnu.edu.ua

Abstract

Cadmium contamination restricts plant growth by disrupting mineral nutrition, photosynthetic metabolism, and redox homeostasis. Fast-growing woody plants may be useful for phytoremediation, but their biochemical tolerance to cadmium must be evaluated before field application. This study examined micropropagated plantlets of the *Paulownia* clone Pao Tong Z07, a hybrid derived from *P. tomentosa*, *P. fortunei*, and *P. kawakamii*, cultivated *in vitro* for 40 days on media containing 0, 0.5, 1.0, 2.5, or 5.0 mg/L Cd^{2+}. Levels of photosynthetic pigments, total polyphenols and flavonoids, activities of superoxide dismutase (SOD), guaiacol peroxidase (GPOX), and ascorbate peroxidase (APX), elemental composition, and leaf histology were evaluated. Concentrations of chlorophyll *a* and carotenoids were lower than control at all Cd concentrations, whereas chlorophyll *b* and anthocyanins did not differ significantly from control. Total polyphenol and flavonoid concentrations were higher than control at 1.0 and 2.5 mg/L Cd^{2+}. SOD activity was higher than control at 1.0 and 2.5 mg/L, GPOX activity was higher than control at 0.5, 1.0, and 2.5 mg/L, while APX activity did not differ significantly among treatments. X-ray fluorescence analysis at 0, 0.5, and 1.0 mg/L revealed redistribution of mineral elements, with chlorine values higher than in the control and manganese, potassium, and phosphorus values lower than control in several organs. Histological sections showed damaged mesophyll cells at 1.0 mg/L and tissue ruptures at 2.5 mg/L. Together, the results indicate a selective antioxidant and phenolic response that partially accompanies cadmium-induced disruption of mineral balance, pigment composition, and leaf tissue integrity in micropropagated *Paulownia*.

Keywords: *Paulownia; cadmium; oxidative stress; antioxidant enzymes; phytoremediation.*

1. INTRODUCTION

Cadmium (Cd) is a non-essential metal with high biological toxicity and environmental persistence. In plants, Cd^{2+} can enter cells through transport systems that normally mediate the uptake of essential divalent cations, and its presence can disturb ion homeostasis, photosynthesis, membrane integrity, and growth (Clemens 2006; Lux *et al.*, 2011). Mineral-nutrient imbalance is an important component of Cd toxicity because Cd can interfere with the uptake and translocation of K, Ca, Mg, Mn, Fe, Zn, and P, with consequences for enzyme function and photosynthesis (Nazar *et al.*, 2012). These effects are particularly

relevant when plants are considered for phytoremediation, because effective candidates must combine metal tolerance with sustained biomass production.

Species and hybrids of *Paulownia* are attractive for phytoremediation because of rapid biomass formation and the capacity to grow under different environmental conditions. Pilot-scale work with *P. tomentosa* demonstrated substantial retention of Cd and other metals, particularly in roots (Doumett *et al.,* 2008), while studies with *Paulownia* hybrids have shown the ability to tolerate and accumulate toxic elements in contaminated substrates and *in vitro* systems (Fathy *et al.,* 2016; Drzewiecka *et al.,* 2021). Nevertheless, accumulation alone does not define a suitable phytoremediator. Metal exposure may alter leaf anatomy, pigment composition, mineral nutrition, and antioxidant metabolism even when plantlets remain viable. For this reason, biochemical and structural responses should be evaluated together.

Cadmium does not directly participate in redox cycling in the same manner as transition metals such as Fe or Cu, but it can promote reactive oxygen species formation indirectly through disruption of electron transport, depletion of redox-active metabolites, and disturbance of antioxidant systems (Clemens 2006; Das and Roychoudhury 2014). Plants counter this imbalance through enzymatic and non-enzymatic mechanisms. Superoxide dismutase converts superoxide radicals to H_2O_2 and molecular oxygen, whereas peroxidases such as guaiacol peroxidase (GPOX) and ascorbate peroxidase (APX) participate in H_2O_2 removal (Shigeoka *et al.,* 2002; Das and Roychoudhury 2014). Phenolic compounds and flavonoids provide an additional redox-buffering component and may also contribute to metal chelation and membrane protection. The response of these systems is often concentration-dependent: moderate stress can activate protective metabolism, whereas stronger or prolonged stress can exceed the capacity of individual defense components.

Although previous studies have investigated heavy-metal uptake, tolerance, and phytoremediation potential in *Paulownia* species and hybrids (Doumett *et al.*, 2008; Fathy *et al.,* 2016; Drzewiecka *et al.,* 2021), the responses of individual clones may differ, and their physiological characteristics require separate evaluation. The present study focused on the micropropagated *Paulownia* clone Pao Tong Z07 and examined its responses to nominal Cd concentrations of 0–5.0 mg/L under controlled *in vitro* conditions for 40 days.

The specific contribution of this study is the integration of photosynthetic pigment measurements, phenolic and flavonoid contents, antioxidant enzyme activities, XRF-derived elemental profiles, and qualitative leaf histology to characterize the Cd-associated response of this particular clone. This combined approach provides an initial physiological and anatomical assessment that may inform the development of preliminary screening strategies for micropropagated woody plants. Such laboratory-based assessments could help identify relevant response indicators for subsequent studies under soil and field conditions. However, the present study does not establish the phytoremediation efficiency of Pao Tong Z07 or validate an *in vitro* screening method as a predictor of field performance.

2. MATERIALS AND METHODS

2.1. Materials

Micropropagated plantlets of the *Paulownia* clone Pao Tong Z07, a hybrid reported to originate from *P. tomentosa*, *P. fortunei*, and *P. kawakamii*, were used in this study

(Johnson 2022; Husak *et al.,* 2024). The plant material was previously established and maintained as a micropropagated culture under sterile *in vitro* conditions.

Plantlets were cultivated on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) containing standard MS macro- and micronutrients and vitamins, supplemented with 30 g/L sucrose, 100 mg/L myo-inositol, 6.0 g/L agar, 0.5 mg/L 6-benzylaminopurine (BA), and 0.02 mg/L indole-3-acetic acid (IAA). The medium pH was adjusted to 5.8 before autoclaving at 121 °C for 30 min. Each glass culture jar contained 25 mL of nutrient medium.

Cadmium was supplied as $CdCl_2 \cdot 2.5H_2O$ to obtain nominal Cd concentrations of 0 (control), 0.5, 1.0, 2.5, or 5.0 mg/L. Each treatment comprised 10 independent culture vessels, with two plantlets cultivated per vessel, resulting in 20 plantlets per treatment. The culture vessel was considered the independent experimental unit, whereas the two plantlets grown within the same vessel were treated as subsamples.

Uniform shoot explants measuring up to 1 cm in height and containing a single bud each were selected from the *in vitro* stock cultures and transferred to the experimental nutrient medium supplemented with the respective Cd concentrations. Thus, all experimental treatments were initiated using explants of comparable size and developmental stage. Cultures were maintained for 40 days in a controlled growth room at 25 ± 2 °C under a 16/8 h light/dark photoperiod, with a light intensity of approximately 50 µmol m^{-2} s^{-1} and a relative humidity of 95–99%.

Since $CdCl_2 \cdot 2.5H_2O$ was used as the Cd source, chloride was simultaneously introduced into the medium and was considered when interpreting the elemental composition of plant tissues.

2.2. Methods

For biochemical and enzymatic analyses, leaf material was collected from one plantlet per selected culture vessel. Samples from different culture vessels were processed independently and were not pooled. The second plantlet grown in the same vessel was considered a subsample and was not treated as an additional biological replicate. Repeated spectrophotometric measurements performed using the same extract were considered technical replicates, and their mean was used as a single biological observation. Thus, technical measurements were not counted as independent biological replicates.

Fresh leaf tissue (100 mg) was homogenized in chilled 96% ethanol at a 1:10 (w/v) ratio in the presence of 10 mg $CaCO_3$. After centrifugation at 8000 × g for 10 min at 4 °C, the supernatant was collected, and the extraction was repeated three times. Absorbance was measured using a ULAB 102 UV spectrophotometer at 664.2, 648.2, and 470 nm for chlorophyll *a*, chlorophyll *b*, and carotenoids, respectively. For anthocyanin determination, one drop of 37% HCl was added to the extract before measurement at 530 nm. Calculated pigment concentrations were expressed as µmol/g fresh weight (FW) (Lichtenthaler and Buschmann 2001; Husak *et al.,* 2026).

Total polyphenols were determined colorimetrically at 765 nm using a gallic-acid calibration and expressed as milligram of gallic acid equivalents (GAE)/g FW (Sánchez-Rangel *et al.,* 2013). Total flavonoids were quantified at 510 nm using a quercetin

calibration and expressed as milligram of quercetin equivalents (QE)/g FW (Ayele *et al.*, 2022).

For antioxidant-enzyme assays, leaf material was homogenized under cooled conditions. For APX and GPOX activity evaluation, tissue was homogenized at 10 mg per 100 µL of medium containing 50 mM potassium-phosphate buffer, 0.5 mM EDTA, and 0.8 mM phenylmethylsulfonyl fluoride, followed by centrifugation for 10 min at 8000 rpm. APX activity was recorded at 290 nm from the ascorbate-dependent reaction initiated by H_2O_2. GPOX activity was recorded at 420 nm from guaiacol oxidation in the presence of H_2O_2. For SOD, tissue was homogenized at a 1:10 tissue-to-medium ratio with 1.5% polyvinylpyrrolidone and centrifuged at 13000 rpm for 15 min at 4°C. SOD activity was assessed at 560 nm from inhibition of nitroblue tetrazolium reduction in a riboflavin-methionine photochemical system (Babitha *et al.*, 2002). Protein concentration was measured by the Bradford assay at 595 nm, and enzyme activities were normalized to protein content (Bradford 1976).

For elemental analysis, roots, stems, and leaves were separated, dried to constant mass, and ground into a homogeneous powder using a mortar. The powdered samples were analyzed directly, without prior ashing or acid digestion, using an EXPERT 3L energy-dispersive X-ray fluorescence (XRF) spectrometer. Elemental profiles were determined for the control and treatments containing nominal Cd concentrations of 0.5 and 1.0 mg/L.

Four independent biological samples were analyzed for each plant organ (roots, stems, and leaves) and treatment (n = 4). Each biological replicate represented material obtained from a separate culture vessel. Samples from different vessels were processed and analyzed independently. Any repeated instrumental measurements of the same powdered sample were considered technical replicates and were not treated as independent biological observations.

Elemental mass fractions were calculated automatically using the instrument's quantitative analysis software. Major elements were reported as percentages (%), whereas trace elements, including Cd, were reported in parts per million (ppm). Both units refer to the software-calculated elemental composition obtained from the same quantitative model. Examination of the original instrumental reports confirmed that the reported elemental fractions, including oxygen, summed to approximately 100%.

Therefore the reported elemental values represent software-calculated mass fractions within the instrument-derived composition rather than independently validated absolute concentrations on an original plant dry-weight basis. This limitation applies to both the major-element percentages and the Cd values expressed in ppm.

The uncertainties accompanying individual XRF results (±) were generated by the instrument software and represent internal instrumental/model-derived estimates rather than biological variability. Statistical comparisons between treatments were based on the four independent biological observations per organ and treatment, not on the instrumental uncertainty estimates.

Because the XRF measurements were not independently validated using matrix-matched certified reference materials, the resulting values were interpreted as comparative elemental profiles rather than definitive measurements of absolute tissue concentrations, total Cd accumulation, or organ-specific Cd burdens.

For histological examination, fully developed leaves of comparable size were collected from the apical region of *Paulownia* plantlet shoots after 40 days of cultivation. Samples were obtained from three independent culture vessels per treatment, with one leaf collected from each vessel (n = 2 biological samples per treatment). At least three transverse sections were examined per leaf, corresponding to a minimum of nine sections per treatment.

Leaf samples were fixed in 10% neutral buffered formalin for 24 h at room temperature, washed, dehydrated through a graded ethanol series, cleared, and embedded in paraffin. Transverse sections of 5 µm thickness were prepared using a rotary microtome, mounted on glass slides, deparaffinized, rehydrated, and stained with hematoxylin and eosin.

The sections were examined using an Evolution LUM LS-8530 light microscope in transmitted-light mode. Histological observations focused on epidermal continuity, palisade and spongy mesophyll organization, vascular bundle appearance, intercellular spaces, cell deformation, tissue discontinuities, and trichome occurrence.

The assessment was qualitative, without quantitative histomorphometric measurements or statistical comparisons among treatments. Representative photomicrographs were selected to illustrate typical anatomical features. Potential tissue discontinuities introduced during section preparation were considered when interpreting the observations.

Biochemical data were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test for comparisons of each Cd treatment with the control. Statistical significance was accepted at $p < 0.05$. Each treatment initially comprised ten independent culture vessels containing two plantlets each. The culture vessel was considered the experimental unit, and plantlets grown within the same vessel were treated as subsamples rather than independent biological replicates. The experimental datasets contained 7–8 observations for pigment measurements, 6–8 observations for total polyphenol and flavonoid measurements after the documented outlier screening, and four observations per enzyme-treatment group. Repeated spectrophotometric measurements of the same extract were considered as technical replicates and averaged before statistical analysis. Outliers in the original analysis were screened using Dixon's and Chauvenet's criteria. Calculations were performed using StatOptima and MS Excel. For XRF-derived elemental data, statistical comparisons were based on four independent biological replicates per organ and treatment (n = 4). One-way ANOVA followed by Dunnett's multiple-comparison test was used to compare Cd-treated groups with the corresponding controls, with statistical significance accepted at $p < 0.05$. Instrument-derived uncertainty estimates were not used as independent observations in the statistical analysis.

3. RESULTS

Cadmium exposure was associated with clear concentration-dependent differences in pigment and antioxidant-related variables (Table 1). Chlorophyll *a* was lower than control at every Cd concentration: 2.37 ± 0.34, 3.04 ± 0.22, 2.59 ± 0.21, and 2.91 ± 0.32 µmol/g FW at 0.5, 1.0, 2.5, and 5.0 mg/L, respectively, compared with 4.21 ± 0.38 µmol/g FW in the control ($p < 0.05$). Carotenoid content was also lower than control in all Cd treatments ($p < 0.05$). In contrast, chlorophyll *b* and anthocyanin contents did not differ significantly from the control.

Non-enzymatic antioxidant metabolites showed a different response pattern. Total polyphenol content at 1.0 and 2.5 mg/L Cd^{2+} was 12.7 ± 2.2 and 19.3 ± 2.3 mg GAE/g FW, respectively, and was higher than the control (7.97 ± 1.36 mg GAE/g FW; $p < 0.05$). At 2.5 mg/L, the mean polyphenol value was approximately 2.4-fold higher than control. Total flavonoid content was likewise higher than control at 1.0 mg/L (1.91 ± 0.14 mg QE/g FW) and 2.5 mg/L (3.46 ± 0.65 mg QE/g FW), with the latter approximately 4.3-fold higher than control (0.810 ± 0.137 mg QE/g FW; $p < 0.05$). Values at 0.5 and 5.0 mg/L did not differ significantly from control.

Table 1. Biochemical responses of *Paulownia* plantlets to Cd^{2+} exposure for 40 days

Parameter	Unit	0	0.5	1.0	2.5	5.0
Chlorophyll *a*	µmol/g FW	4.21 ± 0.38	2.37 ± 0.34*	3.04 ± 0.22*	2.59 ± 0.21*	2.91 ± 0.32*
Chlorophyll *b*	µmol/g FW	2.67 ± 0.33	2.25 ± 0.32	2.31 ± 0.26	2.06 ± 0.13	2.09 ± 0.22
Carotenoids	µmol/g FW	1.28 ± 0.13	0.725 ± 0.078*	0.653 ± 0.090*	0.577 ± 0.109*	0.780 ± 0.087*
Anthocyanins	µmol/g FW	4.17 ± 0.38	4.11 ± 0.40	3.78 ± 0.28	3.76 ± 0.19	3.87 ± 0.27
Polyphenols	mg GAE/g FW	7.97 ± 1.36	6.82 ± 1.49	12.7 ± 2.2*	19.3 ± 2.3*	6.56 ± 0.55
Flavonoids	mg QE/g FW	0.810 ± 0.137	0.788 ± 0.124	1.91 ± 0.14*	3.46 ± 0.65*	1.23 ± 0.22
SOD	U/mg protein	0.848 ± 0.216	0.976 ± 0.149	1.32 ± 0.18*	1.28 ± 0.11*	0.764 ± 0.130
GPOX	U/mg protein	9.48 ± 0.56	24.9 ± 3.1*	15.3 ± 1.2*	20.3 ± 1.3*	7.25 ± 1.84
APX	U/mg protein	0.469 ± 0.034	0.490 ± 0.054	0.588 ± 0.103	0.512 ± 0.014	0.459 ± 0.164

Values are mean ± SEM. *Significantly different from the control according to ANOVA followed by Dunnett's test ($p < 0.05$). FW, fresh weight.

The enzymatic response was selective. SOD activity at 1.0 and 2.5 mg/L Cd^{2+} was higher than control by approximately 55% and 51%, respectively ($p < 0.05$), whereas the values at 0.5 and 5.0 mg/L did not differ significantly from control. GPOX activity was higher than control at 0.5, 1.0, and 2.5 mg/L ($p < 0.05$), with the highest mean activity at 0.5 mg/L. At 5.0 mg/L, GPOX activity was not higher than control. APX activity remained statistically similar to the control across the entire Cd range.

XRF analysis of dried powdered plant material revealed treatment-associated differences in software-calculated normalized elemental profiles at 0.5 and 1.0 mg/L Cd^{2+} (Table 2). The XRF software-calculated Cd mass fractions in root samples were 143 ± 11 ppm and 370 ± 14 ppm at nominal medium Cd concentrations of 0.5 and 1.0 mg/L, respectively. These values represent model-derived relative elemental fractions and should not be interpreted as independently validated absolute Cd concentrations expressed per kilogram of original dry root biomass. In roots, the relative potassium fraction decreased from 56.3% in the control to 50.4% and 46.4% at 0.5 and 1.0 mg/L Cd^{2+}, respectively. Similar differences were observed in the relative fractions of Mn, P, and Cl, although their magnitude varied among organs and treatments.

Importantly, the reported percentages represent normalized elemental fractions calculated by the instrument software, rather than absolute percentages of the original dry plant biomass. Therefore, the observed differences indicate changes in the relative XRF-derived

elemental profiles and cannot independently establish changes in absolute mineral accumulation or depletion.

Table 2. Software-calculated normalized elemental mass fractions in dried powdered roots, stems, and leaves of *Paulownia* Pao Tong Z07 plantlets.

Organ	Element	0 mg/L	0.5 mg/L	1.0 mg/L
Root	Cl	2.01 ± 0.13	9.05 ± 0.07*	9.41 ± 0.07*
	K	56.3 ± 3.4	50.4 ± 0.2*	46.4 ± 0.2*
	Mn	2.40 ± 0.15	0.729 ± 0.013*	0.965 ± 0.015*
	P	2.33 ± 0.15	1.86 ± 0.03*	1.89 ± 0.03*
	Cd	Not Detected	143 ± 11	370 ± 14
Stem	Cl	3.32 ± 0.06	8.86 ± 0.06*	10.3 ± 0.7*
	K	59.8 ± 0.2	55.1 ± 0.2*	56.3 ± 3.8*
	Mn	0.443 ± 0.010	0.345 ± 0.007*	0.246 ± 0.018*
	P	3.01 ± 0.04	2.20 ± 0.03*	1.84 ± 0.13*
Leaves	Cl	3.01 ± 0.06	4.24 ± 0.08*	5.76 ± 0.07*
	K	51.2 ± 0.3	50.9 ± 0.557	47.7 ± 0.2*
	Mn	0.741 ± 0.016	0.459 ± 0.013*	0.571 ± 0.013*
	P	2.94 ± 0.04	3.00 ± 0.05	2.97 ± 0.04

Values are presented as software-calculated elemental mass fractions obtained from dried powdered plant material. Statistical comparisons were performed using independent biological replicates, with the culture vessel considered the experimental unit. Instrument-derived uncertainties were distinguished from biological variability. *Significantly different from the control according to one-way ANOVA followed by Dunnett's test ($p < 0.05$).

Histological observations supported the biochemical evidence of Cd stress. Control leaves showed a continuous epidermis and organized mesophyll. At 0.5 mg/L Cd^{2+}, the overall tissue pattern remained comparatively preserved. At 1.0 mg/L Cd^{2+}, localized regions of damaged mesophyll cells were evident, and at 2.5 mg/L the sections showed more extensive tissue disorganization together with visible ruptures (Fig. 1).

4. DISCUSSION

The combined data show that Cd exposure affected several physiological layers simultaneously, but the responses were not uniform across endpoints. The most consistent photosynthetic signal was the lower chlorophyll *a* and carotenoid content relative to the control. Cadmium-related impairment of pigments can arise from disturbed nutrient supply, altered chloroplast metabolism, and oxidative damage. Earlier work with Cd-exposed plants has linked metal stress with changes in mineral nutrition and photosynthetic performance (Nazar *et al.*, 2012; Zhang *et al.*, 2014), while high metal loads in *Paulownia* have also been associated with structural and physiological damage to photosynthetic tissues (Azzarello *et al.*, 2012). In the present plantlets, the pigment response was accompanied by lower K and Mn values in several organs and by histological lesions at the higher examined

concentrations, supporting a connection between ion imbalance and impaired leaf function. It should also be considered that the plantlets were cultivated under mixotrophic *in vitro* conditions, with sucrose supplied in the nutrient medium and relatively low light intensity. These conditions may influence chloroplast development, pigment metabolism, and antioxidant responses. Therefore, the observed Cd-associated changes should be interpreted within the context of this controlled *in vitro* system and should not be directly extrapolated to fully photoautotrophic plants grown under greenhouse or field conditions.

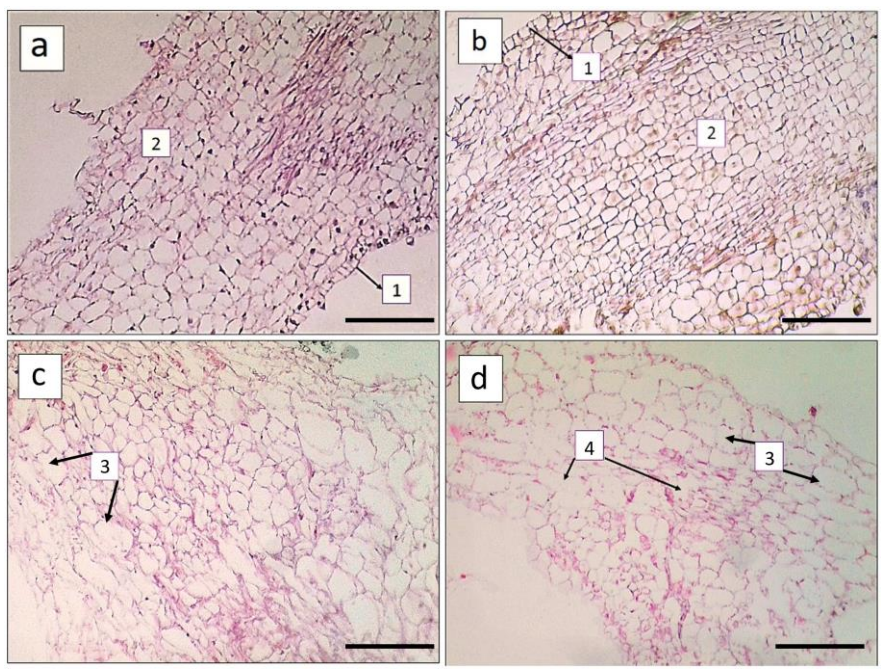


Fig. 1. Histological sections of *Paulownia* leaf tissues exposed to 0 (a), 0.5 (b), 1.0 (c), and 2.5 (d) mg/L Cd^{2+}. 1, epidermis; 2, intact mesophyll cells; 3, damaged mesophyll cells; 4, tissue ruptures. Scale bars = 100 µm.

An important consideration in interpreting the elemental data is the normalization procedure applied by the XRF software. Examination of the original instrumental reports confirmed that the software-calculated elemental fractions, including oxygen, summed to approximately 100%. However, these calculations did not provide a complete determination of all components of the original plant dry matter, particularly carbon and hydrogen associated with the organic matrix.

Consequently, potassium values approaching 60% represent the relative contribution of potassium within the software-derived elemental composition and should not be interpreted as indicating that potassium constitutes 60% of the total dry biomass. Furthermore, changes in normalized elemental fractions may partly reflect simultaneous changes in other components of the calculated composition. Thus, the observed treatment-associated differences in K, Mn, P, and Cl are interpreted as changes in relative elemental profiles rather than definitive evidence of altered absolute mineral concentrations or uptake.

The same analytical consideration applies to Cd. The Cd values reported in ppm were calculated by the XRF software using the same quantitative composition model as the major-element fractions. The measurements were performed directly on dried powdered plant material, without prior ashing. Accordingly, the values of 143 and 370 ppm represent Cd mass fractions within the software-calculated elemental composition of the analyzed powder. Although these values are expressed in ppm, they cannot be directly equated with independently validated Cd concentrations of 143 and 370 mg/kg of original dry root biomass, because the exact normalization procedure and quantitative accuracy of the model for the plant matrix were not independently verified. Although the higher software-calculated Cd value at 1.0 mg/L indicates a higher model-reported Cd fraction, the present measurements do not independently establish absolute Cd accumulation, uptake efficiency, or root-to-shoot translocation.

The relative XRF-derived elemental profiles showed notable treatment-associated differences in Mn, K, and P. The software-calculated Mn fraction was lower than in the control in roots, stems, and leaves at both analyzed Cd concentrations, whereas the relative fractions of K and P were lower mainly in roots and stems. These patterns may be associated with Cd-related disturbances in mineral homeostasis, although changes in normalized elemental fractions alone cannot establish changes in absolute nutrient concentrations. Cd^{2+} can compete with essential ions for uptake pathways or indirectly modify transporter activity and membrane properties (Clemens 2006; Lux *et al.,* 2011; Nazar *et al.,* 2012). Such interactions are physiologically relevant because Mn participates in photosynthetic oxygen evolution and serves as a cofactor for one class of SOD, K contributes to osmotic regulation, stomatal function, enzyme activation, and charge balance, while P is essential for energy metabolism. The relative P fraction in leaves remained close to the control values, indicating that the observed XRF-derived elemental responses differed among organs rather than reflecting a uniform change in the relative abundance of all mineral elements.

Chlorine showed a distinct treatment-associated pattern. In roots, the software-calculated Cl fraction was approximately 4.5-fold and 4.7-fold higher than in the control at 0.5 and 1.0 mg/L Cd^{2+}, respectively. In stems, the relative values were approximately 2.7- and 3.1-fold higher than in the control, whereas in leaves they were 1.4- and 1.9-fold higher. Thus, the strongest relative difference was observed in roots, followed by stems and leaves. Importantly, Cd was supplied as $CdCl_2 \cdot 2.5H_2O$, meaning that increasing Cd concentrations were accompanied by additional chloride in the culture medium. Therefore, the observed changes in the relative Cl fractions may partly reflect the increased chloride supply rather than a Cd-specific physiological response. Moreover, because the XRF-derived elemental fractions were normalized within the instrument's quantitative model, these differences should not be interpreted as direct evidence of absolute Cl accumulation in plant tissues.

The antioxidant data indicate selective, rather than generalized, enzyme activation. SOD was higher than control at 1.0 and 2.5 mg/L, and GPOX was higher than control from 0.5 to 2.5 mg/L, whereas APX remained statistically similar to control. Such divergence is biologically plausible because antioxidant enzymes differ in cellular localization, substrate affinity, regulation, and sensitivity to metal stress (Shigeoka *et al.,* 2002; Das and Roychoudhury 2014). Studies in other species also show that Cd can produce distinct response profiles among H_2O_2-detoxifying enzymes rather than a uniform shift in all

activities (Schneiderová and Šmejkal 2015). The SOD and GPOX maxima at intermediate Cd concentrations, together with the absence of a higher value at 5.0 mg/L, suggest that these components were most responsive within a moderate stress range.

The phenolic response complements the enzyme data. Polyphenols and flavonoids were higher than in control plantlets at 1.0 and 2.5 mg/L Cd^{2+}, and the largest means occurred at 2.5 mg/L Cd^{2+}. *Paulownia* leaves are naturally rich in diverse phenolic constituents (Zhang *et al.*, 2014), and phenolics can contribute to redox buffering through radical scavenging, electron donation, and interactions with metal ions. The coincidence of higher phenolic content with higher SOD and GPOX activities at intermediate Cd^{2+} concentrations is consistent with coordinated enzymatic and non-enzymatic defense. At 5.0 mg/L, polyphenols were not higher than in control plantlets, despite continued pigment impairment, which suggests that the protective metabolic response did not simply scale with external Cd concentration.

The histological observations provide an anatomical counterpart to these biochemical responses. Leaves at 1.0 mg/L Cd^{2+}-contained localized damaged mesophyll, while the 2.5 mg/L Cd^{2+} sections displayed more extensive disruption and tissue ruptures. Similar structural sensitivity has been reported for *Paulownia* exposed to excessive Zn, where high metal concentrations altered cellular ultrastructure and photosynthetic function (Azzarello *et al.*, 2012). In the present study, tissue damage became apparent within the same concentration range in which antioxidant and phenolic defenses were strongly expressed. This combination is compatible with an active stress response that mitigates, but does not fully prevent, cellular injury.

The main contribution of the present study is the characterization of the integrated physiological, biochemical, and anatomical response of the micropropagated *Paulownia* clone Pao Tong Z07 to Cd exposure under controlled *in vitro* conditions. Unlike studies focused primarily on metal accumulation or phytoremediation performance, this work combines pigment composition, phenolic metabolism, antioxidant enzyme activities, exploratory elemental profiling, and qualitative leaf anatomy within a single experimental system. The results reveal a clone-specific response pattern in which changes in antioxidant-related and phenolic variables occur alongside reduced photosynthetic pigment levels and visible anatomical alterations.

From an applied perspective, this approach may contribute to the development of preliminary *in vitro* screening strategies for micropropagated woody plants before more resource-intensive soil-based and field experiments. However, the present investigation involved only one clone and did not assess field growth, independently validated absolute Cd accumulation, or phytoremediation efficiency. Therefore, the measured responses should be regarded as preliminary physiological and anatomical indicators rather than validated predictors of Cd tolerance or field phytoremediation performance. Future research should include comparisons among multiple clones, growth and biomass measurements, quantitative Cd analysis, and validation under contaminated-soil and field conditions.

5. CONCLUSIONS

Micropropagated *Paulownia* Pao Tong Z07 plantlets responded to Cd^{2+} through coordinated changes in mineral composition, photosynthetic pigments, antioxidant enzymes, phenolic

metabolites, and leaf structure. Chlorophyll *a* and carotenoids were lower than control throughout the Cd range. SOD and GPOX activities, together with total polyphenols and flavonoids, were higher than control primarily at intermediate Cd concentrations, whereas APX remained stable. XRF analysis showed lower Mn, K, and P values in several organs and a strong treatment-associated Cl signal, while histological sections showed mesophyll damage and tissue rupture at 1.0-2.5 mg/L. The response profile indicates that antioxidant and phenolic defenses are activated within a concentration range that already produces measurable structural stress. These findings provide a preliminary, clone-specific physiological and anatomical characterization of Cd responses in micropropagated *Paulownia* Pao Tong Z07. The combined *in vitro* approach may serve as an initial step toward developing screening strategies for woody plant clones, but its predictive value for field performance remains to be established. Further studies comparing different clones and assessing biomass production, validated Cd accumulation, bioconcentration, and translocation factors, and performance in contaminated soils and field conditions are required before the phytoremediation potential of Pao Tong Z07 can be evaluated.

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Comparative evaluation of aluminium cryo-plate methods for the cryopreservation of autochthonous plum genotypes in Albania

Yllka Lala[1], Tatjana Vujović[2], Efigjeni Kongjika[3], Valbona Sota[1,3]

[1]*Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Albania*

[2]*Fruit Growing Institute, Čačak, Serbia*

[3]*Research Center of Biotechnology and Genetics, Academy of Sciences of Albania, Tirana, Albania*

Yllka.Lala@qttbfushekruje.gov.al

Abstract

This study aimed to optimize and evaluate vitrification (V cryo-plate) and dehydration (D cryo-plate) techniques for the cryopreservation of two autochthonous plum (*Prunus domestica* L.) genotypes, 'Tropojane' and 'Vlonjate'. Shoot tips excised from *in vitro*-grown plantlets were precultured for 3 days at 23 °C in darkness on QL basal medium supplemented with 0.3 M sucrose. Subsequently, explants were transferred for 5 days to QL medium containing 0.5 M sucrose under dark conditions at 4 °C. After preculture, shoot tips were placed in calcium alginate within the wells of the aluminium cryo-plate, and polymerization was achieved by the addition of 0.1 M calcium chloride solution. In the D cryo-plate protocol, dehydration was performed by air-drying the cryo-plates under laminar flow at 23 °C for 2, 2.5, and 3 hours. In the V cryo-plate method, dehydration was achieved using plant vitrification solution 2 (PVS2), applied for 20, 30, and 40 minutes. Cryo-plates were directly immersed in liquid nitrogen (−196 °C). Rewarming was carried out rapidly, and explants were transferred to recovery medium. The results indicated a genotype-dependent response to the applied cryo-plate methods. The highest efficiency was obtained with the D cryo-plate method at 2.5 hours in the 'Vlonjate' genotype, reaching 52.5% survival and 45% regeneration. Under the same conditions, 'Tropojane' achieved 37.5% regeneration. In the V cryo-plate method (30 minutes), 'Tropojane' exhibited higher regeneration (35%) compared to 'Vlonjate' (27.5%). The D cryo-plate method demonstrated superior performance across both genotypes, indicating its suitability for long-term germplasm conservation. These findings highlight genotype-specific responses and emphasize the importance of protocol optimization to improve cryopreservation efficiency.

Keywords*: plum genotypes, cryogenic storage, germplasm conservation, D cryo-plate, V cryo-plate.*

1. INTRODUCTION

The conservation of fruit tree genetic resources is important for maintaining valuable local germplasm and genetic diversity. Cryopreservation offers a long-term option for preserving valuable plant material with little maintenance (FAO, 2014). The conservation of cultivars and local genotypes of European plum (*Prunus domestica* L.) relies on vegetatively propagated plant material to preserve their genotype-specific characteristics. Field collections provide plants for evaluation and propagation, but their long-term maintenance requires continuing care and leaves them vulnerable to loss. Aluminium cryo-plates allow shoot tips to be cryopreserved after dehydration by vitrification (V cryo-plate) or air drying (D cryo-plate), followed by immersion in liquid nitrogen (Yamamoto *et al.*, 2011; Niino *et al.*, 2014). Both methods have been applied to *Prunus* genotypes in the region, including *P. domestica* (Vujović *et al.*, 2015). To the best of our knowledge, this is the first study in

Albania to evaluate both cryo-plate procedures using the autochthonous plum genotypes 'Tropojane' and 'Vlonjate'.

For shoot tips to recover after cryopreservation, enough water must be removed through dehydration under laminar airflow to limit ice formation without damaging the tissues needed for regrowth. Alginate secures the small explants to an aluminium plate, allowing the plate and its attached shoot tips to be moved rapidly between treatment solutions and liquid nitrogen. The duration of dehydration is therefore critical: too little may leave freezable water in the tissue, whereas extended air drying or exposure to vitrification solution may reduce its ability to grow (Yamamoto *et al.*, 2011; Niino *et al.*, 2014).

Experience with other crops shows the breadth of cryo-plate applications and the need to adjust the treatment to the plant material. V cryo-plates have been used for strawberry and mint (Yamamoto *et al.*, 2012a, 2012b), and the V and D methods have been compared in strawberry and mulberry (Gupta and Tewari, 2020; Tanaka *et al.*, 2019). D cryo-plates have been studied in *Juncus decipiens* Nakai, *Diospyros kaki* Thunb., and *Vaccinium* spp. (Niino *et al.*, 2014; Matsumoto *et al.*, 2015; Dhungana *et al.*, 2017), as well as in polyembryonic masses of *Phoenix dactylifera* L. (Salma *et al.*, 2014). V cryo-plates have also been investigated in *Vitis* spp. (Bettoni *et al.*, 2019). The diversity of explants and pretreatments in these studies precludes assuming that one exposure time will suit plum shoot tips.

The most relevant evidence comes from *Prunus*. Vujović *et al.* (2015) recovered shoots of *P. cerasifera* and the European plum cultivar 'Požegača' after both cryo-plate procedures. The cultivar 'Crvena Ranka' was subsequently studied (Vujović *et al.*, 2021), and a study of eight indigenous *P. domestica* genotypes documented variation in regrowth across genotypes and treatments (Vujović *et al.*, 2023). Evaluating the intended accessions directly is especially pertinent when building a conservation protocol for local germplasm.

We evaluated V and D cryo-plates for the Albanian plum genotypes 'Tropojane' and 'Vlonjate'. Three PVS2 exposure times and three air-drying periods were compared using shoot-tip survival and regeneration after immersion in liquid nitrogen. Our aim was to identify promising treatment durations for each genotype as a basis for conserving local plum germplasm.

2. MATERIALS AND METHODS

2.1. Plant material and preculture

Shoot tips measuring 1.5–2.0 mm in length were excised from *in vitro*-grown plantlets of two autochthonous plum (*Prunus domestica* L.) genotypes, 'Tropojane' and 'Vlonjate'. Following excision, shoot tips were precultured in darkness on Quoirin and Lepoivre (QL) basal medium (Quoirin and Lepoivre, 1977) supplemented with 0.3 M sucrose for three days at 23 °C. Subsequently, explants were transferred to QL medium containing 0.5 M sucrose and maintained in darkness at 4 °C for five days. The same preculture conditions were applied to both cryopreservation methods.

The preculture media were supplemented with 0.7% agar, and their pH was adjusted to 5.7 before sterilization.

2.2. Mounting on aluminium cryo-plates

Following preculture, individual shoot tips were positioned in the wells of aluminium cryo-plates. Approximately 4 µL of 2% (w/v) sodium alginate, prepared in calcium-free QL basal medium supplemented with 0.4 M sucrose, was applied to each well. Alginate polymerization was achieved by adding 0.1 M calcium chloride solution prepared in QL basal medium containing 0.4 M sucrose. After 20 min, excess solution was removed by gently tapping the cryo-plates against sterile filter paper. The alginate matrix ensured that the shoot tips remained attached to the cryo-plates throughout the subsequent cryopreservation procedures.

2.3. Loading and dehydration using V and D cryo-plate methods

Following mounting, cryo-plates carrying the precultured shoot tips were exposed for 20 min to 2 mL of loading solution (LS) consisting of 2 M glycerol and 0.4 M sucrose prepared in QL basal medium. The loading treatment was performed at room temperature.

For the V cryo-plate method, loading solution was replaced with plant vitrification solution 2 (PVS2), and the cryo-plates were exposed at 0 °C for 20, 30, or 40 min. The conventional PVS2 formulation commonly used in plant cryopreservation comprises 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO), and 0.4 M sucrose in basal medium, as described in the literature following Sakai *et al.* (1990).

For the D cryo-plate method, dehydration was performed under laminar airflow at 23 °C for 2, 2.5, or 3 h. The two methods shared the preculture, mounting, and loading stages but differed in their dehydration procedures.

2.4. Cryopreservation, rewarming, unloading, and recovery

Following dehydration, aluminium cryo-plates carrying the shoot tips were placed in 2 mL cryotubes and immersed in liquid nitrogen (LN; −196 °C) for at least 30 min. The cryotubes were closed, and the cryo-plates were protected from direct LN contact within the closed cryotubes.

Following cryopreservation, V cryo-plates were rapidly transferred to unloading solution consisting of 1.2 M sucrose dissolved in hormone-free Quoirin and Lepoivre (QL) basal medium (Quoirin and Lepoivre, 1977), at room temperature for 20 min. For the D cryo-plate method, cryotubes containing the cryo-plates were rapidly rewarmed in a water bath at 40 °C for 90 s. The D cryo-plates were then removed from the cryotubes and immersed for 25 min at room temperature in an unloading solution containing 0.8 M sucrose in hormone-free liquid QL basal medium.

After rewarming and unloading, shoot tips were transferred to QL basal recovery medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP) and 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA). The medium additionally contained 3% sucrose, 0.7% agar, and its pH was adjusted to 5.7.

Cultures were maintained in darkness for seven days, followed by incubation at 25 ± 2 °C under a 16 h photoperiod with a photosynthetic photon flux density of 50 µmol m^{-2} s^{-1}.

2.5. Experimental design and control treatments

For each genotype, three dehydration durations were tested: 20, 30, and 40 min for the V cryo-plate method and 2, 2.5, and 3 h for the D cryo-plate method. Each treatment was

performed in two independent replicates, with 20 explants per replicate and 10 explants per cryo-plate. Overall, 120 shoot tips on 12 cryo-plates were used per genotype for each method.

Control explants were established for each genotype and treatment duration. Control shoot tips underwent the same preculture, mounting, loading, dehydration, rewarming-equivalent and unloading procedures as the cryopreserved samples, with LN exposure omitted.

The controls were then transferred to the same recovery medium and maintained under identical culture conditions. This experimental design permitted the effects of LN exposure to be distinguished from the effects of the preceding cryoprotection and dehydration treatments.

2.6. Assessment of survival and regeneration

Shoot-tip survival was evaluated three weeks after transfer to the recovery medium, whereas regeneration was assessed after eight weeks. Survival was defined as the maintenance of green, viable tissue and/or visible tissue expansion, while regeneration was defined as the formation of a new, clearly distinguishable shoot originating from the original explant. Survival and regeneration percentages were calculated for each genotype, cryo-plate method, and treatment duration as the proportion of surviving or regenerating shoot tips relative to the total number of shoot tips subjected to the respective treatment.

3. RESULTS

3.1. Effects of cryo-plate method and treatment duration on shoot-tip survival and regeneration

Survival and regeneration of *Prunus domestica* L. shoot tips varied according to genotype, cryo-plate method, and treatment duration (Figure 1). Both ‘Tropojane’ and ‘Vlonjate’ showed differences in their responses to dehydration using the D cryo-plate method and exposure to plant vitrification solution 2 (PVS2) using the V cryo-plate method.

With the D cryo-plate method, the highest survival and regeneration percentages were obtained after 2.5 h of air dehydration in both genotypes. Under these conditions, ‘Tropojane’ exhibited 50% survival and 37.5% regeneration, whereas ‘Vlonjate’ showed slightly higher values of 52.5% survival and 45% regeneration. Shorter dehydration (2 h) resulted in lower regeneration percentages of 20% in ‘Tropojane’ and 25% in ‘Vlonjate’. Similarly, extending dehydration to 3 h reduced regeneration to 22.5% and 32.5%, respectively. Thus, regeneration peaked at an intermediate dehydration duration of 2.5 h in both genotypes, with lower values observed following either shorter or longer treatments.

With the V cryo-plate method, the highest regeneration percentages were recorded following 30 min of PVS2 exposure, reaching 35% in ‘Tropojane’ and 27.5% in ‘Vlonjate’. Extending exposure to 40 min resulted in a slight decrease in regeneration to 30% and 25%, respectively. These findings indicate that, within the tested treatment durations, 30 min of PVS2 exposure was more favorable for shoot regeneration in both genotypes.

Comparison of the two cryo-plate methods at their respective optimal tested durations revealed differences between genotypes. In ‘Tropojane’, maximum regeneration was relatively similar between the D and V cryo-plate methods (37.5% and 35%, respectively), corresponding to a difference of only 2.5 percentage points. In contrast, ‘Vlonjate’ exhibited

a more pronounced difference, with regeneration reaching 45% using D cryo-plates compared with 27.5% using V cryo-plates, a difference of 17.5 percentage points. Furthermore, 'Vlonjate' showed higher maximum regeneration than 'Tropojane' with the D cryo-plate method, whereas the opposite pattern was observed with the V cryo-plate method. Overall, the highest regeneration percentage recorded in the study was 45%, obtained for 'Vlonjate' following 2.5 h of air dehydration using D cryo-plates.

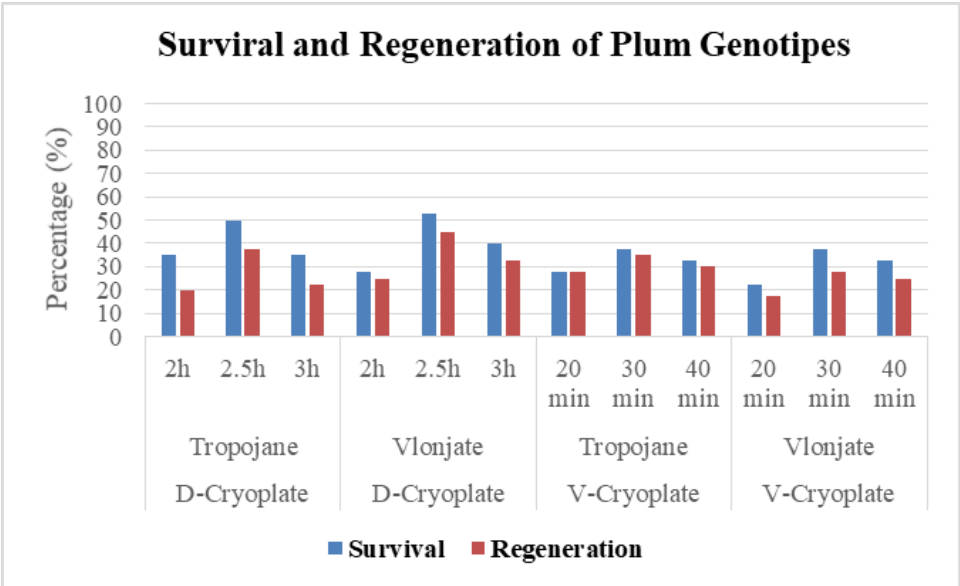


Fig. 1. Comparison of survival and regeneration percentages of shoot tips of two *Prunus domestica* L. genotypes (Tropojane and Vlonjate) following cryopreservation using the D cryo-plate and V cryo-plate techniques under different treatment durations. Each treatment was performed in two independent replicates, with 20 shoot tips per replicate. Survival percentages were assessed two weeks after cryopreservation, whereas regeneration percentages were determined after five weeks to evaluate shoot tip regrowth.

3.2. Post-cryopreservation shoot recovery and plantlet development

In addition to the quantitative differences in survival and regeneration, post-cryopreservation recovery was documented through successive stages of shoot development under *in vitro* conditions (Figure 2). Following dehydration and cryopreservation using the D and V cryo-plate methods, shoot tips were transferred to recovery medium to evaluate their capacity to resume growth and regenerate shoots.

Representative observations showed shoot regeneration in both 'Tropojane' and 'Vlonjate' after three weeks of recovery culture (Figure 2.3). Regenerated shoots were also observed in 'Vlonjate' after four weeks (Figure 2.4), followed by further shoot development during continued *in vitro* cultivation (Figure 2.5). In 'Tropojane', plantlet development was documented after two months of culture, including the formation of a well-developed root system (Figure 2.6).

These observations complement the regeneration percentages presented in Figure 1 by illustrating the progression from cryopreserved shoot tips to regenerated shoots and, in the representative 'Tropojane' material, a rooted plantlet. The photographic documentation provides evidence of continued growth in the recovered plant material beyond the initial regeneration stage.

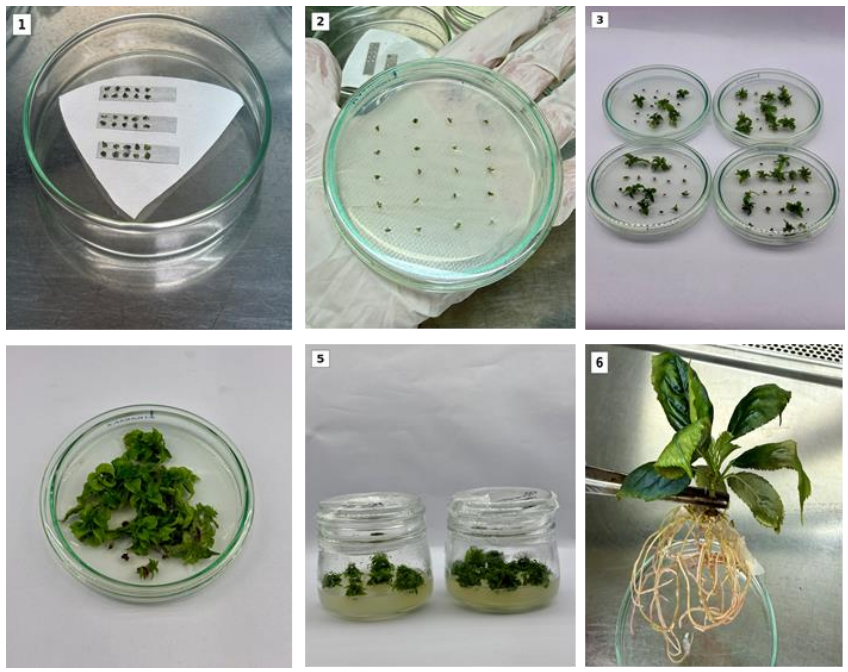


Fig. 2. Representative images of plant material and regeneration of *Prunus domestica* L. following cryopreservation using V and D cryo-plate methods. (1) Mounting of shoot tips on aluminium cryo-plates before dehydration; (2) transfer of explants to recovery medium after cryopreservation using V and D cryo-plate methods; (3) shoot regeneration after three weeks of recovery culture, with V cryo-plate samples on the left and D cryo-plate samples on the right, 'Tropojane' in the upper row and 'Vlonjate' in the lower row; (4) regenerated 'Vlonjate' shoots after four weeks of recovery culture; (5) further growth of regenerated plum shoots under *in vitro* culture conditions; (6) development of a 'Tropojane' plum plantlet after two months of culture, showing a well-developed root system.

4. DISCUSSION

The present study demonstrated that both D and V cryo-plate techniques can support post-cryopreservation regeneration in the two Albanian plum genotypes, although their responses differed according to treatment duration and genotype. The highest regeneration percentages were achieved with the D cryo-plate method following 2.5 h of air dehydration. The lower regeneration observed after shorter and longer dehydration periods suggests that an appropriate balance between water removal and the preservation of tissue viability is important for successful cryopreservation. Insufficient dehydration may leave tissues vulnerable to freezing injury, whereas excessive dehydration may cause cellular damage

and reduce their regenerative capacity. The observed responses therefore highlight the importance of optimizing dehydration conditions for individual genotypes.

These findings are consistent with previous studies demonstrating the applicability of cryo-plate techniques to plum germplasm conservation. Vujović *et al.* (2015) reported regrowth of up to 47.5% with D cryo-plates and 44.6% with V cryo-plates in the plum cultivar 'Požegača'. In the same study, regrowth in P. cerasifera reached 77.5% and 56.1%, respectively. The maximum D-method regeneration percentages obtained in the present study were slightly lower than those reported for 'Požegača', particularly in 'Tropojane', although the value obtained for 'Vlonjate' was comparable. These results suggest that the two Albanian genotypes exhibit regenerative responses within a range previously reported for other plum germplasm, supporting the applicability of the D cryo-plate approach to their long-term conservation.

Nevertheless, the relative effectiveness of D and V cryo-plate techniques is not consistent across plum genotypes. In 'Crvena Ranka', regrowth reached 30–40% with D cryo-plates and 50–66.7% with V cryo-plates under the vitrification treatments tested (Vujović *et al.,* 2021). Furthermore, Vujović *et al.* (2023), investigating eight indigenous plum genotypes, demonstrated that the air-dehydration duration associated with the highest D-method regrowth differed among accessions, with some responding best after 2 h and others requiring longer exposure. Taken together with the present results, these observations suggest that cryopreservation responses are strongly influenced by genotype-dependent physiological characteristics, emphasizing the need for genotype-specific protocol optimization rather than the application of a single standardized treatment.

The response to PVS2 exposure observed with V cryo-plates further supports the importance of treatment optimization. The decrease in regeneration following prolonged exposure from 30 to 40 min may reflect a balance between the protective effects of vitrification solutions and their potential chemical or osmotic toxicity. A similar dependence on vitrification solution composition and treatment conditions has been reported in other woody species. For example, Ružić *et al.* (2014) observed regrowth of 8–17% following conventional PVS2 vitrification in the cherry rootstock 'Gisela 5', whereas treatment with a modified PVS2 formulation (PVS A3) increased regrowth to 39–56%. Although the experimental systems differ, these findings illustrate how modifications in vitrification treatments can substantially influence post-cryopreservation recovery.

The treatment durations required for successful cryopreservation also vary considerably among woody species. In mulberry, shoot tips derived from *in vitro* cultures were air-dehydrated for 2.5 h using D cryo-plates, whereas shoot tips obtained from new growth were exposed to PVS2 for 50 min using V cryo-plates (Tanaka *et al.,* 2019). With the D cryo-plate method, favorable recovery was obtained after only 30 min of air dehydration for shoot tips from dormant persimmon buds and after 1 h for blueberry shoot tips (Matsumoto *et al.,* 2015; Dhungana *et al.,* 2017). Successful shoot regrowth has also been demonstrated in grapevine using V cryo-plates (Bettoni *et al.,* 2019). Such differences likely reflect variation in explant characteristics, physiological state, and sensitivity to dehydration or vitrification treatments. Consequently, dehydration and PVS2 exposure periods reported for other species cannot be transferred directly to the Albanian plum genotypes without experimental validation.

Additional evidence of species- and cultivar-dependent variation is available from studies on strawberry and date palm. In strawberry cultivar 'Earliglow', D cryo-plate treatment produced maximum regrowth of 40%, comparable to the highest regeneration percentages obtained in the present study (Gupta and Tewari, 2020). In contrast, Yamamoto *et al.* (2012a) reported mean regrowth of 81% across 15 strawberry cultivars using V cryo-plates. These differences demonstrate that high recovery rates are achievable with cryo-plate techniques, although their effectiveness depends on the biological material and experimental conditions. In date palm, Salma *et al.* (2014) reported the absence of recovery of cryopreserved polyembryonic masses following D cryo-plate treatment without sucrose preculture, highlighting the importance of appropriate pretreatment conditions. Although this observation concerns a different type of plant material, it reinforces the broader principle that successful cryopreservation requires optimization of several interconnected protocol stages.

An important consideration in evaluating cryopreservation efficiency is the distinction between survival and regeneration. Survival reflects the capacity of explants to remain viable following cryogenic exposure, whereas regeneration provides a more stringent measure of their ability to resume organized shoot development. Accordingly, the recovery of green or viable tissues does not necessarily indicate successful regeneration. In the present study, the highest survival percentages were accompanied by lower regeneration percentages, indicating that not all surviving shoot tips developed into new shoots. The representative images in Figure 2 further demonstrate that regenerated material can undergo subsequent shoot growth and, in the case of 'Tropojane', root development under *in vitro* culture conditions. However, additional experiments are necessary to determine whether regenerated shoots consistently exhibit normal multiplication, rooting, and subsequent acclimatization.

From a conservation perspective, the successful regeneration of both 'Tropojane' and 'Vlonjate' represents a useful step toward developing cryopreservation strategies for Albanian autochthonous fruit-tree germplasm. These local genotypes constitute valuable genetic resources whose preservation may contribute to maintaining agricultural biodiversity and supporting future breeding and cultivation programs. Further research should focus on refining pretreatment and dehydration conditions, evaluating regeneration across additional genotypes, and assessing the long-term growth performance of recovered plants. In the context of increasing environmental pressures and climate-related risks to field germplasm collections, cryopreservation could provide an important complementary approach to conventional ex situ conservation of Albania's fruit-tree genetic resources.

5. CONCLUSIONS

The present study demonstrated the successful cryopreservation and regeneration of shoot tips from two Albanian autochthonous plum (*Prunus domestica* L.) genotypes, 'Tropojane' and 'Vlonjate', using both D and V cryo-plate methods. The highest regeneration percentages were achieved with the D cryo-plate method following 2.5 h of air dehydration, reaching 37.5% in 'Tropojane' and 45% in 'Vlonjate'. Nearly all regenerated shoots subsequently developed roots, indicating that post-cryopreservation recovery extended beyond initial shoot regeneration to plantlet development. These findings highlight the

potential of cryo-plate techniques for the long-term conservation of Albanian plum genetic resources and provide a foundation for further protocol optimization and application to other autochthonous fruit-tree genotypes.

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WORKING GROUP 3

Scaling up and automation

Fifteen years of research on hybrid larch somatic embryogenesis: Progress, bottlenecks and perspectives for forestry

Andrea Rupps[1], Emma Ehmke[1], Madlen Walther[1], Juliane Raschke[1], Ute Tröber[2], Ricardo Joffe[2], Silvan Liebsch[2], Thomas Hoppe[3], Birgit Reiche[4], Sophie Maiwald[5], Anna Rudzinski[5], Tony Heitkam[5]

[1]*Dept. of Plant Evolution and Biodiversity, Späth-Arboretum, Institute of Biology, Humboldt-Universität zu Berlin, Späthstr. 80/81, 12437 Berlin, Germany*

[2]*Staatsbetrieb Sachsenforst, Dept. 42 Forest Genetics and Forest Plant Breeding, Bonnewitzer Str. 42, 01796 Pirna OT Graupa, Germany*

[3]*Baumschulen Oberdorla GmbH, Burgstr. 57, 99986 Vogtei OT Oberdorla, Germany*

[4]*TU Dresden, Institute of Forest Botany and Forest Zoology, Pienner Str. 19, 01737 Tharandt, Germany*

[5]*RWTH Aachen University, Institute of Biology I, 52074 Aachen, Germany*

andrea.rupps@hu-berlin.de

Abstract

Over the last 15 years, somatic embryogenesis (SE) has been investigated as a tool for clonal propagation of hybrid larch (*Larix* × *eurolepis*). While SE offers unique opportunities for mass propagation of superior genotypes, transferring laboratory protocols to operational forestry remains challenging. Our work has revealed several bottlenecks during all steps of the SE process chain: initiation and maintenance of embryogenic cultures, maturation, acclimation and reliable genotype traceability. Here, we summarize some of our approaches to address these bottlenecks with the aim to transfer the SE process towards forestry applications. Research has focused on optimizing critical steps of the SE process, with particular attention paid to culture stability and maturation capacity during prolonged maintenance. Parallel subculturing resulting in different clonal lines and early cryopreservation were implemented to prevent losses in embryogenic embryo yield. To improve plant production, synchronized germination and adjustments of acclimation conditions were evaluated. In parallel, molecular approaches were developed for clone characterization, identification and as a prerequisite for clone approval. Marker systems based on repetitive DNA elements were established and used to verify genotype identity across different developmental stages and more than 110 genotypes. These approaches have transformed hybrid larch SE from an experimental propagation method into an increasingly integrated platform for plant production, clone characterization and field evaluation.

Keywords: *genotype characterisation; genetic markers; field evaluation*

1. INTRODUCTION

Future forestry requires planting material that combines desirable genetic characteristics with reliable propagation and performance across changing environmental conditions. Hybrid larch (*Larix* × *eurolepis* Henry) is of particular interest because of its rapid juvenile growth, robustness and drought-related advantages. However, conventional seed-based propagation does not provide the same degree of genetic control and clonal uniformity as vegetative propagation. For selected genotypes, a reliable clonal propagation system could contribute to the production of trees with superior properties. Somatic embryogenesis (SE) enables the regeneration of complete plants from somatic cells and provides a promising

approach for the large-scale clonal propagation of selected conifer genotypes. For hybrid larch, an SE protocol was established based on earlier work on *Larix decidua* Mill. and subsequently adapted in order to multiply selected genotypes (Nagmani and Bonga, 1985; Kraft and Kadolsky, 2018).

Despite this potential, the establishment of an SE protocol does not automatically result in an operational production system for forestry so we aimed for an improved pipeline from culture initiation up to nursery- and field-testing *Larix* × *eurolepis* (i) maintaining stable embryogenic cultures and overcoming losses during initiation and maintenance, (ii) obtaining sufficient numbers of mature embryos at a defined point in time, (iii) achieving reliable transfer of regenerated plantlets to *ex vitro* conditions, and (iv) maintaining unambiguous genetic identity throughout propagation and field evaluation.

In this article, we draw on fifteen years of experience to highlight selected critical bottlenecks that have emerged throughout our work. Among the various challenges identified, we focus on four key areas, describing the progress made, the practical approaches developed to address them, and the limitations that remain.

2. MATERIALS AND METHODS

2.1. Plant material and initiation of embryogenic cultures

We used immature zygotic embryos at the precotyledonary to cotyledonary stage of hybrid larch (*Larix* × *eurolepis* Henry) originating from controlled crossings or open pollination of selected mother trees for the initiation of SE (protocol, see Kraft and Kadolsky, 2018). Briefly, seeds were surface-sterilized, dissected under sterile conditions, and the megagametophytes containing the immature embryos were placed on 1/2 LMp (Kim, 2015) initiation medium. Cultures were kept in darkness at 23 °C and monitored for the development of embryogenic tissue. Established embryogenic cultures were maintained and proliferated on MSG-1 medium. For long-term conservation, successfully established embryogenic cultures were cryopreserved. Somatic embryos were matured on maturation medium and transferred to germination medium for conversion into plantlets according to Kraft and Kadolsky (2018) with modified KNO_3 content (according to Gupta and Holmstrom, 2005).

2.2. Maintenance of embryogenic cultures and maturation

To analyze the influence of culture duration on maturation outcome, different embryogenic culture lines were maintained in parallel and sampled after different periods of subculture. The number and developmental quality of mature embryos were recorded for the respective culture lines. The duration of subculture was related to the production of normally developed cotyledonary embryos. Parallel subculturing of embryogenic lines was used as a practical approach to reduce the risk that maturation capacity would be lost in a single continuously maintained line. In addition, successfully established embryogenic cultures were cryopreserved at an early stage to provide a backup of valuable genotypes.

2.3. Germination and acclimation

To improve the efficiency and uniformity of plantlet production, synchronized germination was applied. Plantlets were transferred to soil and acclimatized at once under controlled humidity before subsequent nursery cultivation.

Acclimation conditions were adjusted with respect to duration, water supply and embling age. The establishment and subsequent development of regenerated plantlets were monitored during the acclimation and nursery phases. Mortality after acclimation was recorded under the current nursery procedure.

2.4. Genotype characterization and fingerprinting

Unambiguous identification of genotypes based on morphological characteristics is not very reliable, particularly during the embryonic and early growth stages. Therefore, a molecular characterization is required to ensures that plant material can be verified throughout all stages of development.

Short interspersed nuclear elements (SINEs) are non-autonomous retrotransposons that can occur at high copy numbers. They are widely dispersed, occur within or close to other repeats as well as in coding regions, and show pronounced sequence and copy-number variation even between closely related species (Seibt *et al.*, 2020), making them suitable candidates for repetitive-element-based markers. To identify both known and species-specific SINEs in *Larix kaempferi*, the recently published genome assembly (GCA_027924585.1) was screened de novo using SINE-Finder (Wenke *et al.*, 2011), as similarity-based searches alone depend on existing repeat libraries and may therefore miss lineage-specific elements. Candidates were detected based on characteristic structural features, including RNA polymerase III promoter boxes A and B, a 3′ poly(A) or poly(T) stretch, and flanking target-site duplications (TSDs), yielding 438,973 initial hits. Candidate families were subsequently quantified by BLAST, followed by manual curation to remove truncated, degraded or otherwise incomplete copies, including sequences lacking characteristic promoter regions. For each curated family, multiple-sequence alignments, consensus sequences, and pairwise sequence-identity matrices were generated to assess within-family conservation. Copy number, sequence conservation, and structural integrity were then used to select suitable SINE families for primer design to function as molecular markers.

In a standard PCR reaction, these primers can be used in different combinations to amplify DNA segments which are located between two adjacent SINE copies. These Inter-SINE amplified polymorphisms (ISAPs) can be visualized using agarose gel electrophoresis. Due to the dispersed distribution of SINE copies throughout the genome, ISAP-markers form fingerprint-like banding patterns and the resulting length polymorphisms can be detected and assigned to the respective genotype in a database. The utility of ISAP markers for the identification and differentiation of clones has already been demonstrated for potato (Seibt *et al.* 2012) and poplar (Reiche *et al.* 2021). After selection of informative primer combinations, all successfully induced embryos were genotyped. In addition, stability tests were conducted at various developmental stages. Currently, eight primer combinations are available that will enable the unambiguous verification and identification of hybrid larches.

3. RESULTS

3.1. From SE protocol establishment to a hybrid larch SE platform

Over the last years, a reproducible SE system for hybrid larch was established and progressively transferred towards practical application. Starting from the initiation of the

first embryogenic cultures of *Larix decidua* in 1993 (based on Nagmani and Bonga, 1985), the work resulted in an effective SE protocol, a cryopreserved clone collection containing more than 300 genotypes, and the establishment of field trials for the evaluation and approval of selected clones (Fig. 1).

These developments demonstrate that the basic SE pipeline can be run at a scale relevant to practical clonal production. However, the long-term experience also revealed that individual steps remain sensitive to genotype, culture duration and production conditions. The following sections therefore focus on specific bottlenecks identified during this development and the approaches used to mitigate them.



Fig. 1. Timeline from the establishment of SE cultures to clone collection, field-testing and clone approval

3.2. Identification and mitigation of bottlenecks along the SE pipeline

The long-term development of hybrid larch SE revealed recurring constraints at several stages of the process chain (Fig. 2). During initiation and maintenance, excessive callus formation led to culture losses and reduced the embryogenic potential. Because activated charcoal (AC) reduced callus growth in embryogenic cultures of *Pseudotsuga menziesii* (Walther *et al.* 2022) its integration into the maintenance protocol of Larix × eurolepis is currently being evaluated.

During maturation, embryo yield can be strongly affected by the duration of subculture, highlighting culture age as an important factor for maintaining maturation capacity (Li *et al.* 2013). Apart from cryopreservation, maintaining several culture lines in parallel was therefore adopted as a practical precaution: if maturation capacity declines in one line, other lines may still provide sufficient embryos at the required time.

At the conversion and acclimation stage, synchronized germination was implemented to facilitate more uniform plantlet production. Acclimation success was further influenced by the duration of acclimation, water supply and embryo age. Under the current nursery procedure, mortality after acclimation was approximately 10–20%, while around 135,000 plantlets were available at the nursery level.



Fig. 2. The hybrid larch SE pipeline showing specific bottlenecks identified at each stage and the approaches developed to address them

3.3. Time in subculture affects maturation success

The maturation experiment showed how time in culture can influence the predictability of embryo production. As expected from previous observations (e.g. Li *et al.*, 2013), longer periods were negatively associated with maturation success in terms of embryo number. The results showed that independent culture lines differed in the number of normally developed cotyledonary embryos and that maturation capacity changed with increasing culture time (Fig. 3). Thus, maintaining a single culture line over an extended period does not guarantee a stable supply of mature embryos.

Accordingly, parallel subculturing was adopted to minimize the risk of losing high-performing lines and to maintain access to cultures with sufficient maturation capacity. Combined with early cryopreservation, this approach provides a practical strategy to manage variation in embryo production and to preserve valuable embryogenic material.

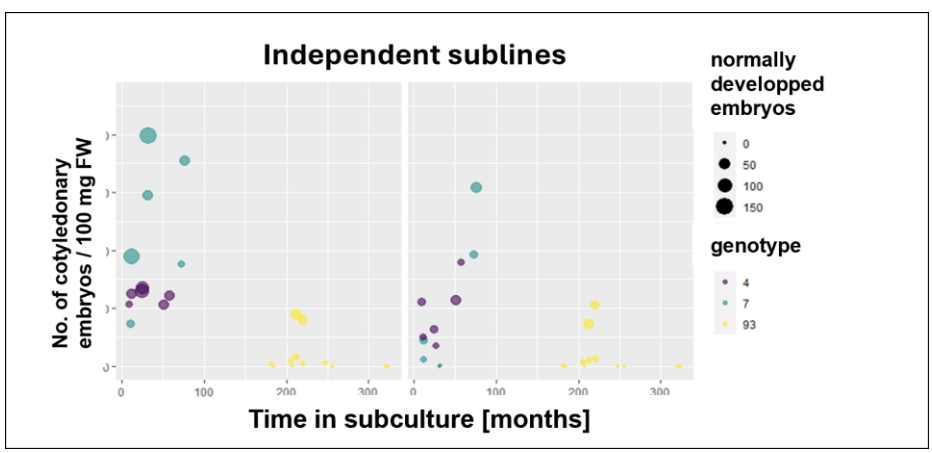


Fig. 3. The scatterplots show the number of normally developed and deformed mature embryos against the duration in subculture (months); point size represents the number of normally developed embryos

3.4. Molecular fingerprinting supports genotype identification and clonal deployment

Molecular ISAP-fingerprinting was used to characterize hybrid larch genotypes and to support their unambiguous identification. Eight polymorphic SINE-based primer

combinations were selected to distinguish between more than 110 genotypes. All fingerprints are stored in the BioNumerics database and can be accessed to identify unknown plant material or to verify the identity of tissues and plants. At least five ramets were examined of each genotype to ensure the stability and reproducibility of the fragment patterns. Additionally, different cell lines and developmental stages -from embryonic cultures to seedlings - were analyzed to verify identity of the fingerprints.

As an example, the analysis of 13 genotypes using two ISAP primer combinations revealed distinct molecular profiles and enabled the genotypes to be differentiated but ramets of genotypes 017.24 and 048.24 show similarity of 100% in the fragment patterns (Fig. 4).

Molecular characterization therefore provides an additional level of traceability linking *in vitro* material to subsequent nursery production and field-testing.

Together with growth and drought-response data and field trials across different environments, molecular fingerprinting contributes to the characterization and selection of clones for future deployment.

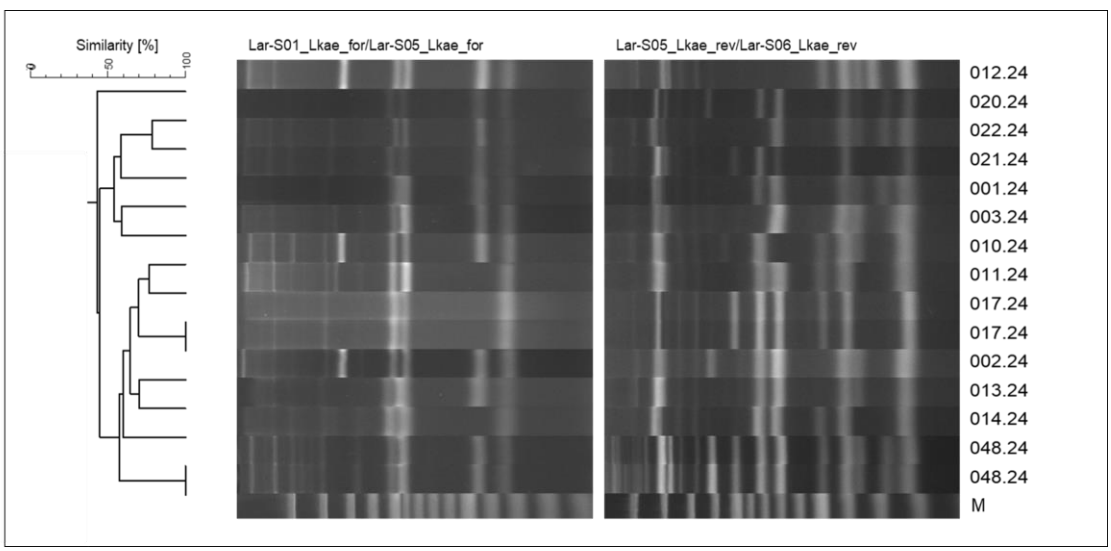


Fig. 4. Molecular fingerprinting of different hybrid larch genotypes (numbers shown on the right side) using two primer combinations (shown above the fragment patterns). The dendrogram on the right was constructed using UPGMA-method based on Jaccard- similarities of the band classes

4. DISCUSSION

Hybrid larch SE research has taken several years to evolve from an experimental regeneration technique into an integrated platform that connects genotype selection, clonal propagation, molecular characterization, nursery production and field-testing. At the same time, the long-term perspective makes clear that successful implementation does not depend on solving a single technical step. Instead, several recurring bottlenecks have to be managed simultaneously.

4.1. Initiation and maintenance: increasing stability and reducing callus growth

The establishment and maintenance of embryogenic cultures is strongly genotype-dependent. In particular, callus formation can compromise culture stability and embryogenic potential. The current evaluation of activated charcoal therefore represents a practical approach to reducing this observed cause of culture decay. This intervention can improve culture performance, but the biological mechanisms underlying differences in initiation and long-term embryogenic competence remain insufficiently understood.

4.2. Maturation: managing culture effects and embryo number

The results showed that culture duration is an important determinant of maturation performance. Longer periods in subculture were associated with lower numbers of normally developed embryos, supporting previous observations that maintenance conditions and culture age can influence embryogenic potential (Li *et al.*, 2013).

The parallel-line approach represents a fail-safe strategy for production: maintaining several lines with different culture histories increases the likelihood that at least one line retains sufficient maturation capacity when embryos are required. Early cryopreservation further reduces the risk of permanently losing valuable genotypes. At the same time, the strong influence of time in subculture raises the fundamental question about the (genetic) mechanisms responsible for declining embryogenic competence during prolonged culture.

4.3. Acclimation: improved for nursery scale

During acclimation the problem is the reliable conversion of emblings into nursery plants. Synchronized germination and subsequent acclimation improved the uniformity of plantlet production, while acclimation duration, water supply and embryo age affected establishment.

The current mortality of approximately 10–20% reflects the improved procedure. The availability of approximately 135,000 plantlets at nursery level proves that the current procedure is already capable of supporting production at a relevant scale.

Therefore, the acclimation problem can be regarded as partially addressed. Further optimization should aim not only at increasing survival but also at reducing variation in plant quality and production costs.

4.4. Fingerprinting: genotype traceability and verification

Genotype identification represents a quality-control challenge that can be addressed directly with molecular markers. Morphological identification is supported by clear molecular assignment during embryogenic and also adult stages as long-term propagation and planting increases the risk of losing or confusing labels.

The ISAP system provides an independent genetic identifier that can be stored in a database and used to verify plant material. The analysis of more than 110 genotypes demonstrates that the approach is applicable at a considerably broader scale than the 13 genotypes shown as an example in Fig. 4. The additional analysis of different developmental stages and multiple ramets supports the reproducibility and stability of the fingerprints.

This creates an important link between laboratory propagation and later forestry applications: plant material can be genetically verified.

5. CONCLUSIONS

Taken together, these four approaches highlight different levels of progress and remaining challenges across the SE pipeline: Initiation and maintenance remain strongly genotype-dependent and require further optimization; maturation can be managed through parallel culture lines and cryopreservation, but remains influenced by time in culture; acclimation has reached a production-relevant level but still involves losses; and molecular fingerprinting provides a robust tool for genetic traceability.

The SE system is sufficiently developed to support large-scale clonal production, nursery cultivation and field evaluation, but a fully standardized and cost-efficient transfer to operational forestry has not yet been achieved.

The remaining limitations also define the next research questions. A better understanding of culture age, somaclonal variation, genotype-dependent recalcitrance during initiation and the mechanisms underlying loss of maturation capacity could allow future protocols to move beyond empirical optimization towards more predictable control of the SE process.

At the same time, the practical relevance of the system is demonstrated by the transition from laboratory research to large-scale nursery production and field evaluation. The combination of molecular traceability, clonal propagation and multi-environment testing provides a setting in which selected hybrid larch genotypes can be evaluated not only for their performance *in vitro* but also for their potential value in future forestry.

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Multiplication of *Melaleuca alternifolia* shoots in temporary immersion: Effect of genotype, cytokinin and bioreactor type

Bruce Christie[1], Anxela Aldrey[2], Conchi Sánchez[2], Jesús M Vielba[2], M José Cernadas[2], Saleta Rico[2], Nieves Vidal[2]

[1]Greenplant Co, Palmerston North 4410, New Zealand

[2]Department of Plant Production, Misión Biológica de Galicia, Sede Santiago de Compostela. CSIC, Avda de Vigo s/n, 15705, Santiago de Compostela, Spain

brucechristie101@gmail.com

Abstract

Melaleuca alternifolia (Maiden & Betche) Cheel is an Australian tree widely cultivated for its essential oil. This study investigates the feasibility of culturing melaleuca shoots with liquid medium in temporary immersion bioreactors. We carried out a preliminary evaluation of the effect of the genotype, the type of bioreactor, the type of cytokinin, and the duration of the subculture. The results showed that melaleuca shoots can be successfully cultured in liquid medium, and extending the subculture duration from 4 to 7 weeks increased shoot production and quality. We obtained high multiplication coefficients with all the genotypes, bioreactors and cytokinins, although we observed differences between them. Shoots developed spontaneous roots in the bioreactors, making it possible to acclimatize them without auxin treatment. This study represents a starting point to develop an efficient protocol for culturing melaleuca shoots in bioreactors.

Keywords: *meta-topolin, Plantform™, RITA®, tea tree.*

1. INTRODUCTION

The Myrtle family comprises a large group of more than 120 genera and about 4000 species, many of which are woody plants originating predominately in the warm/temperate parts of the southern hemisphere with only one genus (Myrtus) indigenous to Europe. *Melaleuca alternifolia* (Maiden & Betche) Cheel or "tea tree" is native to New South Wales, Australia, where it grows almost always in moist areas and selection of genotypes with higher oil content is possible (Homer *et al.,* 2000). Australia is the largest producer of pharmaceutical grade oil in an internationally expanding market. *Melaleuca* spp. typically have limited seed production and are often recalcitrant to vegetative propagation by cuttings, which is a potential restriction on industry growth. In this context, the application of micropropagation techniques represents a promising approach for mass-production of this species, with several studies reporting good results using semisolid medium (Chen *et al.,* 2016; Gonzales-Alvarado and Cardoso, 2024; Tian *et al.,* 2025). Temporary immersion bioreactors based on liquid medium have the potential of increasing plant production and quality with reduced costs (Vidal *et al.,* 2026), but to the best of our knowledge in the case of melaleuca production they have been explored only scarcely, with two reports using liquid medium in jars (de Oliveira *et al.*, 2010) or in bioreactors (Scheidt *et al.,* 2011).

The objectives of this study were to evaluate the effect of the cytokinin type and the bioreactor type on the multiplication of melaleuca shoots and their competence for rooting and acclimatization. We used ten genotypes established from seedings obtained from a single mother tree and cultured them in commercial bioreactors RITA® or Plantform™ for 4 or 7 weeks, either with N^6-benzyladenine (BA) or meta-topolin (mT). All genotypes could be cultured in liquid medium with high multiplication coefficients, with increased shoot production and quality when subculture duration was extended from 4 to 7 weeks. Although there were some differences in shoot development regarding the bioreactor and the cytokinin type, roots were developed spontaneously in the multiplication medium and plantlets acclimated successfully. Next experiments will study the effect of genotype and culture treatments on the production of bioactive compounds.

2. MATERIALS AND METHODS

2.1. Materials

We used ten melaleuca genotypes established in semisolid Murashige & Skoog (MS) medium (Murashige and Skoog, 1962) four months after seed germination. Shoots were cultured in MS with 2.22 µM BA, 2.69 µM α-naphthaleneacetic acid (NAA), 3% sucrose and 0.7 % agar for four years before starting the experiments in liquid medium (LM).

2.2. Methods

For temporary immersion, 15 mm apical sections were grown in MS with 1.11 µM cytokinin (BA or mT), 2.69 µM NAA and 3% sucrose in RITA® or Plantform™ bioreactors. Shoots were immersed 6 times per day for two min; in Plantform™ additional aerations of 2 min were applied after every immersion. Each RITA® contained 200 mL of LM and 16 explants, whereas Plantform™ contained 500 mL and 40 explants. The bioreactors were sub-divided with aluminium foil to hold four genotypes each (Figure 1). The number of explants for each genotype and treatment was four in RITA® and ten in Plantform™. The effect of the cytokinin type was evaluated in RITA® bioreactors, and the effect of the bioreactor was evaluated only with shoots cultured with BA.

The acclimatization step was performed using shoots cultured during seven weeks. Well developed shoots with spontaneously formed roots were transferred to soil-less substrate (peat:perlite) in trays inside plastic boxes.

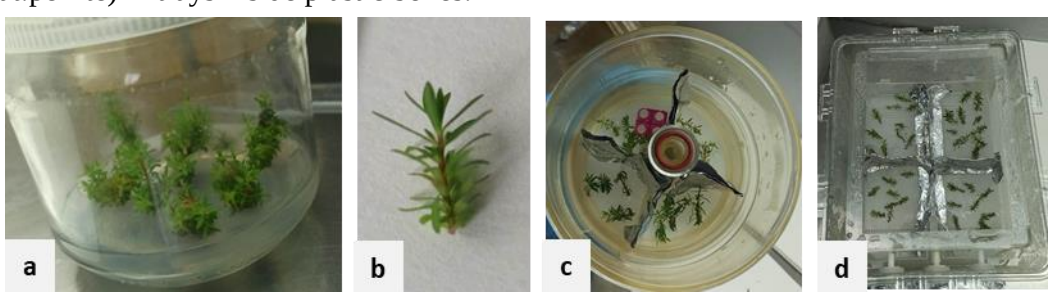


Fig. 1. Initial plant material for experiments in bioreactors: a) shoots growing in semisolid medium; b) donor explant from semisolid medium used to initiate the culture in bioreactors; c,d) RITA® (c) and Plantform™ (d) bioreactors subdivided with aluminium foil to hold 4 genotypes each.

2.3. Data recording

For data recording the following parameters were analyzed after four and seven weeks in LM: (a) the number of shoots longer than 8 mm per explant (SN); (b) the length of the longest shoot per explant (SL); (c) multiplication coefficient, defined as the number of apical or nodal sections useful for multiplication obtained per initial explant (MC); (d) the number of rootable shoots obtained per initial explant, defined as non-hyperhydric shoots longer than 20 mm and with an active apex (RS). In the graphs, the columns represent the means and the bars represent the standard errors. Due to the small sample size of some experiments, and the differences in the number of shoots of RITA® and Plantform™ bioreactors we could not apply statistical methods at this stage of the study.

3. RESULTS

Melaleuca shoots grew and developed successfully in bioreactors. The effect of extending the subculture period from 4 to 7 weeks is illustrated in Figure 2 with a photograph of a Plantform™ bioreactor at 4 and 7 weeks. Increasing the subculture duration positively affected all the growth parameters. On average (all genotypes, treatments and culture systems), SN was increased in 4 units, MC in 7 units, RS in 3 units and SL in 11 mm. In next graphics we present the data corresponding to 7 weeks of culture.

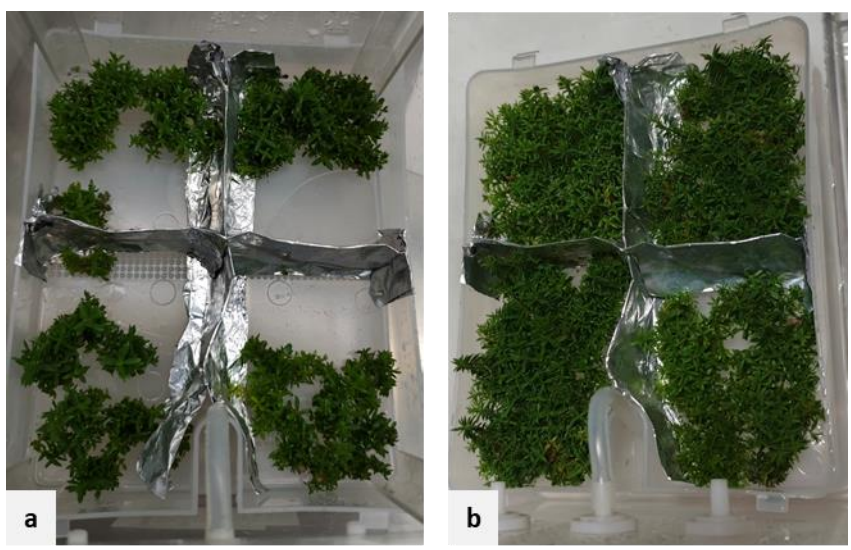


Fig. 2. Effect of extending the culture duration in Plantform™ bioreactors. a) Shoots after 4 weeks, b) shoots after 7 weeks

The influence of cytokinin type was less clear than the subculture duration. All genotypes grew well with BA or mT, but we observed some differences in the behavior of some of them. For example, shoots from clone 8 did not grow more than 3-4 mm in medium with BA when cultured in RITA®, but produced good shoots with mT (Figure 3, Figure 4). On the contrary, clone 12 grew much better with BA than with mT, producing more shoots, higher MC and more rootable shoots (Figure 3a-d).

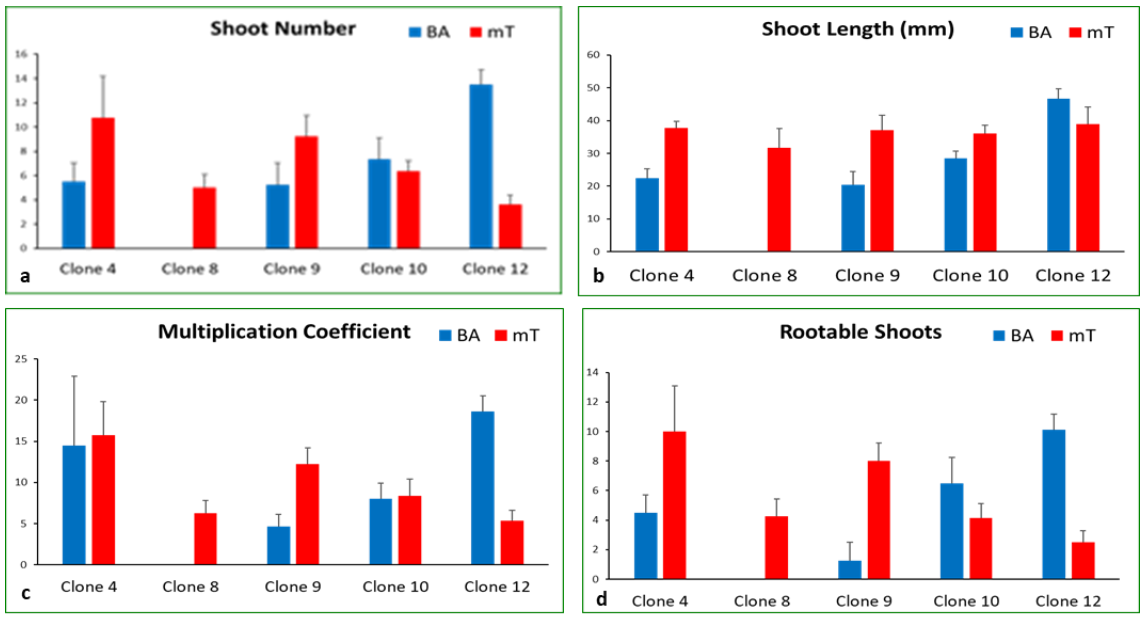


Fig. 3. Effect of the cytokinin type in shoots of 5 melaleuca clones cultured in RITA bioreactors. Shoots were cultured with 1.11 µM cytokinin (BA or mT), 2.69 µM NAA and 3% sucrose for 7 weeks.

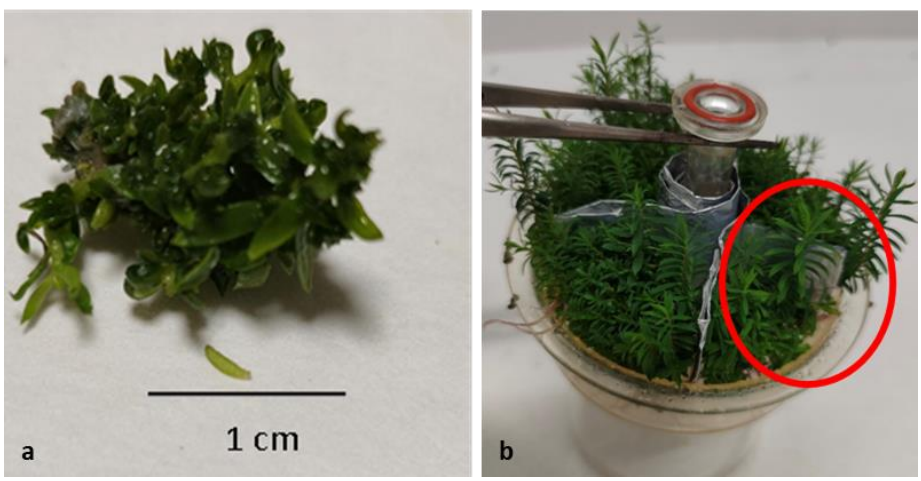


Fig.4. Shoots of clone 3 in RITA® after 7 weeks of culture with BA (a) or with mT (b, red circle)

The effect of the bioreactor was evaluated with shoots cultured with BA and it is shown in Figure 5.

On average, tea tree shoots responded similarly in RITA® and Plantform™, with MC values ranging between 9 and 36. In particular cases (like clone 3), RITA® was clearly superior (MC = 36), but the growth in Plantform™ was still acceptable (MC = 15).

Rooted shoots were produced on many of the shoots during the multiplication step, either with BA or with mT (Figure 6a). We developed a preliminary trial of acclimatization including shoots with or without visible roots (Figure 6b, c), which resulted in an approximate average success of 65% after 2 months (data not shown).

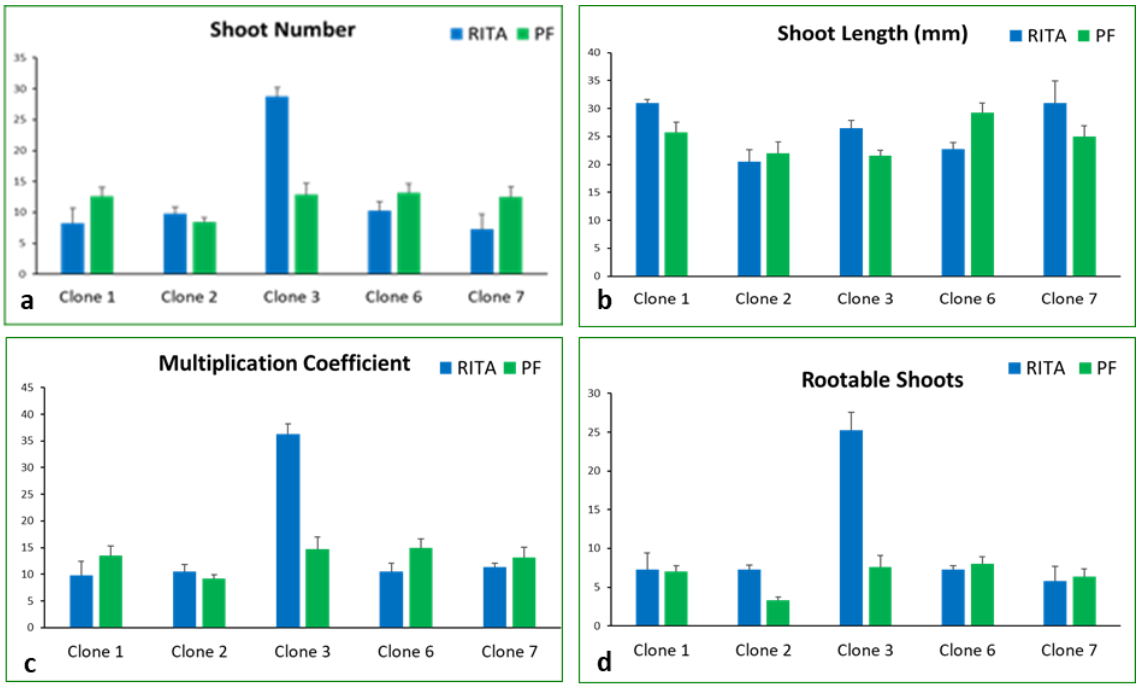


Fig. 5. Effect of the bioreactor type (RITA® or Plantform™ (PF)) in shoots of 5 melaleuca clones cultured with 1.11 µM BA, 2.69 µM NAA and 3% sucrose for 7 weeks.

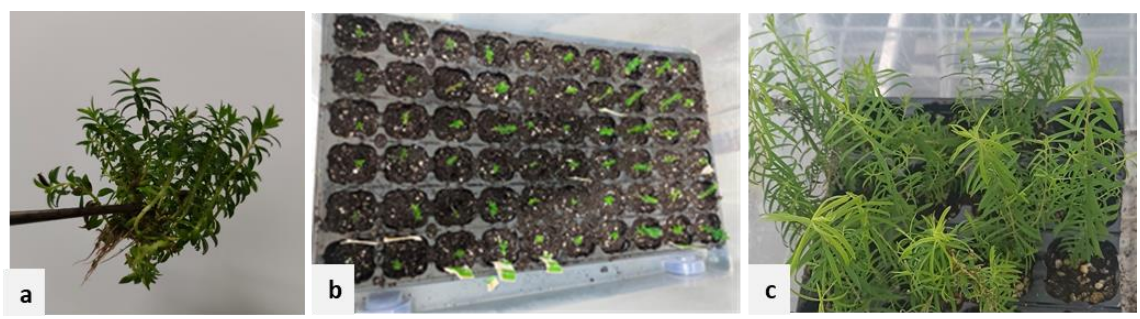


Fig. 6. Acclimatization of melaleuca plantlets obtained in bioreactors. (a) rooted shoots of clone 3 cultured in RITA® with mT. (b,c) Acclimatization at day 0 (b) and 2 months afterwards (c).

4. DISCUSSION

In this study we have shown the feasibility of culturing shoots from ten genotypes of *Melaleuca alternifolia* in two types of bioreactors, RITA® and Plantform™. We obtained good quality shoots in high numbers, as well as acclimatized plantlets. We report higher

multiplication coefficients that previous studies using semisolid medium (Chen *et al.*, 2016; Gonzales-Alvarado and Cardoso, 2024; Tian *et al.*, 2025), and RITA® bioreactors (Scheidt *et al.*, 2011). All these reports used BA as a cytokinin, but here we analyzed the effect of mT as well, which resulted in good growth in all genotypes tested. As a novelty, we explored the use of Plantform™, which can hold more explants and facilitate large-scale propagation.

Our results indicate that extending the culture period to 7 instead of 4 weeks increases productivity. Also, we detected interactions between genotype, cytokinin type and bioreactor type. Being preliminary, our conclusions are limited by the small sample size we used in RITA® bioreactors (four initial apical sections per genotype) and the difference in size with Plantform™ bioreactors (ten initial apical sections per genotype), which did not allow us to perform statistical tests. Our first objective was to apply our methodology to a large number of genotypes (ten) to prepare us for designing a deeper study in the foreseeable future. These studies are already in progress, and include increased sample size, as well as testing more cytokinin levels both on semisolid and liquid medium.

5. CONCLUSIONS

Culture of *Melaleuca alternifolia* shoots in bioreactors is a promising approach for large-scale propagation of this species. In this preliminary study, we showed that both RITA® and Plantform™ are suitable vessels for propagation, and that the cytokinin type (BA or mT) can influence multiplication depending on the genotype. More studies will be carried out to detect and understand these interactions.

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Evaluation of CKX inhibitors in the presence of trans-zeatin during micropropagation of *Prunus domestica* L. cv "Tropojane"

Brunilda Çuko[1], Ledina Shkëmbi[1], Stefaan P. O. Werbrouck[2], Maurizio Lambardi[3], Jaroslav Nisler[4], Efigjeni Kongjika[5], Valbona Sota[1,5]

[1]*Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Albania*

[2]*Ghent University, Belgium*

[3]*IBE-Institute of BioEconomy, National Research Council, Italy;*

[4]*Isotope Laboratory, Institute of the Experimental Botany of the Czech Academy of Sciences, Prague, Czechia*

[5]*Research Center of Biotechnology and Genetics, Academy of Sciences of Albania, Tirana, Albania*

cuko.brunilda@gmail.com

Abstract

Cytokinin oxidase/dehydrogenase plays a crucial role in maintaining cytokinin homeostasis in *in vitro* plants. Four compounds identified as CKX inhibitors were studied for their effect on the micropropagation of *Prunus domestica* L. cv. Tropojane. A concentration of 1µM of each compound CKX 19, CKX 20, CKX 52 and CKX 77, was added separately to QL nutrient medium supplemented with 4.4 µM trans-zeatin (tZ) and 0.537 µM naphthaleneacetic acid (NAA). These tests were organized in parallel using semi-solid nutrient medium and a TIS bioreactor "Plantform™" with liquid nutrient medium. After 28 days of culture, the relative growth rate, shoot number and shoot length were evaluated and compared with those of plants grown on nutrient media supplemented with different concentrations of tZ (0, 4.4, 6.7 and 8.9 µM) combined with 0.537 µM NAA in each case. Results demonstrated that the presence of trans-zeatin in both liquid and semi-solid nutrient media promoted the apical dominance of plum shoots and callus formation on the shoot bases. No significant differences were observed when using different concentrations of tZ, nor among the plants grown in the presence of CKX inhibitors. This finding suggests that the exogenous addition of tZ up to 8.9 µM or the inhibition of cytokinin oxidase/dehydrogenase when using CKX19, CKX20, CKX52 or CKX77 does not improve the micropropagation of *Prunus domestica* L. cv. Tropojane. However, regardless of chemical composition of nutrient medium, plants grown in Plantform™ bioreactor showed better performance in all evaluated parameters compared with those grown on semi-solid nutrient medium.

Keywords: *Prunus domestica L., CKX inhibitors, trans-zeatin, TIS bioreactor*

1. INTRODUCTION

Prunus domestica L. cv "Tropojane" is one of the most important plum cultivars cultivated in Albania and is widely used in the food industry due to its high nutritional values. Given its economic and agronomic importance there is a need to develop efficient methodologies for the commercialization and large-scale production of this plum cultivar. Although efficient protocols for the *in vitro* micropropagation of *P.domestica* have been frequently reported (Ambrozic Turk *et al.,* 1992; Yuan *et al.*, 2009; Wolella, 2017; Kastritskaya *et al.,* 2018) limitations still remain, particularly regarding the species- and genotype-dependent responses of tissue culture, multiplication efficiency and the quality of regenerated plants. To overcome these limitations, several studies have investigated the optimization of

different *in vitro* culture parameters, including nutrient medium composition (Baziuk & Kobyletska, 2025), the type and concentration of plant growth regulators (Wolella & Dagnaw, 2017), light sources (Nacheva *et al.,* 2023) and cultivation systems (Gago *et al.,* 2022). Among these approaches, shifting from the conventional micropropagation system in glass jars with semi-solid nutrient medium to temporary immersion bioreactors using liquid nutrient medium has been considered a promising alternative for improving the efficiency and scalability of *in vitro* plant production, although several operational and biological parameters need to be optimized (Etienne & Berthouly, 2002; Carvalho *et al.,* 2018).

Regardless of the cultivation system used, the type and concentration of plant growth regulators (cytokinin-auxin combination) as well as the endogenous regulation of cytokinin homeostasis are important determinants of the morphogenic response of cultured plants. Cytokinin oxidase/dehydrogenase (CKX) enzymes play a crucial role in the degradation of cytokinins within plant cell. Genetic or chemical inhibition of these enzymes represents a promising strategy for improving the micropropagation process. Nisler *et al* (2021, 2022, 2024) presented a series of chemical compounds known as candidate CKX inhibitors (CKX19, CKX20, CKX52, CKX77) which inhibit cytokinin degradation in tobacco leaf disks.

Based on these considerations, this study aims to evaluate the effects of these four candidate CKX inhibitors in combination with exogenous trans-zeatin on the micropropagation of *Prunus domestica* L. cv "Tropojane" using both a conventional micropropagation system and a temporary immersion bioreactor (Plantform™).

2. MATERIALS AND METHODS

2.1. Experimental design

The micropropagation of *Prunus domestica* L. cv "Tropojane" explants was evaluated using three different concentrations of trans-zeatin (tZ) (4.4, 6.7 and 8.9 µM) combined with a fixed concentration of naphthaleneacetic acid (NAA) (0.537 µM) and compared with a control treatment where no plant growth regulators were added. After evaluating how the micropropagation efficiency changes by increasing the trans-zeatin concentration in the nutrient medium, the results were compared with the same evaluations done on plants grown in nutrient medium supplemented with trans-zeatin – NAA combination and 1 µM of each of four candidate CKX inhibitors (CKX19, CKX20, CKX52 and CKX77) applied separately. All experiments described above were conducted in glass jars containing semi-solid nutrient medium and in parallel, in Plantform™ bioreactors to compare the two micropropagation systems.

2.2. Plant material

The plant material used in this study was selected from an *in vitro* virus-free collection of *P. domestica* L. cv "Tropojane" plantlets established and maintained in the Laboratory of *In vitro* Tissue Culture of Plants, Department of Biotechnology, Faculty of Natural Sciences, University of Tirana. Uniform shoot tips measuring 1-2 cm in length grown in semi-solid QL nutrient medium free of plant growth regulators were carefully inoculated into glass jars (10 shoot tips per jar) or horizontally placed in the specific container of the Plantform™ bioreactor (20 explants per bioreactor).

2.3. Nutrient medium

QL (Quoirin & Lepoivre, 1977) nutrient medium was used in all experiments and supplemented with 100 mg/L myo-inositol, 0.537 µM naphthaleneacetic acid (NAA), 3% (w/v) sucrose and 0.7% (w/v) agar for the semi-solid nutrient medium. A volume of 100 mL semi-solid nutrient medium was used in each glass jar and 500 mL of liquid nutrient medium for micropropagation in the TIS bioreactor. The liquid medium was autoclaved inside the Plantform™ bioreactor container at 121°C and 1.05 kg/cm² for 20 min. The specific volumes of trans-zeatin and four candidate CKX inhibitors were filter-sterilized and added to the medium at laminar flow cabinet after autoclaving.

2.4. Growth conditions

After the manipulations under aseptic conditions in a laminar flow cabinet, the plum cultures were transferred to the growth chamber maintained at 23 ± 2°C with a 16-h photoperiod of 28–30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PPFD) fluorescent white light. Plants grown in the Plantform™ bioreactors were immersed in the liquid nutrient medium for 1 min every 8 hours followed by 15 min of aeration.

2.5. Data collection and analysis

After 28 days of cultivation, the fresh weight of the regenerated plantlets was measured and morphological and biometric parameters including shoot length, number of new formed shoots, relative growth rate (RGR) and spontaneous rooting percentage were evaluated. The relative growth rate (RGR) was then calculated as follows:

$$RGR = \frac{ln(FW2) - ln(FW1)}{T2 - T1} X\ 100$$

Equation 1: Relative growth rate (RGR). FW_1 and FW_2 represent the fresh weight at the beginning and end of the cultivation period, respectively, and T_1 and T_2 correspond to the initial and final time points (days).

The experiments were conducted using a completely randomized design (CRD) with three independent replications with 20 explants used per each replication. The collected data were subject to one-way analysis of variance (ANOVA) using OriginPro 2024. Means were compared using Tukey's honestly significant difference (HSD) test at a significance level of $p < 0.05$. Results are expressed as mean ± standard deviation (SD). Different letters indicate statistically significant differences among treatments.

3. RESULTS

3.1. Effects of different concentrations of tZ – NAA combination

Based on visual observations of regenerated plants grown at different concentrations of trans-zeatin (tZ) in combination with NAA it was observed that plants grown in the presence of tZ compared with the control treatment without PGRs, showed a larger leaf area (especially those grown in TIS bioreactor), increased shoot length and callus formation at the shoot bases (Fig. 1).

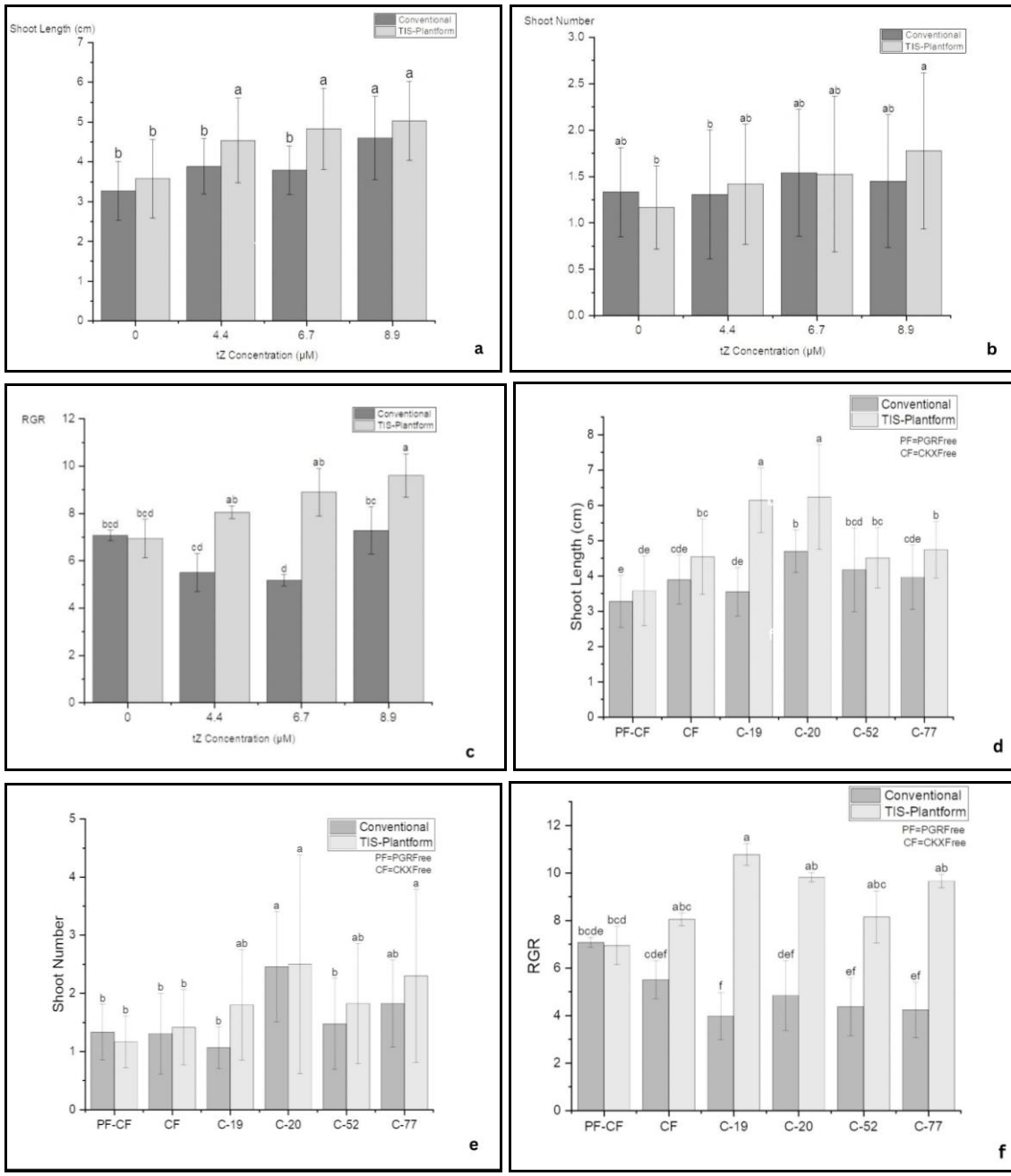


Fig. 1. *Prunus domestica* L. cv "Tropojane" plantlets grown in different concentrations of trans-zeatin. (a, e) control; (b, f) 4.4 µM tZ-NAA; (c, g) 6.7 µM tZ-NAA; (d, h) 8.9 µM tZ-NAA in Plantform™ bioreactor (a-d) and glass jars (e-h).

The effect of trans-zeatin concentration on the micropropagation of Tropojane plum was evaluated based on the shoot length after 28 days of cultivation, the number of new formed shoots and the Relative Growth Rate (RGR) as presented in the following graphs (Fig. 2 a-c).

The statistical analysis of shoots length showed that plants grown in the Plantform™ bioreactor with liquid nutrient medium supplemented with 8.9 µM tZ and NAA reached the highest shoot length (5.03 cm). Similarly, within the group of plants grown in the conventional system with semi-solid nutrient medium, the highest shoot length (4.60 cm) was recorded in plants grown in the presence of 8.9 µM tZ-NAA in the nutrient medium (Fig. 2a). Meanwhile the lowest shoot length was recorded in the control treatment for both TIS and conventional systems, at 3.58 cm and 3.27 cm respectively.

After the cultivation period, an average of 1.0 (PGRFree) to 1.8 (8.9 µM tZ-NAA) newly formed shoots per plantlet was observed. No significant differences in this parameter were detected among plants grown in TIS bioreactor or those grown in glass jars (Fig. 2b). The highest RGR value was recorded in *P.domestica* L. cv "Tropojane" plantlets grown in the Plantform™ bioreactor with liquid QL nutrient medium supplemented with 8.9 µM tZ-NAA (Fig. 2c). In all evaluated treatments, the RGR of plantlets grown in the temporary immersion bioreactor was higher than that of plants regenerated in the conventional micropropagation system.



Note: Means that do not share a letter are significantly different.

Fig. 2. Statistical evaluation of shoot length (a, d), number of newly formed shoots (b, e), and relative growth rate (RGR) (c, f) of regenerated plantlets grown in TIS and conventional micropropagation systems. Panels (a–c) show the effects of different tZ–NAA concentrations, whereas panels (d–f) show the effects of the control and 4.4 µM tZ–NAA treatments supplemented with four candidate CKX inhibitors (C-19, C-20, C-52, C-77).

3.2. Effects of candidate CKX inhibitors combined with tZ-NAA combination

The effect of candidate CKX inhibitors combined with 4.4 µM tZ-NAA on the micropropagation of *P.domestica* L. cv "Tropojane" plantlets was evaluated and compared with a control treatment without PGRs or the tested CKX inhibitors (PF=PGRFree, CF=CKXFree) as well as with a second control treatment in which the QL nutrient medium was supplemented with 4.4 µM tZ-NAA without the addition of candidate CKX inhibitors. Regarding shoot length of the regenerated plantlets, the highest value (6.23 cm) was recorded in plants grown in the Plantform™ bioreactor with liquid QL nutrient medium supplemented with 4.4 µM tZ-NAA and compound 20. Also, plantlets regenerated under this treatment developed the highest number of newly formed shoots (2.5 shoots per plantlet) in both micropropagation systems. The best relative growth rate (RGR=10.77) was obtained when compound 19 was combined with 4.4 µM tZ-NAA in TIS bioreactor. However, this result was not consistent under semi-solid nutrient medium conditions where plantlets grown under this treatment exhibited the lowest RGR value (3.97). Significant differences in shoot length and RGR were observed even when other compounds (C-20, C-52, C-77) were combined with tZ and NAA and added to either liquid or semi-solid nutrient media.

3.3. Root induction

After 28 days of cultivation, the effect of candidate CKX inhibitors on root induction in plum plants was investigated and compared with the control treatment in which no candidate CKX inhibitors were added to the QL nutrient medium supplemented with 4.4 µM tZ-NAA. Based on visual observations it was found that only plantlets grown in TIS bioreactors have developed short and strong roots (Fig. 3).



Fig. 3. Plum plantlets regenerated in Plantform™ bioreactor in the nutrient medium supplemented with 4.4 µM tZ-NAA and candidate CKX inhibitors.

However, root formation was observed only in plants grown in the treatments C-19 + 4.4 µM tZ-NAA (20%) and C-20 + 4.4 µM tZ-NAA (5%). The combination of compound 52 and 77 applied separately with 4.4 µM tZ-NAA as well as 4.4 µM tZ-NAA alone in the liquid QL nutrient medium did not result in root formation.

4. DISCUSSION

Trans-zeatin is a naturally occurring isoprenoid cytokinin. Many studies have reported the application of zeatin application in the *in vitro* tissue culture of *Vaccinium* species (Reed & Abdelnour-Esquivel 1991), *Arbutus andrachne* (Papafotiou, 2010), *Corylus avellana* L (Thomson & Deering, 2011) *Humulus lupulus* (Staats & Jenderek, 2026) mostly confirming its positive effect on shoot proliferation and elongation. In our study, the application of filter-sterilized trans-zeatin on QL nutrient medium promoted apical dominance of plum shoots and callus formation on the shoot bases. When trans-zeatin was added to the liquid nutrient medium for the micropropagation of plum shoots on Plantform™ bioreactor, no significant differences were observed among the different concentrations of the tZ-NAA combination. However, in the conventional micropropagation system using semi-solid nutrient medium, the effect on shoot elongation of plants was observed only at the highest concentration of trans-zeatin and NAA combination (8.9 µM). Similarly, Xi *et al.,* (2022) and Paprštein & Sedlák, (2015) reported the application of 1.5 mg/L to 2 mg/L zeatin for the growth of *Prunus avium* axillary buds and the multiplication of *Vaccinium vitis-idaea* L respectively. The differences noted between the TIS and conventional systems in response to trans-zeatin concentration may be related to its stability and sensitivity to temperature. The addition of filter-sterilized tZ on the semi-solid nutrient medium was done after autoclaving and before agar solidification. Although, Hart *et al.,* (2016) found that trans-zeatin remained stable after one autoclave cycle with 1x MS basal salts, the elevated temperature of semi-solid nutrient medium at the time of tZ addition may have reduced its biological activity of tZ. Our results also suggest that the formation of new shoots and the RGR of P.domestica L cv "Tropojane" is not affected by the presence or the concentration of trans-zeatin -NAA combination in the nutrient medium.

The combination of 4.4 µM tZ-NAA with candidate CKX inhibitors slightly improved shoot length in plum plants but did not have a significant effect on the formation of new shoots or relative growth rate compared with the corresponding control treatments. The presence of CKX inhibitors reduces the activity of cytokinin oxidase/dehydrogenase (CKX) enzyme which may lead to higher levels of cytokinin available within the plant tissues and consequently to an enhanced morphological response on plant development (Nisler *et al.,* 2024). Since our results demonstrated that increasing the trans-zeatin -NAA concentration up to 8.9 µM did not improve micropropagation efficiency as assessed by RGR, the application of potential CKX inhibitors would not be expected to further enhance this parameter. Among the tested candidate CKX inhibitors, compound 20 resulted to be the most effective by increasing the plant shoot length, stimulating the formation of new shoots and inducing root formation. However, it did not significantly affect the relative growth rate of plants compared with the control treatment when tZ-NAA was applied without the addition of candidate CKX inhibitors.

5. Conclusions

This study evaluated the effect of four potential CKX inhibitors on the micropropagation of *Prunus domestica* L. cv "Tropojane" using a nutrient medium supplemented with a fixed concentration of trans-zeatin and NAA in temporary immersion system bioreactor and a conventional micropropagation system. Our results demonstrated that compound 20 exhibited the greatest inhibition potential among the tested CKX inhibitors and that its application combined with exogenous trans-zeatin-NAA improved the micropropagation efficiency of plum plants. Moreover, the use of TIS bioreactors instead of conventional glass jars represents an efficient alternative for the large-scale production of plum plants. However, further studies are needed to optimize the operational and chemical parameters, especially by application of CKX inhibitors in the liquid nutrient medium.

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Improvement of *in vitro* propagation of 'Gisela 5' (*Prunus cerasus* × *P. canescens*) cherry rootstock using the ElecTIS bioreactor

Elektra Papakosta[1], Efigjeni Kongjika[2], Magadalena Cara[3]

[1]*Centre of Agricultural Technology Transfer (ATTC) Vlore, Shamogjin, Albania*

[2]*Albanian Academy of Sciences, Research Center "Biotechnology & Genetics"*

[3]*Agricultural University of Tirana, Faculty of Agriculture and Environment, Department of Plant Protection*

elektrapapakosta@gmail.com

Abstract

Gisela 5' (*Prunus cerasus* × *P. canescens*) is a widely used cherry rootstock commonly propagated *in vitro* to ensure uniform and healthy plant material. This study aimed to improve its micropropagation by comparing conventional agar-based culture with liquid culture in an ElecTIS temporary immersion bioreactor. Explants (1.0–1.5 cm) were sterilized and cultured on Murashige and Skoog medium supplemented with different concentrations of 6-benzylaminopurine (BAP): 0.5, 1.0, 1.5, 2.0 mg L^{-1}, combined with 0.1 mg L^{-1} gibberellic acid (GA_3) and 0.01 mg L^{-1} naphthaleneacetic acid (NAA). After three subcultures, the highest multiplication rate (80%) and mean shoot number (3.19 per explant) were obtained with 1.0 mg L^{-1} 6- benzyl aminopurine BAP. During rooting, 1.5 mg L^{-1} indole-3-butyric acid (IBA) produced the highest mean root number (2.55) and a rooting rate of 75%. The optimal multiplication medium was then tested in the ElecTIS bioreactor under three immersion regimes. An immersion frequency of 8 min every 8 h produced the highest relative growth rate (RGR, 3.07), exceeding those recorded on high-agar or low-agar media (2.31 and 1.88, respectively). These results indicate that ElecTIS improves biomass production and represents an efficient system for the *in vitro* propagation of 'Gisela 5'.

Keywords: *bioreactor; cherry rootstock; micropropagation; temporary immersion system*

1. INTRODUCTION

The clonal cherry rootstock 'Gisela 5' (*Prunus cerasus* × *P. canescens*) is propagated vegetatively using *in vitro* techniques to ensure the production of clean, virus-free plant material. Dwarf or semi-dwarf cherry rootstocks are used to establish suitable high-density planting systems (Long & Kaiser, 2010).

The 'Gisela 5' rootstock is significant for enhancing efficiency and yield in cherry orchards; it is suitable for calcareous and dry soils and exhibits high resistance to cold, excess moisture, and drought. It demonstrates good compatibility with tested cherry cultivars, high productivity in grafted varieties, good fruit quality, and resilience to climate change (Zenginbal *et al*., 2017; Milić *et al*., 2019; Świerczyński & Schroeter-Zakrzewska, 2024). The application of *in vitro* propagation techniques for vegetative rootstocks proves to be an efficient method, relying on the interaction of factors such as the nutrient medium, plant growth regulators (PGRs), temperature, and light.

Studies on cherry rootstocks have been conducted by several authors (Ružić *et al*., 2000; Nacheva & Gerçeva 2009; Clapa *et al*., 2013; Šiško, 2011; Kumar *et al*., 2020). Recently,

liquid culture techniques using temporary immersion systems TIS; (Lambardi, 2012; Welander *et al*., 2014) have been tested to reduce the costs of micropropagating various species. Bioreactors for TIS culture are categorized into single-vessel systems (RITA®, Plantform, ElecTIS) or twin-vessel systems (SETIS™) (Baltazar *et al*., 2026). ElecTIS (patent no. 2617282) is a novel TIS bioreactor design developed through a collaboration between Claudio Depaoli and the CNR-IBE in Sesto Fiorentino (FI). The Elec-TIS components are made of transparent, heat-resistant polypropylene and can be easily autoclaved (Capuana *et al*., 2018).

The primary objective of this study is to improve the scientific protocols for the *in vitro* micropropagation of the 'Gisela 5' cherry rootstock during the proliferation and rooting stages.

2. MATERIALS AND METHODS

2.1. Collection and inoculation of plant material:

Explants (apical and lateral buds) from the 'Gisela 5' rootstock were obtained from the fruit tree collection greenhouse. The explants were collected from new shoots, and inoculation was performed in April. The study was conducted at the *In vitro* laboratory of the Agricultural Technology Transfer Center in Vlora, Albania. For each treatment variant, the experiment involved ten sterile vessels each containing eight explants with three replicates. Data were recorded after three subcultures. The tissue cultures underwent the following stages: inoculation, multiplication, rooting, and acclimatization. After being cut to size (1 - 1.5 cm), the bud explants were washed with clean water, immersed in 70% ethyl alcohol for 2 minutes, disinfected with 10% sodium hypochlorite (NaOCl) for 20 minutes, and rinsed three times with sterilized, disinfected water to eliminate contamination (Munir *et al*., 2015). After disinfection, the explants were cultured on MS (Murashige & Skoog, 1962) nutrient medium containing MS macro- and micro-salts, FeEDTA chelate, and MS vitamins (inositol 100 mg L^{-1}, thiamine 0.1 mg L^{-1}, pyridoxine 0.5 mg L^{-1}, nicotinic acid 0.5 mg L^{-1}, glycine 2 mg L^{-1}), supplemented with varying concentrations (0.5, 1.0, 1.5, 2.0 mg L^{-1}) of 6-benzylaminopurine (BAP) combined with 0.1 mg L^{-1} gibberellic acid (GA_3) and 0.01 mg L^{-1} naphthaleneacetic acid (NAA), as well as 0.7% agar and 3% sucrose; the pH was adjusted to 5.7. After four weeks in the growth chamber, the developed shoots were transferred to the MS multiplication medium.

Proliferation phase: Cherry shoots were subcultured (three times) every 20 days on MS medium supplemented with benzylaminopurine (BAP) at the previously indicated concentrations; the multiplication rate, as well as the average number and length of shoots, were evaluated for each subculture. All trials were conducted in triplicate.

Rooting and Acclimatization Phase: During the rooting phase, the MS medium was tested with varying concentrations of indole-3-butyric acid (IBA): (0.5, 1, 1.5, and 2 mg L^{-1}). Subsequently, the shoot rooting rate, the average number of roots produced per explant, and the average root length were assessed. After 20 days of the rooting phase, the plantlets had developed a robust root system and were then transferred to a greenhouse specifically into an acclimatization tunnel using a substrate composed of sterilized peat (pH 6) and perlite (2:1 ratio).

2.2. Liquid culture with ElecTIS

The MS nutrient medium, containing the optimal hormone combination for the multiplication phase, was also used in liquid culture with an ElecTIS bioreactor, where various immersion frequencies were applied. The relative growth rate (RGR) was subsequently evaluated and calculated as follows:

(Final FW – Initial FW) / days of cultivation × 100 where FW represents fresh weight; the results were compared with cultures grown on a gelled medium (control). The starting material consisted of shoots micropropagated on the MS medium using the optimal parameters determined earlier: 0.1 mg L^{-1} GA_3, 0.01 mg L^{-1} NAA, 1.0 mg L^{-1} BAP, 0.7% agar, and 3% sucrose, with the pH adjusted to 5.7.

Comparisons were made between: (1) a control on a gelled substrate with 7.0 g L^{-1} agar; (2) a control on a semi-gelled substrate with 3.5 g L^{-1} agar both in 500 mL glass vessels containing 20 plants each; and (3) liquid culture using an ElecTIS bioreactor with three immersion cycles: (i) 8 min/8 h with 30 initial plants; (ii) 10 min/8 h with 35 plants; and (iii) 12 min/16 h with 30 plants. For all treatments, experiments were conducted on MS multiplication medium supplemented with 1.0 mg L^{-1} BAP, 0.1 mg L^{-1} GA_3, 0.01 mg L^{-1} NAA. After 40 days, the relative growth rate (RGR) was evaluated for each treatment.

Culture conditions. All experiments were conducted in a growth chamber with a photoperiod of 16 hours of light, a temperature of 24 ± 1°C, and a light intensity of 3500 lux.

Statistical analysis: Statistical analysis was performed using JMP and included analysis of variance (ANOVA, $P \leq 0.05$) and the Tukey-Kramer test.

3. RESULTS AND DISCUSSION

3.1. Micropropagation of Gisela 5 in agarized medium

Explant sterilization. After collection, the explants were disinfected and cut to the appropriate size. The response of each plant explant in the inoculation medium was analyzed and evaluated based on survival and infection percentages. For the Gisela 5 rootstock, inoculation onto MS culture medium after four weeks in the vegetative growth room resulted in a high survival rate (85%). The fungal infection rate was 10%, and the necrosis rate was 5%.

Proliferation Phase: Effect of BAP concentration on shoot proliferation of Gisela 5. This study demonstrated that BAP concentration had a highly significant effect on both the multiplication and elongation of 'Gisela 5' cherry rootstock shoots, as confirmed by one-way ANOVA ($p < 0.0001$). Among the concentrations evaluated, 1.0 mg L^{-1} BAP yielded the highest values for both shoot number (3.19 ± 0.11) and shoot length (2.70 ± 0.10) (Tab.1).

Increasing the BAP concentration beyond this level resulted in a progressive decline in the measured proliferation parameters. The stimulatory effect of BAP on axillary shoot proliferation aligns with the well-known role of cytokinin in promoting cell division and releasing axillary buds from apical dominance. The influence of cytokinin in tissue and organ culture depends on the nutrient medium, species, and explant age (Thorpe *et al*.,

2008; Sedeghi *et al.*, 2015). However, the response of Gisela 5 to BAP appears to depend on the concentration used and the composition of the culture medium (Xu *et al*., 2015; Thakur *et al*., 2016; Sharma *et al*., 2017).

Table 1. Effects of BAP concentrations (0.5, 1.0, 1.5, 2.0 mg L^{-1}) on shoot multiplication parameters and (0.1 mg L^{-1} GA_3, 0.01 mg L^{-1} NAA)

Treatment BAP (mg L^{-1})	**Number of Shoots (Mean ± SE)**	**Length of Shoots (cm) (Mean ± SE)**
V_1 (0.5 mg L^{-1})	2.06 ± 0.12^{c}	1.64 ± 0.11^{b}
V_2 (1.0 mg L^{-1})	3.19 ± 0.11^{a}	2.70 ± 0.10^{a}
V_3 (1.5 mg L^{-1})	2.52 ± 0.13^{b}	2.34 ± 0.09^{a}
V_4 (2.0 mg L^{-1})	1.43 ± 0.08^{d}	1.25 ± 0.08^{c}

Values correspond to Mean ± SE. Treatments followed by different letters are statistically different at p = 0.05

Previous studies on Gisela 5 have reported successful shoot multiplication across a relatively wide range of BAP concentrations. Fallahpour *et al*., (2015) reported that a BAP concentration of 2.0 mg L^{-1} yielded the highest number of shoots when comparing different basal media. Ružić *et al*., (2000) reported better growth and development of the Gisela 5 rootstock on MS medium compared to ½ MS and ¼ MS. Clapa *et al*., (2013) reported very high proliferation with 0.3 mg L^{-1} BAP in modified MS medium, whereas Borsai *et al*., (2020) reported better results on DKW medium with (0.3 - 0.5) mg L^{-1} BAP. Discrepancies between these findings and the results of the present study may be attributed to differences in basal medium composition, explant characteristics, culture conditions, and the physiological state of the plant material. The observed decline in the shoot multiplication rate at BAP concentrations of 1.5 and, particularly, 2.0 mg L^{-1} in this experiment suggests that excessive cytokinin concentration acts as an inhibitor of normal shoot development. High cytokinin levels can induce abnormal proliferation, inhibit shoot elongation, or disrupt the endogenous hormonal balance of the explants (Jimenez *et al*., 2007). Therefore, increasing the BAP concentration does not necessarily lead to a proportional increase in the multiplication rate. A significant finding of this study is that a BAP concentration of 1.0 mg L^{-1} yielded the highest number of shoots while also resulting in the greatest shoot length (Fig. 1).

This is of practical importance, as a high multiplication rate combined with adequate shoot elongation produces higher quality explants for the subsequent rooting phase. The Tukey–Kramer multiple comparison test revealed statistically significant differences among the four treatments ($p < 0.05$), with each treatment belonging to a distinct homogeneous group. These results indicate that the multiplication capacity of Gisela 5 increased with rising BAP

concentrations up to 1.0 mg L^{-1}, whereas further increases in BAP concentration led to a progressive reduction in shoot production.

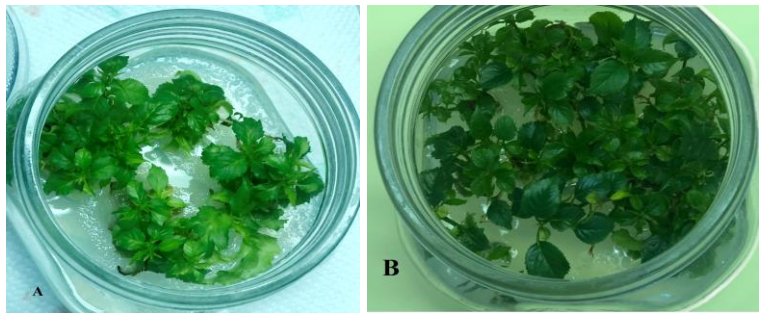


Fig. 1. A, B. Proliferation Phase of Gisela 5 (BAP 1 mg L^{-1})

A high concentration of cytokinins limits the growth of sweet cherries (Ružić & Vujović., 2008). A similar response was observed regarding shoot length. Treatment with 1.0 mg L^{-1} BAP resulted in the greatest shoot elongation, followed by treatments with 1.5 mg L^{-1} and 0.5 mg L^{-1} BAP, while the highest BAP concentration (2.0 mg L^{-1}) yielded the shortest shoots. The highly significant ANOVA result ($p < 0.0001$), combined with the Tukey–Kramer comparisons, confirms that BAP concentration significantly influenced shoot elongation.

Rooting Phase: *Effect of IBA concentration on root induction and development of Gisela 5*

The rooting phase demonstrated a significant effect of IBA concentration on both the number and length of roots. In the rooting phase, the variant with an IBA concentration of (0 mg L^{-1}) resulted in plants without roots. The greatest rooting response was observed at a dosage of 1.5 mg L^{-1} IBA, which yielded the highest mean number of roots (2.66 ± 0.14) and the greatest root length (2.84 ± 0.16), whereas the response declined at the highest concentration tested (2.0 mg L^{-1} IBA). Responses observed at IBA concentrations of 0.5, 1.0, and 2.0 mg L^{-1} were lower, indicating that the relationship between IBA concentration and rooting was non-linear. Conversely, root induction and development increased up to an intermediate IBA concentration and subsequently declined at the highest concentration tested (Tab.2).

Tukey-Kramer comparative analysis revealed significant differences between certain treatment pairs, confirming that the observed variation in rooting performance was attributable to the IBA treatments. However, some treatment pairs did not differ significantly, indicating that their mean rooting responses were statistically comparable and thus belonged to the same homogeneous group. The results suggest that 1.5 mg L^{-1} IBA was the most effective concentration among those evaluated for both root initiation and root elongation in Gisela 5 (Fig. 2A).

The lower rooting response observed at 2.0 mg L^{-1} IBA may indicate that an excessive auxin concentration does not further stimulate rooting and may instead inhibit root development. The stimulating effect of IBA on root formation is significant in the

micropropagation of woody fruit species. However, the concentration required for efficient rooting depends heavily on the genotype and the species' propagation protocol.

Table 2. Effects of IBA concentrations (0.5, 1.0, 1.5, 2.0 mg L^{-1}) on rooting parameters and 0.01 mg L^{-1} NAA

Treatment IBA (mg L^{-1})	**Number of Roots (Mean ± SE)**	**Length of Roots (cm) (Mean ± SE)**
V_1 (0.5 mg L^{-1})	1.41 ± 0.07 c	1.65 ± 0.10 c
V_2 (1.0 mg L^{-1})	2.08 ± 0.09 b	2.31 ± 0.11 b
V_3 (1.5 mg L^{-1})	2.66 ± 0.14 a	2.84 ± 0.16 a
V_4 (2.0 mg L^{-1})	1.81 ± 0.11 bc	1.96 ± 0.11 bc

Values correspond to Mean ± SE. Treatments followed by different letters are statistically different at p = 0.05

Previous studies on 'Gisela 5' have reported successful rooting at broadly similar IBA concentrations. Clapa *et al*., (2013) achieved 82.5% rooting using 1.0 mg L^{-1} IBA, whereas Sharma *et al*., (2020) reported 100% rooting using 0.5 mg L^{-1} IBA under specific culture conditions. Other research has shown that the basal medium can significantly modify the effect of IBA. Fallahpour *et al*., (2015) reported the highest rooting percentage in WPM supplemented with 2.0 mg L^{-1} IBA, while rooting was significantly lower in MS and DKW media under their experimental conditions. The process of rhizogenesis depends not only on the concentration of the auxin IBA but is specifically linked to the species' genotype, physiological state, basal medium composition, and the rooting conditions employed in this study. The decline in root development observed at the 2.0 mg L^{-1} IBA dosage suggests that excess auxin can become inhibitory once a concentration threshold is reached. High auxin concentrations may stimulate excessive callus formation or negatively affect subsequent root elongation.

The study results demonstrate that the rooting response of 'Gisela 5' depends on the optimization of IBA concentration, with 1.5 mg L^{-1} IBA proving to be the most effective concentration under the experimental conditions used. In this study, dosages of 1.0 mg L^{-1} BAP and 1.5 mg L^{-1} IBA yielded the most favorable responses for shoot multiplication and rooting, respectively.

Acclimatization phase. After the shoots have been micropropagated and rooted *in vitro*, it is important to acclimatize them to external environmental conditions. This step involves the gradual transfer of the plants to environments with low humidity to avoid transplant shock. The plant acclimatization rate was 86%. The rooting process is essential for successful acclimatization to soil. Roots can be viewed as a site of polar auxin transport originating

from the shoot, while exogenous auxin acts to induce the *de novo* formation of root primordia from competent cells in the newly formed stem (Pasternak and Steinmacher, 2024).



Fig. 2. A. Rooting phase with IBA 1.5 mg L^{-1}; B. Acclimatized plants previously rooted with IBA 1.5 mg L^{-1} and 1 mg L^{-1}; C. *In vivo* acclimatization of plants rooted with IBA 1.5 mg L^{-1}

3.2. Liquid culture using the Elec-TIS bioreactor

The optimal multiplication medium was subsequently tested in the Elec-TIS bioreactor under three immersion regimes. The results obtained from liquid culture using this type of bioreactor are presented in Table 3. Compared to the other two temporary immersion regimes (10 min/8 h and 12 min/16 h), the immersion frequency of 8 minutes every 8 hours proved to be the best, yielding the highest relative growth rate (RGR) of 3.07 surpassing the rates recorded in solid and semi-solid media (2.31 and 1.88, respectively) (Tab. 3; Fig.3).

Table 3. Relative growth rate (RGR) of Gisela 5 culture media (1.0 mg L^{-1} BAP, 0.1 mg L^{-1} GA_3, 0.01 mg L^{-1} NAA)

Cultivation system	**Immersion frequency**	**Average initial weight**	**Final average weight**	**RGR (Relative Growth Rate)**
Elec-Tis (30 plants)	8 min/8 h	3.46	13.83	3.07
Elec-Tis (35 plants)	10 min/8 h	4.19	12.75	2.46
Elec-Tis (30 plants)	12 min/16 h	5.01	11.07	1.75
Control 1 (high-agar medium) 20 plants		3.28	9.29	2.31
Control 2 (low-agar medium) 20 plants		3.57	8.40	1.88

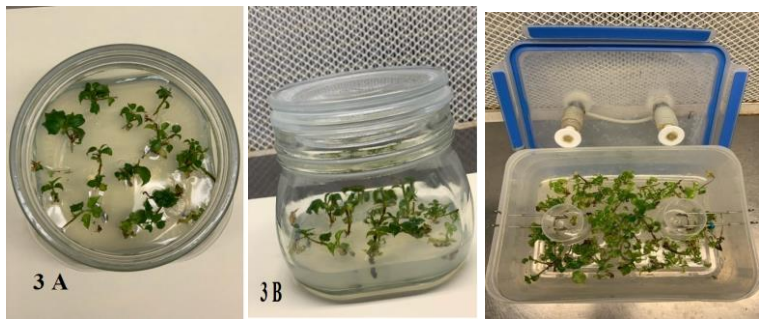


Figure 3. A. Shoots of Gisela 5 in (agar 7,0 g L^{-1};); B. Shoots in (agar 3,5 g L^{-1};); C. Shoots in ElecTIS bioreactor

These results demonstrate that ElecTIS enhances biomass production and represents an efficient system for the '*in vitro*' propagation of the 'Gisela 5' rootstock. The use of a liquid medium in this TIS immersion system eliminates the cost of gelling agents, while the vessel size allows for a higher number of shoots per unit, resulting in longer subculture intervals and a consequent reduction in labor costs, as highlighted by Lambardi (2012), De Carlo *et al*., (2013), Sota *et al*., (2021), Elazab *et al*., (2023).

4. CONCLUSIONS

Micropropagation of the Gisela 5 rootstock yielded satisfactory results using a protocol based on MS medium supplemented with BAP (1.0 mg L^{-1}) for multiplication and IBA (1.5 mg L^{-1}) for rooting. The use of the ElecTIS bioreactor proved highly effective for micropropagation compared to the tested agar media, as evidenced by the higher Relative Growth Rate (RGR) value. Liquid culture in the ElecTIS bioreactor represents an evolution of the traditional *in vitro* culture system on solidified media, offering advantages in terms of culture quality and lower production costs compared to traditional micropropagation.

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Phytochemical composition and antioxidant potential of *Schisandra rubriflora* biomass derived from *in vitro* cultures

Grzegorz Kos[1], Angelika Warzecha[1], Kubica Pawel[1], Michał Dziurka[2], Agnieszka Szopa[1]

[1]*Department of Medicinal Plant and Mushroom Biotechnology, Faculty of Pharmacy, Medical College, Jagiellonian University, Medyczna 9 St., 30-688 Kraków, Poland*

[2]*Polish Academy of Sciences The Franciszek Górski Institute of Plant Physiology, ul. Niezapominajek 21, 30-239 Kraków, Poland*

a.szopa@uj.edu.pl

Abstract

Schisandra rubriflora (Franch.) Rehd. et Wils is an endemic species of the Schisandraceae family with a high potential as a source of biologically active secondary metabolites. However, its phytochemical properties remain relatively poorly investigated due to the limited availability of plant material. The aim of this study was to compare the phytochemical composition, accumulation of selected secondary metabolites and antioxidative potential of *S. rubriflora* leaves, shoots and fruits with those of experimentally established *in vitro* microshoot cultures. Lignans were qualitatively and quantitatively analyzed using UHPLC-MS/MS, while flavonoids and phenolic acids were determined by HPLC-DAD. Antioxidative potential was evaluated using the CUPRAC assay. The analyzed plant materials differed considerably in their phytochemical profiles. Fruits contained the highest total lignan content (1055.65 mg/100 g dry weight), whereas leaves showed the highest levels of total flavonoids (2032.20 mg/100 g DW) and phenolic acids (757.78 mg/100 g DW). Twenty-four lignans were identified in fruits and leaves, while 23 were detected in shoots and microshoot cultures. The *in vitro* cultures accumulated 220.70 mg/100 g DW of lignans, 397.26 mg/100 g DW of flavonoids and 187.71 mg/100 g DW of phenolic acids. The highest antioxidative potential was observed in leaf and shoot extracts, reaching 41.81 and 39.25 mmol Trolox equivalents/100 g DW, respectively, whereas microshoot cultures showed considerably lower activity (3.96 mmol Trolox equivalents/100 g DW). The results demonstrate that *S. rubriflora* tissues differ substantially in their secondary metabolite composition and antioxidative potential. Although *in vitro* cultures accumulated lower amounts of metabolites than *in vivo* plant material, they represent a promising alternative source of bioactive compounds and a useful system for further biotechnological studies.

Keywords: *Schisandra rubriflora; in vitro cultures; lignans; flavonoids; antioxidant potential*

1. INTRODUCTION

Schisandra rubriflora (Franch.) Rehd. et Wils is a woody vine plant belonging to the family Schisandraceae, which is endemic to the western part of Sichuan province of China, where it grows in mountainous areas, 2000-3000 meters above sea level (The World Flora Online 2026). Its fruits are used in traditional Chinese medicine as a toning agent. In recent years, *S. rubriflora*, similarly to other representatives of the genus *Schisandra*, has attracted increasing scientific interest owing to its diverse pharmacological properties. Extracts obtained from *S. rubriflora* have potential antiviral, hepatoprotective, antioxidative and anti-inflammatory properties, which are mostly contributed to their high content of characteristic lignans (Szopa *et al.*, 2019).

However, due to relatively low availability of plant material from *S. rubriflora*, the phytochemical properties of this species have not been thoroughly studied yet (Szopa *et al.*, 2019). In such cases, plant biotechnology gives the opportunity to broaden our knowledge of endemic plants (Khan *et al.*, 2009). Previous studies on *S. rubriflora* have primarily focused on the phytochemical characterization of selected plant organs, while information concerning the metabolic potential of biomass derived from *in vitro* cultures remains limited. Therefore, comparative investigations involving both naturally grown plants and biotechnologically produced biomass are required to evaluate the usefulness of *in vitro* cultures as alternative sources of valuable bioactive compounds.

The aim of the present study was to compare the phytochemical composition, accumulation of lignans, flavonoids and phenolic acids, as well as the antioxidant potential of methanolic extracts obtained from leaves, shoots and fruits of *S. rubriflora* with those derived from experimentally established *in vitro* microshoot cultures. Furthermore, the study was intended to evaluate the potential of *in vitro* cultures as an alternative source of biologically active secondary metabolites and as a model system for future biotechnological investigations of this species.

2. MATERIALS AND METHODS

2.1. Parent plant material

Leaves, shoots and fruits of female specimens of *S. rubriflora* were obtained commercially from CLEMATIS - Źródło Dobrych Pnączy Spółka z o.o., Pruszków, Poland (https://www.clematis.com.pl/).

2.2. In vitro cultures

Experimental *in vitro* microshoot cultures of *S. rubriflora* were established from axillary leaf buds excised from female plants and maintained under controlled culture conditions on solidified Murashige and Skoog (MS) medium supplemented with 1 mg/L 6-benzyladenine (BA) and 1 mg/L indole-3-butyric acid (IBA) (Murashige and Skoog, 1962). Cultures were maintained under controlled growth-room conditions at 25 ± 2°C under a cool-white, fluorescent lamps (PPFD 30 µmol/ m^2 s^1). Biomass was cultivated in Magenta® vessels and subcultured at 30-day intervals. Plant material used for phytochemical and antioxidant analyses was collected after three consecutive growth cycles comprising 20 culture vessels per series. The microshoot cultures exhibited stable growth and served as a source of experimental biomass for subsequent phytochemical profiling and evaluation of antioxidant potential.

2.3. UHPLC-MS/MS analysis

Qualitative and quantitative analysis of lignan content was performed using a UHPLC-MS/MS method. The instrumentation consisted of an Infinity 1260 UHPLC (Agilent, Waldbronn, Germany) and a 6410 QQQ LC/MS tandem quadrupole mass spectrometer (Agilent, Santa Clara, USA) according to (Szopa *et al.*,, 2018; Szopa *et al.*,, 2018). Methanolic extracts were prepared by ultrasonic extraction of freeze-dried leaves, shoots, fruits and *in vitro* biomass of *S. rubriflora* in methanol at a 1:10 (w/v) ratio. Sample size was 2 µL. The analytes were eluted using a gradient of 50% aqueous methanol (solvent A) and methanol (solvent B), both containing 0.1% formic acid—ranging from 20% to 65%

solvent B over 22 minutes, at a mobile phase flow rate of 0.5 mL/min and a temperature of 60°C.

2.4. HPLC-DAD analysis

Flavonoids and phenolic acids were analyzed using the HPLC-DAD method. The instrumentation consisted of a Merck-Hitachi HPLC system equipped with a diode-array detector (DAD). Methanolic extracts were prepared by sonicating 0.3 g of shoots, 0.3 g of leaves, 0.1 g of fruits and 0.1 g of *in vitro* biomass respectively in 3 mL, 3 mL, 2 mL and 1 mL of methanol. The analysis was conducted as described by Ellnain-Wojtaszek and Zgórka (2006) and by Sułkowska-Ziaja *et al.* (2017).

2.5. Antioxidant potential analysis

Antioxidant potential was measured using the CUPRAC method (Apak *et al.*, 2008). Extracts for the analysis were prepared by ultrasonic extraction of 5 mg of each plant material and *in vitro* biomass in 1 mL of methanol. The analysis was performed with a Synergy II microplate reader (BioTek Instruments, USA) controlled by Gen5 software.

2.6. Statistical analysis

All experiments were performed in triplicate using biomass obtained from three independent biological series. The results are expressed as mean values ± standard deviation (SD). Statistical analyses were performed using Statistica software (version 10, StatSoft Inc., Tulsa, OK, USA). Differences among leaves, shoots, fruits and *in vitro* microshoot cultures were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Lignan contents

Twenty-four lignans were identified in the analyzed *S. rubriflora* fruit extracts. The total lignan content in the fruit extracts was 1055.65 mg/100 g DW. The dominant compounds were: schisanhenol (268.02 mg/100 g DW), angeloylgomisin H (185.10 mg/100 g DW), and schisantherin B (118.07 mg/100 g DW)

Table 1. Lignans content in analyzed extracts

Compound name	Compound content in analyzed extracts [mg/100 g DW]			
	Fruit extracts	**Leaf extracts**	**Shoot extracts**	**Microshoot cultures extracts**
Wulignan A_1	19.39±1.35	0.04±0.001	0.03±0.002	0.01
Rubrisandrin A	0.07±0.001	0.06±0.001	0.06±0.002	0.005±0.001
Interiotherin C	0.002	0.001	0.001	0.001
Schisandrin	6.57±0.35	8.15±0.25	4.01±0.005	4.25±0.01
Gomisin D	3.52±0.16	16.45±0.76	8.25±0.42	0.66±0.05
Gomisin J	5.40±0.27	0.97±0.01	0.46±0.01	0.10±0.002

Gomisin A	0.75±0.01	6.40±0.18	2.99±0.02	0.70±0.004
Gomisin G	66.39±12.37	11.13±1.01	5.25±0.04	1.72±0.02
Licarin B	1.98±0.08	0.41±0.06	0.19±0.04	0.10±0.03
Epigomisin O	7.46±0.32	10.62±0.41	4.91±0.06	0.35±0.002
Gomisin O	103.64±26.25	22.90±1.29	12.07±0.20	0.60±0.01
Mesodihydroguaiaretic acid	1.03±0.02	0.34±0.01	0.17±0.001	0.03±0.002
Schisantherin A	27.19±3.00	226.80±16.70	84.35±4.64	1.83±0.01
Schisantherin B	118.07±18.42	291.47±51.98	239.11±38.00	116.68±4.32
Dehydroisoeugenol	0.41±0.004	0.73±0.01	0.33±0.004	0.29
Schisanhenol	268.02±43.12	2.05±0.06	1.13±0.003	2.94±0.01
Schisandrin A	104.32±10.11	0.22±0.002	0.12±0.001	2.55±0.02
Fragransin A_2	0.01±0.002	0.01±0.002	n/d	n/d
Gomisin N	19.20±0.66	1.58±0.02	0.81±0.01	1.15±0.01
6 – O – Benzoylgomisin O	35.28±3.55	134.51±5.91	72.38±4.77	3.14±0.01
Schisandrin C	4.96±0.12	0.44±0.03	0.28±0.002	0.04±0.02
Angeloylgomisin H	185.10±27.55	100.83±7.89	105.80±9.04	72.28±12.57
Angeloylgomisin O	76.88±4.55	17.21±0.26	17.25±0.23	11.26±0.82
Total content	**1055.65±152.26**	**853.33±86.85**	**559.97±57.50**	**220.70±17.91**

n/d – not detected

Twenty-four lignans were identified in the analyzed leaf extracts, with a total content of 853.33 mg/100 g DW. The dominant compounds were schisantherin B (291.47 mg/100 g DW), schisantherin A (226.80 mg/100 g DW), and 6-O-benzoylgomisin O (134.51 mg/100 g DW). Twenty-three lignans were identified in the analyzed shoot extracts. Their total content was 559.97 mg/100 g DW and the dominant compounds were schisantherin B (239.11 mg/100 g DW), angeloylgomisin H (105.80 mg/100 g DW) and schisantherin A (84.35 mg/100 g DW).

Extracts from experimental *in vitro* cultures contained twenty-three lignans, of which schisantherin B (116.68 mg/100 g DW) and angeloylgomisin H (72.28 mg/100 g DW) were dominant. Total lignans content was 220.70 mg/100 g DW.

Overall, fruits contained the highest total content of lignans, followed by leaves and shoots, whereas the lowest lignan concentration was detected in biomass derived from *in vitro* microshoot cultures.

3.2. Flavonoid and phenolic acid contents

The analyzed fruit extracts contained two identified phenolic acids and eight identified flavonoids. The total phenolic acid content was 78.05 mg/100 g DW, while the total flavonoid content was 352.61 mg/100 g DW. The dominant phenolic acid was

cryptochlorogenic acid (69.14 mg/100 g DW), and the dominant flavonoids were kaempferol (138.15 mg/100 g DW) and quercetin (51.66 mg/100 g DW)

Table 2. Flavonoids and phenolic acids content in analyzed extracts

Compound name	**Compound content in analyzed extracts [mg/100 g DW]**			
	Fruit extracts	**Leaf extracts**	**Shoot extracts**	**Microshoot cultures extracts**
Naringin	n/d	62.50±6.19	28.08±0.59	69.48±3.91
Hyperoside	4.78	95.89±6.01	60.36±4.65	13.73±0.64
Rutoside	41.90	593.58±28.06	471.47±43.77	28.70±0.97
Isoquercetin	27.12	432.66±20.16	306.91±18.03	20.59±2.47
Quercetin 3-rhamnoside	51.66	82.76±10.40	93.02±5.48	37.83±0.79
Trifolin	18.80	457.51±49.93	363.77±34.85	27.47±1.01
Quercetin	31.29	128.93±3.96	88.39±8.54	55.59±2.48
Kaempferol	138.15	110.71±4.79	72.19±3.39	72.26±2.74
Isorhamnetin	38.91	58.66±4.40	32.74±4.35	71.61±5.35
Total flavonoid content	**352.61**	**2023.2±133.9**	**1516.93±123.65**	**397.26±20.36**
Neochlorogenic acid	n/d	607.30±26.39	229.41±14.86	80.67±5.83
Chlorogenic acid	8.91	92.55±9.14	17.50±2.26	20.06±1.14
Cryptochlorogenic acid	69.14	57.93±3.38	20.18±1.04	86.98±2.16
Total phenolic acids content	**78.05**	**757.78±38.91**	**267.09±18.16**	**187.71±9.13**

n/d – not detected

Three phenolic acids and nine flavonoids were identified in the analyzed *S. rubriflora* leaf extracts. Total phenolic acids content was 757.78 mg/100 g DW, with neochlorogenic acid being the predominant compound (607.30 mg/100 g DW). Total flavonoids content was 2023.20 mg/100 g DW and the dominant compounds were rutoside (593.58 mg/100 g DW) and trifolin (457.51 mg/100 g DW).

The analyzed shoot extracts contained three identified phenolic acids and nine identified flavonoids. Total phenolic acids content was 267.09 mg/100 g DW, with neochlorogenic acid as the dominant constituent (229.41 mg/100 g DW). Total flavonoids content was 1516.93 mg/100 g DWand the dominant compounds were rutoside (471.41 mg/100 g DW) and isoquercetin (306.91 mg/100 g DW).

Extracts from experimental *in vitro* cultures were found to contain three identified phenolic acids and nine identified flavonoids. Total phenolic acids content was 187.71 mg/100 g DW and total flavonoids content was 397.26 mg/100 g DW. Cryptochlorogenic acid (86.98 mg/100 g DW) was the main phenolic acid, while kaempferol (72.26 mg/100 g DW) and isorhamnetin (71.61 mg/100 g DW) were the main flavonoids.

Overall. leaves accumulated the highest amounts of both phenolic acids and flavonoids, whereas fruits contained the lowest levels of phenolic acids. Biomass derived from *in vitro* cultures exhibited intermediate concentrations of these compounds, exceeding those determined in fruits but remaining lower than those observed in leaves and shoots.

3.3. Antioxidant potential

Antioxidant capacity was expressed as mmol Trolox equivalents per 100 g DW. High antioxidant capacity values were obtained for extracts prepared from *in vivo* plant materials of *S. rubriflora* using the CUPRAC assay. The highest antioxidant activity was observed in leaves (41.81 mmol Trolox eq/100 g DW) and shoots (39.25 mmol Trolox eq/100 g DW). Fruit extracts exhibited markedly lower antioxidant potential (7.87 mmol Trolox eq/100 g DW). Similarly, relatively low antioxidant activity was recorded for extracts obtained from *in vitro* microshoot biomass, reaching 3.96 mmol Trolox eq/100 g DW. A comparison of total lignan. phenolic acid and flavonoid contents in the analyzed extracts and their corresponding antioxidant potentials is shown in Figure 1.

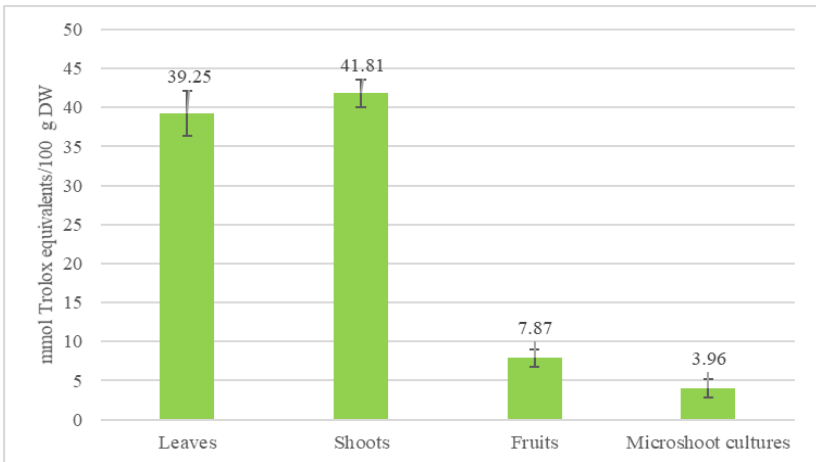


Fig. 1. A comparison of phytochemical composition of methanolic extracts obtained from leaves, shoots, fruits and *in vitro* microshoot cultures of *S. rubriflora*

4. DISCUSSION

The present study demonstrated pronounced organ-specific differences in the accumulation of secondary metabolites in *S. rubriflora*. Although comparable numbers of lignans were detected in all analyzed materials (23-24 compounds), their concentrations varied considerably depending on the origin of the biomass. Fruits represented the richest source of lignans accumulating 1055.65 mg/100 g DW, which was 1.2-fold higher than in leaves, 1.9-fold higher than in shoots and as much as 4.8-fold higher than in *in vitro* microshoot

cultures. The predominance of lignans in fruits corresponds well with the physiological role of these compounds in reproductive organs and agrees with previous reports indicating that fruits constitute the major reservoir of dibenzocyclooctadiene lignans in species of the genus *Schisandra* (Szopa *et al.,* 2018; Sobstyl *et al.,* 2022). The total lignan content recorded in the fruits of *S. rubriflora* was identical to that previously reported by Szopa *et al.* (2018) and exceeded the levels reported for shoots of the same species by almost two-fold. Moreover, fruits of *S. rubriflora* were previously shown to possess lignan concentrations comparable to or even exceeding those reported for certain organs of *Schisandra chinensis*, which further highlights the phytochemical importance of this less explored species (Szopa *et al.*, 2018).

In contrast to lignans, flavonoids and phenolic acids accumulated preferentially in vegetative organs. Leaves contained the highest total flavonoid concentration (2023.20 mg/100 g DW), representing levels 5.7-fold higher than in fruits, 1.3-fold higher than in shoots and 5.1-fold higher than in *in vitro* cultures. Similarly, the concentration of phenolic acids in leaves (757.78 mg/100 g DW) was almost 10-fold higher than in fruits, 2.8-fold higher than in shoots and 4.0-fold higher than in cultured biomass. Such marked differences indicate a clear metabolic specialization of individual organs. While fruits invest predominantly in lignan biosynthesis, leaves appear to be the primary sites of phenolic compound accumulation. This observation is biologically reasonable because flavonoids and hydroxycinnamic acid derivatives play important roles in photoprotection, UV screening, antioxidative defense and protection against environmental stress, functions that are particularly important in photosynthetically active tissues.

The predominance of rutoside, trifolin and isoquercetin in vegetative organs observed in the present study is also consistent with previous phytochemical investigations of *S. rubriflora*. Earlier analyses demonstrated that flavonoid glycosides constitute a substantial fraction of the phenolic profile of leaves and shoots and contribute significantly to their biological activity. Similar distributions have been reported for other *Schisandra* species, including *S. chinensis* and *S. sphenanthera*, in which leaves generally contain higher concentrations of polyphenolic constituents than fruits (Szopa *et al.,* 2019). Likewise, chlorogenic acid derivatives, particularly neochlorogenic and cryptochlorogenic acids, have repeatedly been identified among the dominant phenolic acids in representatives of the family Schisandraceae (Szopa *et al.*, 2019) .

An interesting finding of the present study is that *in vitro* cultures accumulated lower amounts of lignans than all analyzed *in vivo* organs, but simultaneously produced higher levels of flavonoids and phenolic acids than fruits. Specifically, flavonoid content in cultured biomass was 1.13-fold higher than in fruits, whereas phenolic acid content was 2.4-fold higher. These observations suggest that the metabolic activity of cultured tissues is shifted towards the phenylpropanoid pathway. Similar trends have been observed in numerous medicinal plant species cultivated under controlled conditions, where stress associated with artificial culture environments frequently stimulates the biosynthesis of antioxidant phenolics (Dias *et al.,* 2016; Modarres *et al.,* 2018). Although the total lignan concentration in the microshoot cultures was only approximately 21% of that observed in fruits, the cultures maintained the capacity to synthesize a broad spectrum of lignan structures, demonstrating that the complete lignan biosynthetic machinery remained active under *in vitro* conditions.

The relatively high biosynthetic potential of cultured biomass is particularly important from a biotechnological perspective. Previous studies on *S. rubriflora* demonstrated that elicitation with methyl jasmonate, chitosan, yeast extract or ethephon may increase lignan accumulation by more than three-fold compared with non-elicited cultures (Szopa *et al.*, 2022). Consequently, the concentrations obtained in the present study should be treated as baseline values rather than the maximum metabolic capacity of the culture system. Further optimization of medium composition, elicitation strategy and cultivation systems could substantially enhance metabolite production and improve the applicability of *S. rubriflora* cultures as renewable sources of valuable phytochemicals.

The antioxidant activity results further support the observed differences in phytochemical composition. Despite possessing the highest lignan concentrations, fruit extracts exhibited only moderate CUPRAC activity. In contrast, leaves and shoots showed the strongest antioxidant properties, reaching 41.81 and 39.25 mmol Trolox eq./100 g DW respectively. These values were more than five times higher than those recorded for fruits and approximately ten times higher than those observed for *in vitro* biomass. Such relationships strongly suggest that antioxidant capacity in *S. rubriflora* is associated primarily with polyphenolic constituents rather than lignans. This interpretation is supported by the exceptionally high contents of flavonoids and phenolic acids detected in leaves and shoots. Similar observations were reported by Sobstyl *et al.* (2022) who demonstrated stronger antioxidant properties of leaf extracts despite lower lignan concentrations than those observed in fruits. Collectively, these findings indicate that flavonoids and phenolic acids constitute the principal contributors to the antioxidant potential of *S. rubriflora* extracts.

The organ-specific distribution of secondary metabolites is clearly illustrated in Figure 2. While lignans represented the dominant group of compounds in fruit extracts, flavonoids and phenolic acids predominated in leaves and shoots.

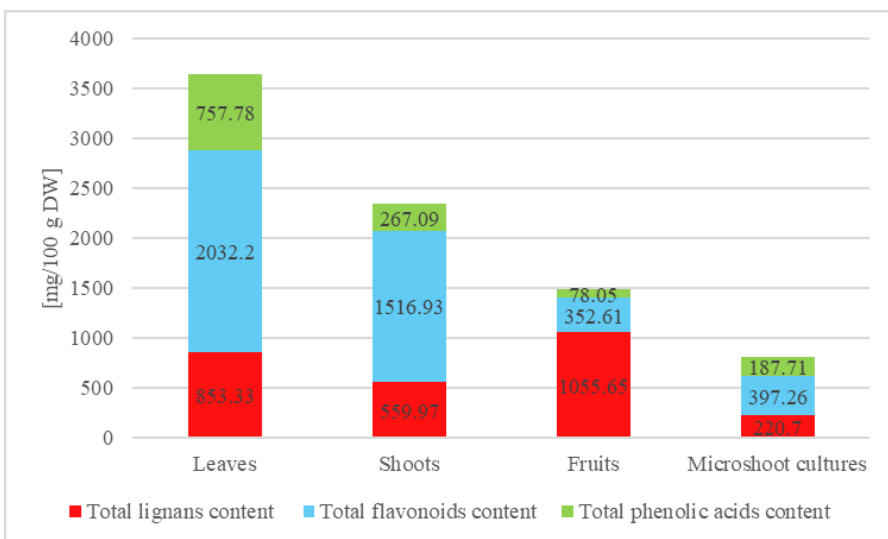


Fig. 2. A comparison of phytochemical composition of methanolic extracts obtained from leaves, shoots, fruits and *in vitro* microshoot cultures of *S. rubriflora*

The figure highlights the contrasting metabolic profiles of reproductive and vegetative organs, demonstrating that fruits accumulated the highest amounts of lignans, whereas leaves were particularly rich in flavonoids and phenolic acids. Moreover, *in vitro* cultures exhibited an intermediate phytochemical profile, characterized by relatively low lignan content but appreciable accumulation of phenolic compounds. These findings indicate substantial specialization of secondary metabolism within different tissues of *S. rubriflora* and emphasize the importance of selecting appropriate plant material depending on the desired group of bioactive constituents.

5. CONCLUSIONS

The present study provides a comprehensive comparison of the phytochemical composition and antioxidant potential of different organs of *S. rubriflora* and biomass derived from *in vitro* microshoot cultures. Pronounced organ-specific differences in secondary metabolite accumulation were demonstrated. Fruits were identified as the richest source of lignans, whereas leaves accumulated the highest concentrations of flavonoids and phenolic acids and exhibited the strongest antioxidant activity. These findings indicate a clear metabolic specialization of individual plant organs and highlight the importance of selecting appropriate plant material depending on the desired group of bioactive compounds.

Although *in vitro* microshoot cultures accumulated lower amounts of lignans than the corresponding *in vivo* organs, they retained the ability to synthesize a broad spectrum of lignans, flavonoids and phenolic acids. Notably, the cultured biomass contained higher levels of flavonoids and phenolic acids than fruits, demonstrating the considerable biosynthetic potential of the culture system. The preservation of metabolite production under controlled conditions confirms that *in vitro* cultures constitute a promising and sustainable alternative source of valuable phytochemicals independent of environmental and seasonal limitations.

The results further suggest that antioxidant activity in *S. rubriflora* is more strongly associated with the accumulation of polyphenolic compounds than with total lignan content. The exceptionally high antioxidant potential observed in leaves and shoots highlights these organs as particularly valuable sources of natural antioxidants.

Overall, the study expands the current knowledge of the phytochemical characteristics of *S. rubriflora* and provides a scientific basis for the future exploitation of both natural plant material and biotechnologically produced biomass. Further research should focus on the optimization of culture condition, elicitation strategies and large-scale cultivation systems to enhance metabolite production and facilitate the practical application of *S. rubriflora* cultures in pharmaceutical, nutraceutical and cosmetic industries.

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Effects of silicon on the micropropagation of peach rootstock GF677 (*Prunus amygdalus* × *Prunus persica*)

Lilyana Nacheva[1], Diyana Aleksandrova[1], Ivaylo Tsvetkov[2]

[1]*Fruit Growing Institute, Agricultural Academy, 12 Ostromila Str., 4004, Plovdiv, BULGARIA*

[2]*Forest Research Institute – BAS, 132, Kliment Ohridski Blvd., Sofia 1756, BULGARIA*

tsvet_i@yahoo.com

Abstract

The rootstock GF 677 (*Prunus amygdalus* × *Prunus persica*) is the most commonly used rootstock for peach in Europe, propagated almost exclusively by tissue culture. It is tolerant to Fe deficiency and especially suited to soils with poor fertility, low water availability, and high $CaCO_3$ content. In the recent years many studies have shown that silicon (Si) has a positive role in improving various aspects of plant micropropagation, cryopreservation, and production of secondary metabolites. The aim of this pilot study was to improve the micropropagation of the GF 677 rootstock by applying silicon (Si) as an additional component of the nutrient medium. The plantlets were cultivated *in vitro* on Murashige and Skoog (MS) nutrient medium, supplemented with 5 µM 6-benzylaminopurine, 0.01 µM indole-3-butyric acid, 30 g L^{-1} sucrose, 6.5 g L^{-1} Phyto agar. Silicon was added to the culture medium as calcium ($CaSiO_3$) or potassium (K_2SiO_3) silicate (1 or 2 mg L^{-1}). Control plants were cultivated on a silicon-free medium. At the end of 3 passages of 4 weeks each, some biometric indicators, fresh and dry biomass were recorded. The results obtained showed that the addition of 1 mg L^{-1} potassium silicate to the culture medium stimulated the accumulation of biomass and the development of new shoots, although the latter was not statistically proven. Incorporation of Si into tissue culture media might have favorable effect on micropropagation of the peach rootstock GF677, but source and concentration must be more thoroughly tested.

Keywords: *tissue culture, in vitro, woody plants*

1. INTRODUCTION

Micropropagation is widely used in agriculture and forestry worldwide, and millions of plants are produced each year. This method includes the advantages of vegetative propagation by preserving and multiplying unique genotypes without the variations characteristic of sexual reproduction. At the same time, it can be used to obtain plants free from economically important viruses and reduces the time required to introduce plant material with improved qualities (Balla and Mansvelt, 2012). Unfortunately, the great advantages of *in vitro* techniques can not be realized on a full scale in the reproduction of woody species, mainly due to their recalcitrance in *in vitro* conditions, the tendency to hyperhydricity, limited ability to rhizogenesis, and low survival rate of microplants during the *ex vitro* acclimatization. Therefore, the search for ways to increase the efficiency of clonal micropropagation of fruit species is still a current task and challenge for researchers.

Peach rootstock GF 677 (*Prunus amygdalus* × *Prunus persica*) is the most commonly used rootstock for peach in Europe (Antonopoulou *et al.*, 2007), propagated almost exclusively by tissue culture. It is tolerant to Fe deficiency and especially suited to soils

with poor fertility, low water availability, and high $CaCO_3$ content (Monticelli *et al.*, 2000; Byrne *et al.*, 2012; Rubio-Cabetas, 2016). Since GF 677 started being propagated commercially by tissue culture, much research has been conducted to improve the multiplication rate and quality of the *in vitro* rooted plantlets (Dimasi-Theriou and Economou, 1995; Ahmad *et al.*, 2003; Molassiotis et al. 2003; Özden *et al.*, 2011; Sepahvand *et al.*, 2012; Aghaye & Yadollahi, 2012; Güney *et al.*, 2016; Borkheyli *et al.*, 2021). The hybrid rootstock GF 677 is extremely dependent on the composition of the nutrient medium, with hyperhydricity and chlorosis being among the main problems (Damiano *et al.*, 2009; Alanagh *et al.*, 2014). Also, according to Tsipouridis *et al.* (2003), low rooting rate, and rosetting of plantlets (rooted plantlets after acclimation, which did not grow or remain dormant) also contributed to the high cost of the planting material.

Silicon (Si) is the most abundant mineral element in soil and although being not considered essential for some plants, it both promotes positive effects for many species and is able to reduce many environmental stress effects (Currie and Perry, 2007). Numerous studies have shown that Si treatment improves the growth and yield of various plants, especially when they are subjected to abiotic (Balakhnina and Borkowska, 2013; Zhu and Gong, 2014) and biotic stress (Van Bockhaven *et al.*, 2013). Si increases stress tolerance by altering antioxidant enzyme activity and endogenous hormone levels; improving the biosynthesis of lignin, chitinase, phenols, phytoalexins, and glucanase; increasing plant nutrient uptake; altering the cation binding capacity of the plant cell wall; improving plant cell strength; maintaining stomatal structure and relative water content; and reducing heavy metal uptake (Vaculík *et al.*, 2009; Sahebi *et al.*, 2015). In this regard, Si has been reported to be deposited in the epidermis of plant leaves to modulate transpiration, leading to reduced water loss (Mitani and Ma, 2005).

Si added to plant tissue culture media improves organogenesis, embryogenesis, growth parameters, morphological, anatomical and physiological characteristics of leaves, increases tolerance to low temperature and salinity, protects cells against metal toxicity, prevents oxidative phenolic browning and reduces the incidence of hyperhydration in various plants (Braga *et al.*, 2009; Soundararajan *et al.*, 2013; Sivanesan and Jeong 2014). Therefore, Si has significant potential for application in a wide range of plant tissue culture studies, such as micropropagation, organogenesis, somatic embryogenesis, cryopreservation and production of secondary metabolites. The plants included in these experiments are mostly herbaceous species. Information about the effects of Si on tree crops is scarce and none is available for woody fruit species.

The aim of this pilot study was to improve micropropagation of Gf 677 by application of Si in the nutrient medium.

2. MATERIALS AND METHODS

The experiment was carried out with peach rootstock GF 677 (*Prunus amygdalus* × *Prunus persica*). The cuttings were collected from a virus-tested three-year-old tree growing in an insect-protected greenhouse of the Fruit Growing Institute, Plovdiv, Bulgaria. Explants (apical and stem segments) were taken in mid-April 2023 from young shoots. After removing the leaves, they were surface sterilized by immersion in 70% (v/v) ethanol for 30 seconds and in 5% (w/v) calcium hypochlorite solution containing 2–3

drops of Tween for 10 minutes. The shoots were then rinsed four times (10 min each) in sterile distilled water. Nodal segments were isolated from the shoots and inoculated in test tubes (⌀ 25 mm, height 100 mm) onto 5 mL solid MS (Murashige and Skoog, 1962) medium, supplemented with 5 µM 6-benzylaminopurine (BAP), 0.01 µM indole-3-butyric acid (IBA), 30 g L^{-1} sucrose and 6.5 g L^{-1} Phyto agar (Duchefa, The Netherlands). The medium (pH 5.6) was autoclaved at 121 °C for 20 minutes. Clean and viable explants were transferred to fresh nutrient medium every four weeks for two passages. Thereafter, the explants were maintained in 100 mL of the medium described above in glass jars (600 mL volume) with glass lids (10 explants per jar) and were subcultured every 4 weeks.

The explants were cultivated in a growth chamber under controlled conditions - temperature 22±2°C, 16 h photoperiod (60 µmol m^{-2} s^{-1}) supplied by cool white fluorescent lamps.

2.1. In vitro shoot proliferation on nutrient media with different Si sources

Experimental material (nodal shoot explants 1-1.5 cm long with 2-3 leaves) from steadily proliferating *in vitro* cultures was transferred onto the aforementioned culture medium and type of culture vessels. The effect of two different Si sources applied in two doses was investigated, namely potassium silicate (K_2SiO_3, 1 or 2 mg L^{-1}, resp. K1 and K2) and calcium silicate ($CaSiO_3$ 1 or 2 mg L^{-1}, resp. Ca1 and Ca2). Plantlets grown on the same nutrient medium without Si were used as a control. For each treatment, 50 explants were used (5 jars, each containing 10 explants).

After 3 passages of 4 weeks each, some biometric indicators, photosynthetic pigments content, fresh (FW) and dry biomass (DW), were recorded. The multiplication index (MI) was calculated as the number of proliferated shoots from one explant. For the biometric analyses, 30 explants from each variant were measured using a digital caliper. The dry mass (DW) of the plants was determined after drying (at 105 °C) to a constant weight. Photosynthetic pigments were extracted from leaves. Fresh plant material (250 mg) was ground with 10 ml of 80% aqueous acetone. After centrifugation of the extract at 10 000 rpm for 10 min, 1 mL was mixed with 2 mL acetone. For each variant, two parallel samples were prepared. The content of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Car) (mg g^{-1} FW) were determined spectrophotometrically according to Lichtenthaler and Wellburn (1983).

2.2. Rooting on nutrient media with different Si sources

Apical shoots (about 10 mm long, with 2-3 leaves) from the proliferating *in vitro* cultures without Si were transferred to rooting medium (pH 5.6) containing half concentration of MS macronutrients, full concentration of micronutrients and vitamins, 1.5 µM IBA, 20 g L^{-1} sucrose and 6.5 g L^{-1} Phyto agar and above mentioned additives of potassium (K_2SiO_3) or calcium ($CaSiO_3$) silicate (1 or 2 mg L^{-1}). Microcuttings were grown in glass jars under light treatments and temperatures described above. Data on rooting percentage, stem length, number of roots per rooted microcutting, and root length were recorded three weeks after the start of the experiment. Rooting experiments were conducted with 30 microcuttings for each treatment (three jars × ten explants) and were repeated twice.

The results were analyzed using one-way analysis of variance (ANOVA), followed by a Duncans's multiple range test (SPSS ver. 26.0, IBM)

3. RESULTS

3.1. Multiplication stage

The inclusion of Si in the culture medium affected the growth of microplants of the peach rootstock GF 677 (Figures 1, 2). The plantlets cultivated with 1 mg L^{-1} potassium silicate (K_2SiO_3) in the nutrient medium (variant K1) had the highest fresh (FW) and dry (DW) biomass weight compared to the control plants and the other treatments (Figures 1, 2). A greater multiplication index (MI) was also recorded in these plants, although there was no proven statistical difference with the control and the calcium silicate treatments (Figure 2).

At the higher potassium silicate concentration (2 mg L^{-1}, K2), a suppressed growth was observed, manifested by lower MI, biomass weight, and stem length (Figures 1, 2). FW and DW of plants from calcium silicate treatments (Ca1, Ca2) did not differ significantly from those of the control plants (Figures 1, 2)

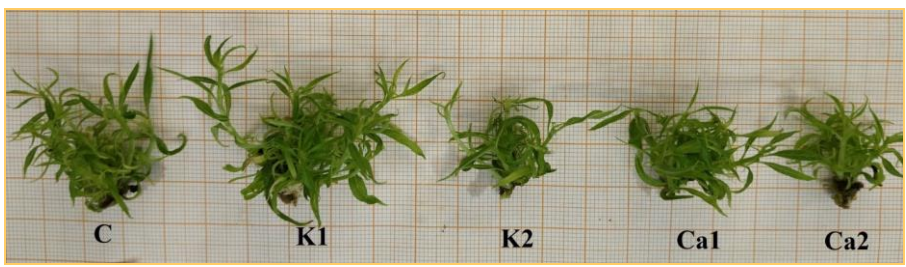


Fig. 1. GF677 shoot culture at multiplication stage on nutrient media with different sources and concentrations of Si. In this and the following figures and tables: C – control, without Si; K1 and K2 - potassium silicate (K_2SiO_3), 1 or 2 mg L^{-1}, resp.; Ca1 and Ca2 - calcium silicate ($CaSiO_3$), 1 or 2 mg L^{-1}, resp.

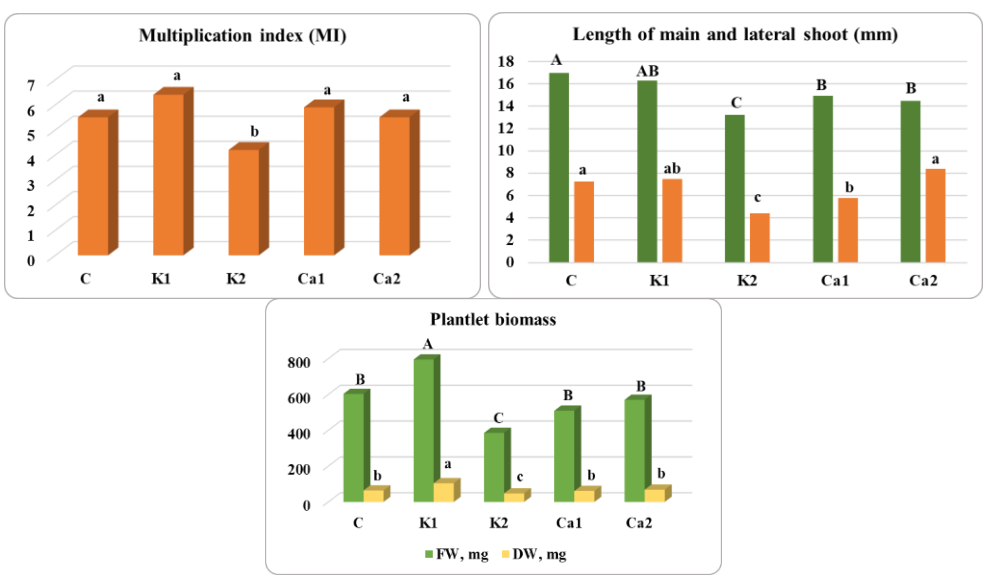


Fig. 2. Growth characteristics of GF677 shoots at multiplication stage on nutrient media with different sources and concentrations of Si. In this and the following figures and tables: C – control, without Si; K1 and K2 - potassium silicate (K_2SiO_3), 1 or 2 mg L^{-1}, resp.; Ca1 and Ca2 - calcium silicate ($CaSiO_3$), 1 or 2 mg L^{-1}, resp.

The content of chlorophyll *a*, total chlorophyll and carotenoids in the leaves of plantlets cultivated with potassium silicate (K_2SiO_3) (K1 and K2) was higher than that of the control and the other treatments (Table 1).

Table 1. Photosynthetic pigments content (mg g^{-1}FW) in the leaves of GF 677 *in vitro* shoots grown on nutrient media with Si. Chl - Chlorophyll, Car – Carotenoids. In this and the following figures and tables: C – control, without Si; K1 and K2 - potassium silicate (K_2SiO_3), 1 or 2 mg L^{-1}, resp.; Ca1 and Ca2 - calcium silicate ($CaSiO_3$), 1 or 2 mg L^{-1}, resp.

Variants	Chl(a)	Chl b(b)	Chl(a+b)	Car	Chl a/Chl b)	Chl/Car
Control	0.49±0.01 c	0.19±0.01 a	0.67±0.02 c	0.18±0.01 b	2.56±0.14 b	3.64±0.03 a
K1	0.87±0.16 a	0.30±0.10 a	1.17±0.16 a	0.32±0.09 a	2.89±0.08 a	3.58±0.08 ab
K2	0.66±0.01 b	0.23±0.01 a	0.89±0.01 b	0.26±0.00 a	2.92±0.06 a	3.49±0.02 b
Ca1	0.36±0.02 c	0.14±0.00 b	0.50±0.02 c	0.13±0.01 b	2.59±0.07 b	1.41±0.05 c
Ca2	0.40±0.02 c	0.15±0.00 b	0.56±0.02 c	0.15±0.01 b	2.59±0.13 b	3.79±0.18 a

3.2. Rooting stage

Silicon in the nutrient medium also affects the rooting of plantlets (Figures 3 and 4). A higher rooting percentage was reported for variant K1 compared to the control and the other treatments (Figure 4). At both concentrations of calcium silicate (variants Ca1 and Ca2), the rooting percentage decreases. In these two variants, however, a trend toward a higher number of roots is observed, but the difference compared to the other variants is not statistically significant (Figure 4).



Fig. 3. GF677 plantlets at rooting stage on nutrient media with different sources and concentrations of Si. In this and the following figures and tables: C – control, without Si; K1 and K2 - potassium silicate (K_2SiO_3), 1 or 2 mg L^{-1}, resp.; Ca1 and Ca2 - calcium silicate ($CaSiO_3$), 1 or 2 mg L^{-1}, resp.

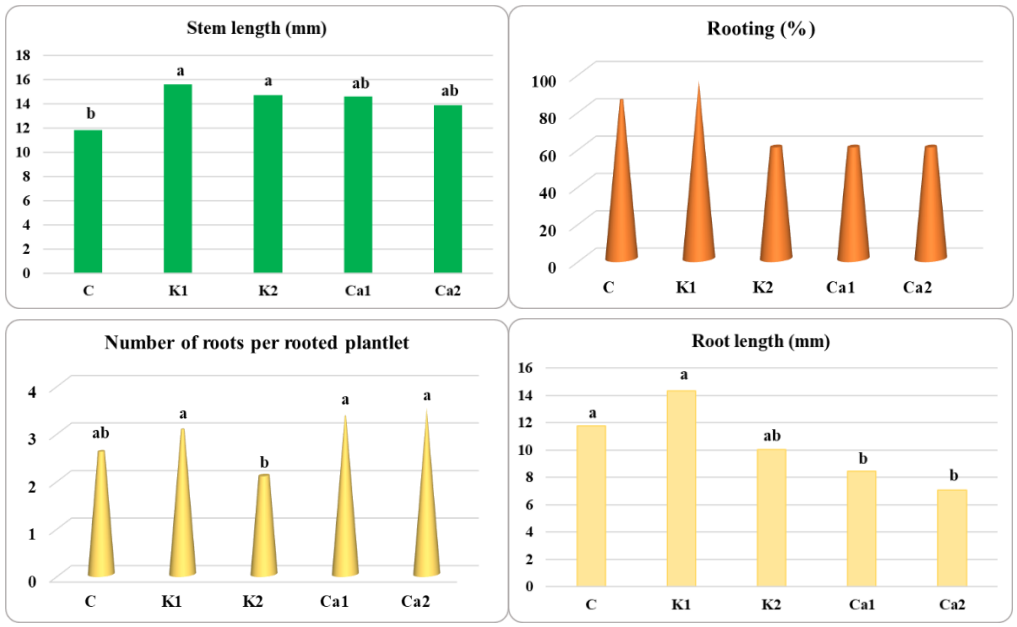


Fig. 4. GF677 plantlets at rooting stage grown on nutrient media with different sources and concentrations of Si. In this and the following figures and tables: C – control, without Si; K1 and K2 - potassium silicate (K_2SiO_3), 1 or 2 mg L^{-1}, resp.; Ca1 and Ca2 - calcium silicate ($CaSiO_3$), 1 or 2 mg L^{-1}, resp.

Adding Si to the culture medium as a potassium silicate (variants K1 and K2) significantly increased the stem length as compared to the control variant. (Figure 4). In the variants of the nutrient medium enriched with Si in the form of calcium silicate, the mean stem length of the plantlets was slightly greater compared to the control, but the difference was not statistically significant (Figure 4).

4. DISCUSSION

The results obtained showed that the addition of 1 mg L^{-1} potassium silicate (K1) to the culture medium stimulated the accumulation of biomass, the development of new shoots, and the rooting of GF677 plantlets. Our results are in agreement with the findings of Soares *et al.* (2011) that cultivation of *Cattleya loddigesii* on medium with K_2SiO_3 (5 mg L^{-1}) improves both microplant growth and root number. In contrast, our studies showed that the higher concentration of potassium silicate (2 mg L^{-1}) had a substantially suppressive effect on the microplants growth.

Potassium silicate (K_2SiO_3) stimulated the accumulation of photosynthetic pigments in the leaves of GF 677 plantlets (Table 1). Similarly, Braga et al. (2009) reported that Si could promote beneficial changes in plants, such as greater development of photosynthetic tissues and chlorophyll production.

Our results regarding the application of calcium silicate at concentrations of 1 and 2 mg L^{-1} did not confirm the findings of Zhuo (1995) concerning enhanced leaf growth in

Phalaenopsis plants grown on a nutrient medium enriched with $CaSiO_3$, nor the data on chlorophyll accumulation in bananas (Asmar *et al.,* 2011).

According to Camargo *et al.* (2007), the use of Si as a nutrient media supplement for during *in vitro* cultivation can increase the content of hemicellulose and lignin cell wall stiffness and the survival of plants during acclimatization.

Optimal Si concentration required for plant growth and development varies between different genotypes within the same species and between individual plant species (Lim *et al.,* 2012).

All these studies show that enrichment of culture media with Si stimulates the *in vitro* growth and morphogenesis of plant tissues, but further studies are needed to optimize the sources of silicon and the most applicable concentrations according to the plant material used.

5. CONCLUSIONS

The results obtained showed that the addition of 1 mg L^{-1} potassium silicate to the culture medium stimulated the accumulation of biomass and the development of new shoots, although the latter was not statistically proven. Incorporation of Si into tissue culture media might have favorable effect on micropropagation of the peach rootstock GF677, but silicate sources and favorable concentrations should be thoroughly tested. The presented results would be useful in attempts for improving the micropropagation of this valuable peach rootstock.

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Preserving historic trees for Pückler Park Branitz, Germany, by *in vitro* propagation – The INVITRA project

Juliane Raschke[1], Aisha Mehar[1], Liis Viljak[1], Anika Dreilich[1], Christian Böhm[2], Juliane Ribbeck-Lampel[2], Andrea Rupps[1]

[1]Department of Plant Evolution and Biodiversity, Späth-Arboretum, Institute of Biology, Humboldt-Universität zu Berlin, Späthstr. 80/81, 12437 Berlin, Germany

[2]Stiftung Fürst-Pückler-Museum Park und Schloss Branitz, Robinienweg 5, 03042 Cottbus, Germany

juliane.raschke@hu-berlin.de

Abstract

Landscaping parks with their numerous heritage trees face several problems - long-term cultivation on the one hand, climate change on the other, leading to the demise of trees due to prolonged droughts, late frosts, and extreme heat. Branitz Park, a unique cultural landscape shaped by Fürst Pückler in 1845/46 experiences loss of several valuable and partly unique trees. A collaboration between the Foundation Pückler Museum - Branitz Park and Palace (SFPM) and the Späth-Arboretum of Humboldt-Universität zu Berlin plans to ensure the conservation of historically valuable and ideally resilient tree genotypes of both landscape parks. Aiming to maintain a variety of species, we apply various cultivation techniques to produce true-to-type trees - from classical horticultural approaches such as rooting cuttings, to applying tissue culture protocols for recalcitrant species. We are trying to establish *in vitro* cultures by establishing micro-cuttings, embryogenic callus or the initiation of somatic embryogenesis, depending on species and available tissue. Further to set up a living collection and preserve the trees for the future.

Keywords: *woody plants; tissue culture; micro propagation; ex situ conservation*

1. INTRODUCTION

Historic trees are an integral part of many gardens, parks and designed landscapes. Their significance extends beyond their biological value: individual trees may document historical planting schemes, rare cultivars, horticultural preferences and the development of a particular garden monument (Kühn *et al.,* 2017). Because these trees are living organisms, their conservation differs fundamentally from the preservation of built structures. Growth, ageing, changing environmental conditions and interactions with pests and pathogens continuously influence their condition. Climate change adds further pressure - through longer periods of drought, higher mean temperatures, more frequent heat extremes and storm events. Mild winters and earlier vegetation periods may additionally increase vulnerability to late frosts and pathogens (Kühn, 2024). Particularly valuable old individuals may therefore be at risk, precisely because of their age and historical significance.

The Branitzer Park, Eastern Brandenburg, Germany, is a prominent example of these challenges. The park was designed by Hermann Fürst von Pückler-Muskau from 1845/46 onwards. Many of the trees still present today were deliberately selected and planted during the development of the landscape. They form part of the historical composition and document the use of woody plants and design principles of the nineteenth century. The loss of individual trees therefore represents not only the loss of a living organism, but potentially

also the loss of a historically specific genotype or cultivar that may be difficult or impossible to replace. The conservation of such material requires an initial identification and documentation of trees, whose historical origin, characteristic morphology, cultivar properties or relevance for the appearance of the garden justify active conservation. At the same time, the selection of future conservation material should consider adaptation to local environmental conditions. The Späth-Arboretum of Humboldt-Universität zu Berlin provides ideal conditions for addressing these challenges. With around 1,100 tree and shrub species, it hosts an exceptionally diverse woody plant collection and draws on more than 40 years of experience in *in vitro* culture. The Pückler Park complements this expertise with long-standing practical experience in field cultivation, woody plant propagation and the management of historically significant tree populations. Those two locations also share challenging site conditions, as both are characterized by sandy soils with high water and nutrient permeability, which can limit plant establishment and increase vulnerability to drought. The combination of complementary expertise and comparable environmental conditions provides a strong basis for the Park Branitz–Späth Arboretum, Berlin cooperation and for developing and testing innovative approaches to the conservation of historically significant woody plants under changing climatic conditions (Rupps *et al.*, 2026, in press).

Vegetative propagation offers a direct route to maintaining the characteristic properties of selected mother trees. Conventional techniques such as cuttings, grafting and layering are well established in horticulture, but their success can be strongly limited by tree age, physiological condition and species-specific rooting capacity (Read & Bavougian, 2012). In some cases, historically valuable trees provide little suitable propagation material, and sufficient numbers of genetically identical offspring cannot be produced by conventional methods. These limitations are especially relevant when rare genotypes need to be secured before the loss of the original tree.

In vitro culture can complement conventional propagation by providing controlled conditions for the establishment and multiplication of selected genotypes. Starting from a small amount of plant material, successful cultures can potentially generate numerous genetically identical plants and can also provide material for living *in vitro* collections and cryopreservation (Guan *et al.*, 2016; Nagel *et al.*, 2024). However, the establishment of a reliable tissue-culture system is itself highly genotype- and species-dependent. Sterilization, medium composition, plant growth regulators, light and temperature conditions may all need to be adjusted for a particular explant and developmental stage. For difficult woody plants, a single standardized protocol is therefore unlikely to be sufficient (Guan *et al.*, 2016; Read & Bavougian, 2012).

The INVITRA project ("*In vitro* propagation of traditional and adaptable species") addresses this challenge by combining the horticultural expertise of Stiftung Fürst-Pückler-Museum Park und Schloss Branitz with the long-standing *in vitro* experience and extensive woody plant collection of the Späth-Arboretum at Humboldt-Universität zu Berlin. The objective is not to replace conventional horticulture, but to establish complementary propagation and conservation routes for historically significant genotypes. Our article summarizes the selection of conservation material and the first results of a broad screening of classical and *in vitro* propagation approaches.

2. MATERIALS AND METHODS

2.1. Materials

Approximately 100 individual trees representing 62 species and 14 plant families were selected as potential conservation material. The selection included historically significant, rare or otherwise particularly valuable individuals from the Branitzer Park and the Späth-Arboretum collections. Genera represented in the screening included, among others, *Acer, Alnus, Carpinus, Celtis, Fagus, Fraxinus, Gleditsia, Larix, Parrotia, Quercus, Robinia, Sophora, Tilia* and *Ulmus*. The selected trees were documented with respect to their identity as well as provenance, and material was collected at several time points during 2025 and 2026.

Plant material was selected with the aim of obtaining tissue that was as ontogenetically young and physiologically responsive as possible (Read & Bavougian, 2012). Depending on species and tree condition, starting material included flower or shoot buds, young shoots, young leaves and softwood cuttings. Where appropriate, attempts were also made to obtain younger or potentially more responsive material through conventional propagation, including grafting.

2.2. Methods

Conventional propagation was investigated in parallel with *in vitro* approaches. Softwood and other cuttings were tested under different rooting conditions and substrates. Grafting experiments were initiated using scions from historically valuable trees, with the objective of generating younger material that might show improved responsiveness in subsequent tissue-culture experiments. These approaches were considered complementary rather than competing strategies.

For *in vitro* establishment, plant material was subjected to sterilization procedures adapted to the sensitivity and contamination risk of the respective explants. Generally closed buds were treated with 70% ethanol for 1 min followed by sodium hypochlorite (NaClO, 5%, 3 min), 70% ethanol for 30 s and rinsing with sterile water. More sensitive material was treated with 5% NaClO for 15 min, followed by 70% ethanol for 1 min and a subsequent treatment with sodium dichloroisocyanurate (NaDCC, 0.01%) for 30 min before rinsing. These procedures were adjusted where necessary according to the response of individual explants.

Culture media were selected and modified according to the plant material and intended developmental response. Media used for initiation and maintenance were based mainly on WPM and MS formulations and included combinations of plant growth regulators and additives such as BA, zeatin, thidiazuron (TDZ), indole-3-butyric acid (IBA), 2,4-D and picloram. The large number of species and limited experimental resources required a broad screening strategy in which the number of media tested per species was restricted. The objective of this approach was to identify promising combinations and visible responses that may subsequently be investigated in greater depth.

Three principal *in vitro* routes were considered. First, micro-cuttings were used with the aim to establish and multiply young shoots or buds. Once a shoot culture has been established, repeated subdivision could provide a basis for multiplication and subsequent rooting. Second, meristematic or other actively dividing tissues were screened for callus formation and organogenic responses. Third, somatic embryogenesis was considered where

organogenesis or conventional shoot multiplication appeared unsuitable, particularly for taxa for which embryogenic systems have been an established or potentially relevant route. Cultures were monitored for changes in tissue structure and development, and promising material was retained for further characterization.

Initial responses were assessed qualitatively according to the development of shoots, callus-like tissue, tissue growth and the potential for further multiplication or differentiation. The screening was designed primarily to identify promising starting points rather than to establish final, species-specific production protocols. Consequently, absence of a visible response under the initial conditions was treated as an indication for further protocol development rather than as evidence that a genotype cannot be propagated *in vitro*.

3. RESULTS

The broad screening confirmed substantial differences in the response of historic woody plant material to both conventional and *in vitro* propagation approaches. The response depended not only on species, but also on the genotype, developmental stage and type of explant used. This variability was apparent already during the establishment phase and influenced the choice of subsequent approaches.

Micro-cutting experiments produced initial positive responses in 18 species. These responses included the development and continued growth of shoots or buds under *in vitro* conditions and were observed for several different genera. Among the material showing initial responses were genotypes of *Acer, Sophora* and *Robinia.* The establishment of a response at this stage demonstrates the feasibility of using micro-cutting as a route for selected historic genotypes, although multiplication and rooting still require further optimization for many of the cultures.

Bud- and leaf-derived explants from 14 species produced callus or potentially callus-like tissue. The morphology of the responses varied considerably, ranging from compact tissue to more proliferative or heterogeneous structures. Examples included material from *Celtis, Acer, Quercus* and *Betula*. The observed responses indicate that the explants remained capable of substantial developmental change under the tested culture conditions, but they do not by themselves demonstrate successful organogenesis or somatic embryogenesis.

Among the cultures obtained so far, genotypes of *Ulmus* and *Fagus* were particularly promising with respect to tissue structure and multiplication potential. Two *Ulmus* genotypes and two *Fagus* genotypes showed responses considered sufficiently promising to justify continued development. In these cultures, further work is focused on distinguishing proliferating calli from organogenic or embryogenic tissue and on identifying culture conditions that support stable multiplication and regeneration.

Somatic embryogenesis remains an important potential route for genotypes and species for which direct shoot multiplication is difficult (Guan *et al.*, 2016). In the present screening, however, the development of callus-like or embryogenic-looking tissue was not considered sufficient evidence of somatic embryogenesis. For the most promising cultures, successful maturation and/or histological characterization is required before a somatic embryogenic pathway can be confirmed (Fehér, 2019). This distinction is important because

morphologically similar callus structures may differ fundamentally in their developmental potential.

The results therefore represent a set of different stages of progress rather than a single standardized propagation protocol. For some genotypes, the work has progressed towards multiplication; for others, the principal aim remains to establish a stable culture and the identification of a potentially suitable explant and culture condition. This reflects the large biological diversity of the starting material and the need to develop conservation strategies on a genotype-specific basis.

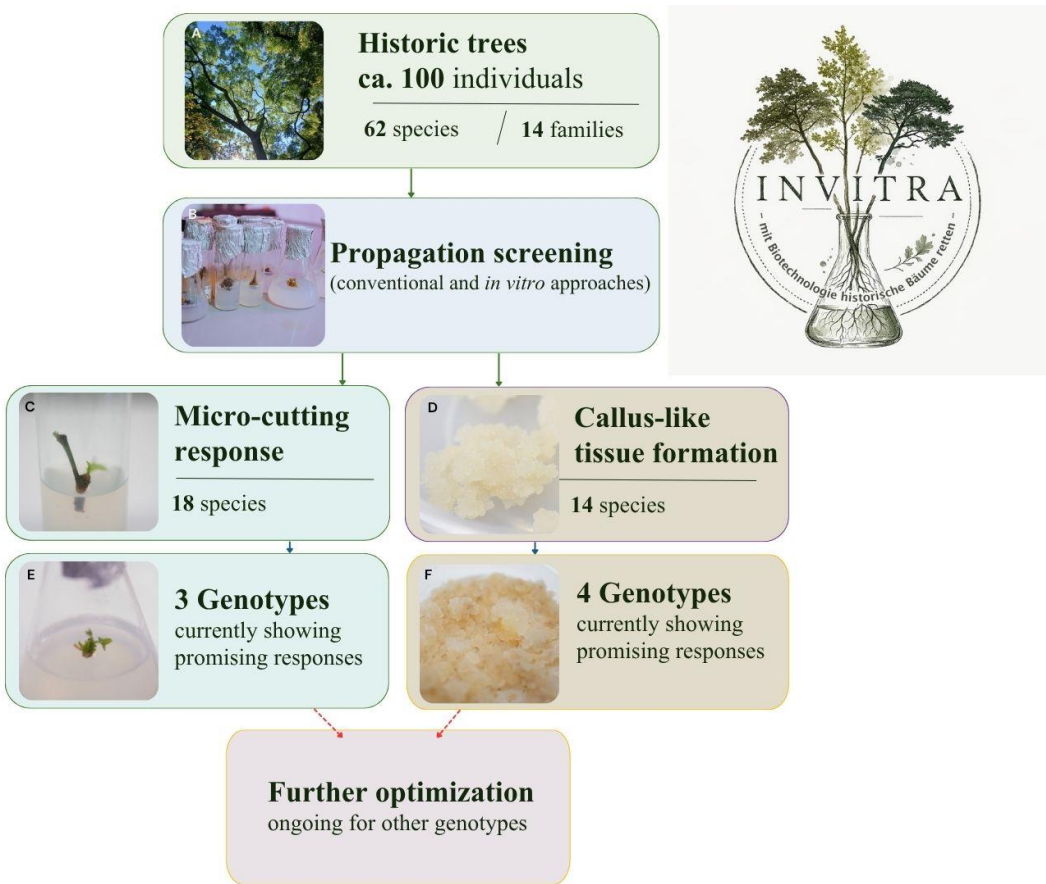


Fig. 1. Workflow and current propagation responses from historic trees in Pückler Park Branitz and Späth-Arboretum Berlin.

Approximately 100 trees representing 62 species and 14 families were screened using conventional and *in vitro* propagation approaches. Micro-cutting responses were obtained for 18 species, while callus-like tissue formation was observed in 14 species. The screening identified four promising genotypes for further "callus- propagation" and conservation as well as three promising genotypes for multiplication via shoot culture.

A *Fraxinus excelsior "Verrucosa"*; B Plant material of various species 6+ weeks after initiation; C *Robinia pseudoacacia*; D *Fagus lucida after multiplication phase*; E *Sophora japonica* L.*;* F *Ulmus lamelosa* after 5 weeks of treatment change was initiated.

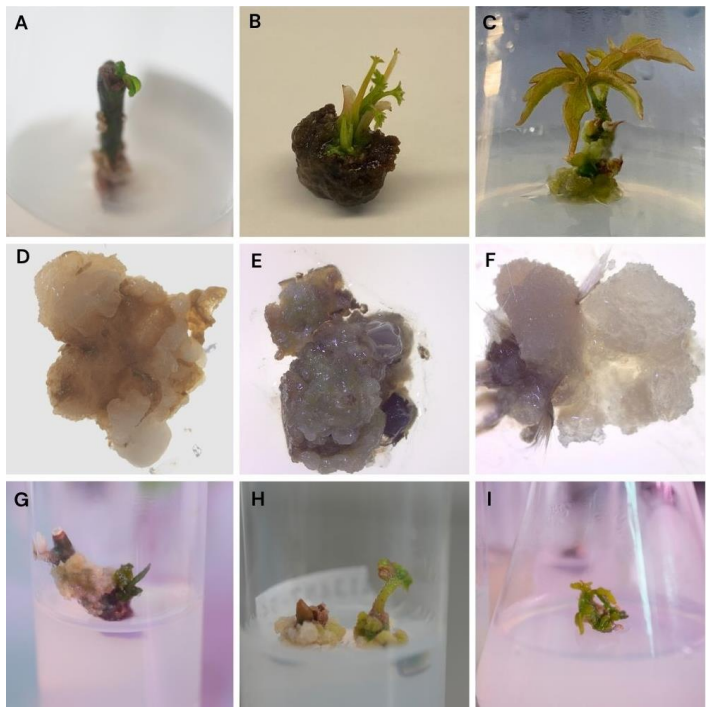


Fig. 2. Representative *in vitro* responses observed during the screening of *in vitro* cultures of historic trees. Examples include an explant establishment of *Gleditsia caspica* DESF. (A); a shoot development in a responding micro-cuttings of *Betula maximowicziana* (B) and *Acer saccharinum ssp. grandidentatum* (C); callus-like tissue formation in *Quercus spec*. (D); *Quercus castaneifolia* (E)*;* and *Fagus lucida* (F); growing callus structures with shoot development in *Robinia x ambigua* POIR. cv. *'Bella-Rosea'* (G) and *Celtis laevigatavar. Reticulata* (H); as well as a beginning shoot regeneration in *Robinia pseudoacacia* (I). Further optimization is required to assess their potential for sustained multiplication and regeneration.

4. DISCUSSION

The results illustrate the central difficulty of conserving historic woody genotypes through *in vitro* propagation: the method is potentially powerful, but its successful application cannot be separated from the biology of the individual genotype. The screening of approximately 100 trees across 62 species was deliberately broad because the first priority was to secure material from a large number of potentially valuable trees. This approach increases the probability of finding responsive genotypes, but it also means that the initial culture conditions cannot be expected to represent optimized protocols for every species.

The initial responses obtained in 18 species demonstrate that micro-cutting can provide a useful complementary route to conventional propagation for selected historic trees. The principal advantage is the possibility of multiplying genetically identical material from a limited amount of starting tissue under controlled conditions. At the same time, the results show that establishment of a shoot response is only one step in the process. Stable multiplication, rooting and subsequent transfer to *ex vitro* conditions must still be achieved before a culture can contribute to a practical conservation collection. The formation of callus-like tissue in 14 species is similarly encouraging but must be interpreted cautiously.

Callus formation is not equivalent to successful regeneration (Fehér, 2019). Tissue may proliferate without acquiring the developmental competence required for organogenesis or somatic embryogenesis. Histological characterization and successful maturation or regeneration are necessary to confirm the developmental identity of the most promising cultures. The particularly promising responses observed in *Ulmus* and *Fagus* demonstrate the value of continuing a broad initial screening while progressively concentrating resources on the most responsive material. The strategy can therefore be viewed as a funnel: many genotypes are screened under a limited set of conditions, promising responses are retained, and increasingly genotype-specific protocols are developed for selected cultures.

Conventional propagation remains an essential component of the conservation strategy. Cuttings and grafting may provide the most direct route for some trees and can also generate younger material that may respond better in tissue culture. *In vitro* propagation should therefore not be considered a substitute for horticultural expertise. Instead, the results support an integrated approach in which conventional propagation, micro-cutting, organogenesis and somatic embryogenesis are selected according to the biological properties of each genotype. A further challenge is the transition from *in vitro* culture to *ex vitro* growth. Plants developing under controlled conditions must adapt to changes in water availability, nutrition, gas exchange and the physical environment when transferred to soil or another horticultural substrate. This acclimatization step can be difficult and may require repeated optimization. Consequently, the successful establishment of an *in vitro* culture does not automatically result in a plant that can be handed over to a nursery or conservation collection. Practical horticultural capacity and long-term care are therefore integral parts of the conservation process. The project also provides a basis for long-term risk reduction through the establishment of living and cryopreserved collections. Living collections allow continued observation, propagation and horticultural evaluation, whereas cryopreservation can maintain revitalizable material independent from external environmental conditions (Nagel *et al.*, 2024). Combining these forms of conservation can reduce the risk that the loss of an old mother tree results in the irreversible loss of its genotype. Finally, the work demonstrates the importance of close cooperation between heritage conservation, horticulture and plant biotechnology. The selection of historically relevant trees requires knowledge of the cultural and historical context; the propagation of the material requires practical horticultural expertise; and the development of *in vitro* systems requires specialized biological methods. None of these components alone is sufficient for the long-term conservation of difficult woody plant genotypes. The INVITRA approach therefore provides a model that can potentially be adapted to other historical parks, gardens and arboreta, while recognizing that protocols and conservation priorities must remain site- and genotype-specific.

5. CONCLUSIONS

The INVITRA project demonstrates the potential of combining conventional horticultural propagation with *in vitro* techniques to secure historically valuable woody genotypes. The broad screening identified initial micro-cutting responses in 18 species and callus or potentially callus-like responses in 14 species, with particularly promising cultures among selected *Ulmus* and *Fagus* genotypes. At the same time, the results emphasize that *in vitro* conservation of historic trees is a developmental process rather than a single standardized

method. Species- and genotype-specific responses, the need for further characterization of callus and embryogenic structures, and the difficult transition from *in vitro* culture to *ex vitro* growth all represent important steps that must be addressed before a robust conservation pipeline is established. The next phase should focus on the most promising genotypes: validating their regenerative capacity, refining multiplication and rooting methods, as well as developing dependable acclimatization procedures. By working closely with European colleagues, sharing experience, possibly even facilities and plant material - where appropriate - we may be better placed to protect and multiply valuable tree resources efficiently and support their resilience in a rapidly changing environment. In parallel, the systematic documentation of mother trees and the establishment of living and cryopreserved collections can provide a long-term safeguard for valuable genetic resources. By combining these approaches, historically significant trees can be conserved not only as individual monuments, but also as living genetic resources capable of supporting future restoration, replacement planting and climate-adapted conservation.

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Salt tolerance of *Artemisia maritima* L. under *in vitro* conditions: A comparison of agar-solidified and temporary immersion cultivation systems

Liva Purmale-Trasune[1,2], Ksenija Nikitina[2], Gederts Ievinsh[2]

[1]*Bulduri Biotechnology Center, Bulduri Technical School, 6 Viestura Str., LV-2010 Jurmala, Latvia*

[2]*Department of Ecology, Faculty of Medicine and Life Sciences, University of Latvia, 1 Jelgavas Str., LV-1004 Riga, Latvia*

liva.purmale@bulduri.lv

Abstract

Soil salinity is a significant abiotic factor limiting plant growth, highlighting the importance of halophyte research. *Artemisia maritima* L. (sea wormwood) is a coastal plant species with considerable potential as a source of bioactive compounds. To evaluate salt tolerance under *in vitro* conditions, Murashige & Skoog medium supplemented with 0, 1.0, 2.5, and 4.0 g/L Na^+ was used for cultivation of proliferating shoot explants of *A. maritima*, both in agarized and liquid media. As no information was available on optimal cultivation regimes for *A. maritima* in Plantform™ temporary immersion system, three immersion/aeration regimes were tested: (I) immersion for 3 min every 3 h followed by 5 min of aeration; (II) immersion for 10 min every 12 h with 10 min of aeration every 2 h; (III) immersion for 15 min every 24 h with 5 min of aeration every 2 h. After four weeks, the experiment was terminated, and plant fresh weight, dry weight, water content, Na^+ and K^+ concentration, and electrical conductivity were determined. The highest fresh weight was obtained in the temporary immersion system 24 h regime at 1 g/L Na^+, whereas growth decreased in all cultivation types at 4 g/L Na^+. Tissue water content was generally higher in temporary immersion system compared to agarized culture medium. Increasing Na^+ concentration in the medium resulted in higher Na^+ concentration and electrical conductivity in plant material, while K^+ concentration remained relatively stable, suggesting effective ion regulation. Moderately low Na^+ concentration had no negative effect on *A. maritima* growth confirming the species salt tolerance.

Keywords: *sea wormwood, halophyte, salt stress, Plantform™*

1. INTRODUCTION

Soil salinity is a significant abiotic stress factor that restricts plant growth, and it is becoming an increasingly pressing issue globally (Shrivastava & Kumar, 2015). Consequently, plant species that naturally occur in saline habitats attract considerable research interest. *Artemisia maritima* (sea wormwood) is a perennial halophytic species thriving in temperate climate zones (Kumar *et al.,* 2011; Andersone-Ozola *et al.,* 2026). In the Baltic Sea region, it thrives close to the sea (Banaszczyk *et al.,* 2025). In Sweden, the species has been characterized as "competitive only under moderate to high salinity" conditions (Tyler *et al.,* 2021). *A. maritima* has long been recognized as a valuable source of bioactive compounds and essential oils (Bora & Sharma, 2010). Furthermore, recent studies have highlighted its potential for utilization in phytoremediation of saline and contaminated environments (Andersone-Ozola *et al.,* 2026).

Although micropropagation protocols for *A. maritima* have been established (Nabi *et al.,* 2022), there is currently a lack of information on the application of temporary immersion systems (TIS) for this species and on its salt tolerance under *in vitro* conditions where shoots are unrooted. It is suggested that clonal growth has a special adaptive role in the resilience of *A. maritima* given the ability of roots to form shoot buds, as plants potentially retain significant regeneration ability when soil salinity decreases (Andersone-Ozola *et al.,* 2026).

The aim of this study was to investigate the salt tolerance of *A. maritima* under *in vitro* conditions by comparing cultivation of proliferating shoot explants on agar-solidified medium and TIS at varying Na^+ concentrations.

2. MATERIALS AND METHODS

For the experiments, previously established *in vitro* cultures of *Artemisia maritima* were used (Andersone-Ozola *et al.,* 2026). Na^+ gradient (0, 1.0, 2.5, 4 Na^+ g/L) was created using NaCl, for pH adjustment KOH was used. As there were no recommendations for *A. maritima* Plantform™ TIS regimes, three protocols were tested and compared to agarized medium, which was used as control. TIS regimes tested were: (I) immersion for 3 min every 3 h followed by 5 min of aeration (Liu *et al.,* 2004); (II) immersion for 10 min every 12 h with 10 min of aeration every 2 h (Purmale *et al.*, 2024); (III) immersion for 15 min every 24 h with 5 min of aeration every 2 h. After 4 weeks the experiment was terminated and plant fresh weight, dry weight, tissue electrical conductivity (EC), concentration of Na^+ and K^+ were measured as described before (Purmale *et al.,* 2022). Hyperhydricity and necrosis were scored on a 0–2 scale (0 = absent, 2 = high). Water content was calculated as (fresh weight − dry weight) x100 / fresh weight.

Results were analyzed by R (v. 4.5.1), and Excel (Microsoft Office 365). Statistical significance of fresh weight, dry weight, and water content differences was evaluated by one-way ANOVA using Tukey post-hoc analysis ($p < 0.05$). Two- way ANOVA followed by Tukey HSD, $p < 0.05$ was used to calculate if there were changes of Na^+, K^+, EC levels in plant material depending on cultivation regime and Na^+ concentration in the medium.

3. RESULTS

Four weeks after the start of the experiment, data were collected and analyzed. Visual observations revealed that the elevated Na^+ concentrations (2.5 and 4.0 g/L) were excessive, leading to hyperhydricity and necrosis. Additionally, explants cultivated in the TIS appeared larger and more vigorous compared to those cultivated on agar-solidified medium (Fig. 1).

Measurements confirmed that fresh weight was generally higher in TIS conditions, particularly in control and treatments with 1.0 g/L and 2.5 g/L Na^+, compared to agarized medium. For dry weight, differences between treatments were less pronounced than for fresh weight. Nevertheless, TIS treatments still exhibited higher dry mass values. Water content between treatments did not show statistically significant differences, but water content tended to be higher in TIS treatments and in treatments with higher Na^+ concentration in the medium (Fig. 2).

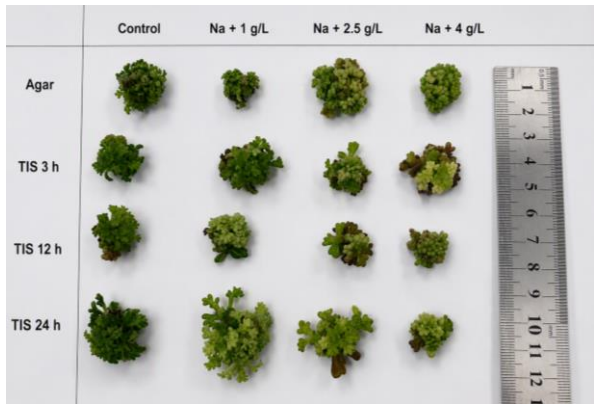


Fig. 1. Representative explants cultured under four growth regimes (agarized medium, TIS 3 h, TIS 12 h, and TIS 24 h) and exposed to different Na^+ concentrations (control, 1, 2.5 and 4 g/L). One representative explant from each treatment combination is shown. Scale bar is indicated by the ruler.

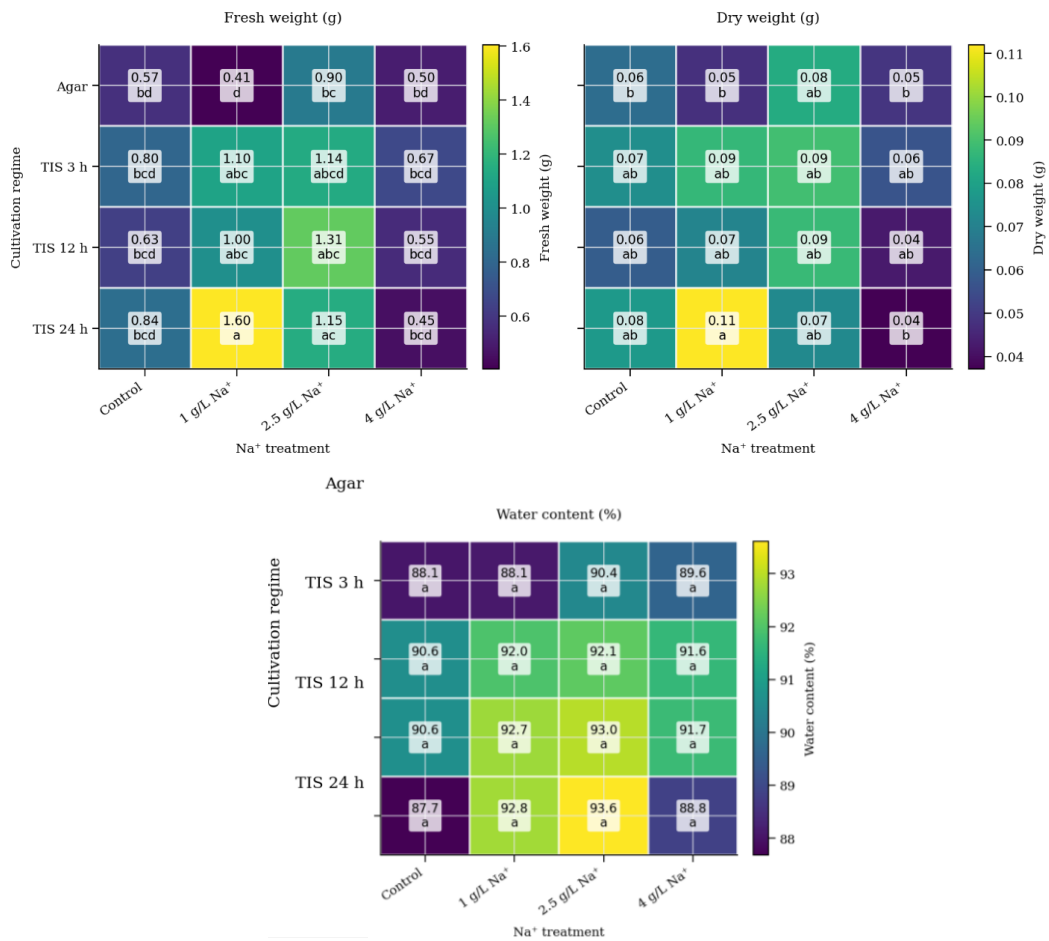


Fig. 2. Effect of cultivation system (agarized medium and TIS with three different regimes) and Na^+ treatment (control, 1, 2.5, 4 g/L) on biomass accumulation and water content. In the heat maps, values are presented as mean ± standard error (SE), followed by the statistical group letter (Tukey, $p \leq 0.05$).

Both necrosis and hyperhydricity increased markedly with salinity and were influenced by the cultivation system and immersion duration (Fig. 3). The least intensity of necrosis was observed on agarized medium. The regime with immersion every 3 h was the worst of the TIS regimes tested resulting in explant hyperhydricity.

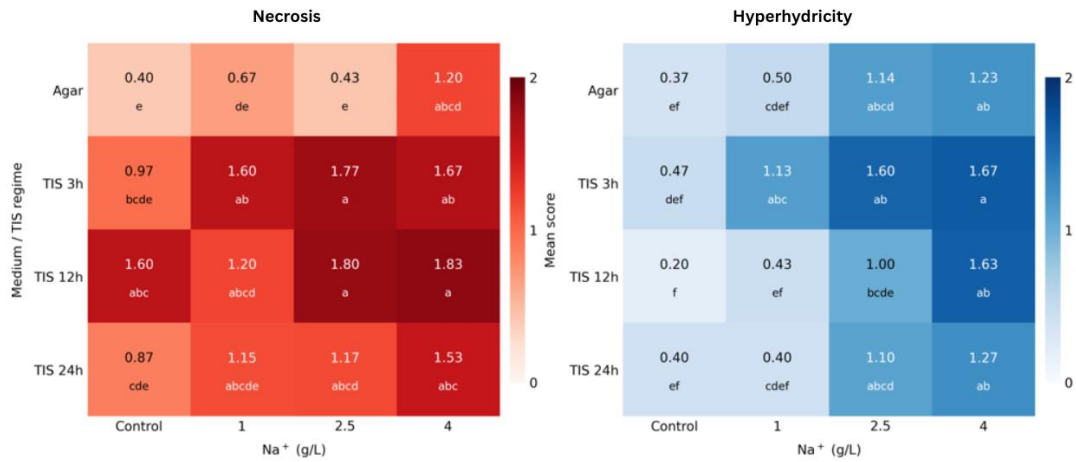


Fig. 3. Heat maps showing necrosis (left) and hyperhydricity (right) in agarized medium and TIS under increasing Na^+ concentration. Values represent mean scores on a 0–2 scale (0 = absent, 2 = high). Letters display groupings based on Tukey's HSD test comparing all 16 treatment combinations; treatments sharing at least one common letter are not significantly different at $p \leq 0.05$

Two-way ANOVA revealed that Na^+ concentration in the culture medium significantly affected Na^+ accumulation ($p < 0.0001$), K^+ concentration ($p = 0.00118$), and EC ($p < 0.0001$) in explant tissues. The cultivation regime had a significant effect on Na^+ concentration in plant tissues ($p = 0.0447$), whereas its effects on K^+ concentration ($p = 0.0534$) and EC ($p = 0.120$) were not statistically significant. However, significant interaction effects between Na^+ concentration in the medium and cultivation regime were detected for K^+ concentration ($p = 0.00587$) and EC ($p = 0.0126$), indicating that the responses of these parameters to salinity depended on the cultivation conditions.

Na^+ accumulation in plant tissues was primarily driven by increasing Na^+ concentrations in the culture medium, while the cultivation regime exerted a comparatively smaller influence. Although K^+ concentration was significantly affected by Na^+ treatment, the correlation between Na^+ and K^+ concentrations in plant tissues was weak (Pearson's $r = -0.15$), suggesting only a limited relationship between the two ions. Nevertheless, the significant interaction effect indicates that K^+ responses to salinity varied between cultivation regimes.

Electrical conductivity was strongly influenced by Na^+ treatment level, as reflected by the very strong positive correlation between Na^+ concentration and EC in plant tissues (Pearson's $r = 0.95$). These results suggest that salinity was the dominant factor affecting ion accumulation and tissue EC (Fig. 4).

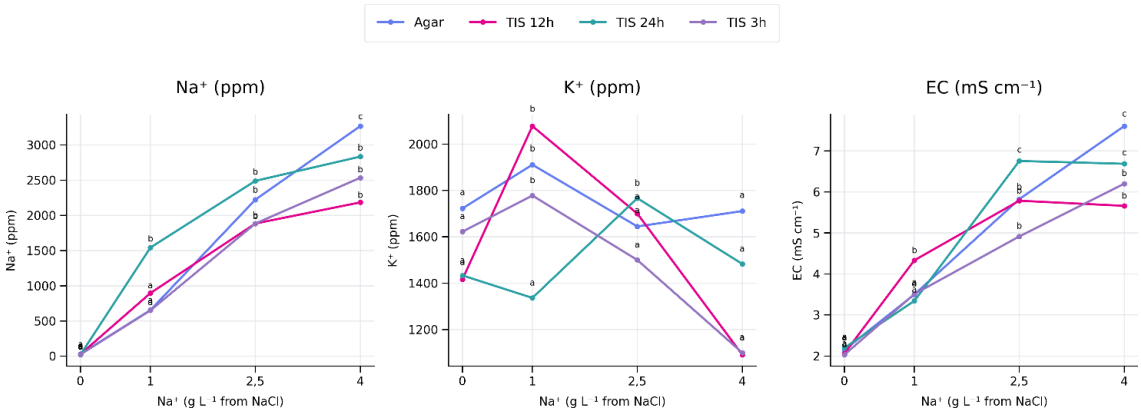


Fig. 4. Effect of Na^+ concentration (in a form of NaCl) in the medium on Na^+ and K^+ content and EC in explant tissues under different cultivation regimes. Different letters indicate statistically significant differences among all treatment combinations, considering both Na^+ concentration and cultivation regime, according to the Tukey HSD test ($p < 0.05$).

4. DISCUSSION

Halophytes typically employ one of two primary strategies to cope with salinity. They either avoid excessive salt accumulation in aboveground tissues by retaining ions in the roots or secreting them from leaves, or they tolerate high internal salt concentrations by compartmentalizing ions within specific tissues and cellular compartments (Ievinsh, 2023). A previous study investigated the salt tolerance of *A. maritima* under *ex vitro* conditions (Andersone-Ozola *et al.*, 2026). In contrast to intact plants, the ones cultivated *in vitro* in the present study did not develop roots that could contribute to the regulation of ion uptake. Despite the absence of roots, *A. maritima* shoot explants maintained active growth with 1.0 g/L Na^+ and avoided severe reductions in accumulation of K^+. Plants acquire cations, particularly K^+, through active transport mechanisms (Pardo & Quintero, 2002). Under saline conditions, excessive concentration of Na^+ can disrupt this process because K^+ transporters do not sufficiently discriminate between the two ions. As a result, high external Na^+ concentration can impair K^+ uptake and promote Na^+ accumulation in plant cells, ultimately leading to ion toxicity. In the case of *A. maritima*, a weak correlation between Na^+ and K^+ concentrations indicated that increasing Na^+ level did not directly displace K^+ from plant tissues. This ability to preserve K^+ status despite elevated salinity may contribute to maintenance of metabolic activity and cellular function in *A. maritima*, as maintenance of K^+ homeostasis is considered important for salinity tolerance (Assaha *et al.*, 2017).

The enhanced explant biomass accumulation observed at 1.0 g/L Na^+, particularly in the TIS 24 h treatment, aligns with the halophytic nature of *A. maritima*. Some halophytes can effectively utilize moderate amounts of Na^+ for osmotic adjustment, thereby minimizing the energetic expenditures associated with the synthesis of organic osmolytes (Nawaz *et al.*, 2014). Consequently, low salinity may enhance plant growth, a phenomenon corroborated by numerous studies (Flowers & Colmer, 2008; Purmale *et al.*, 2024). However, at elevated Na^+ (2.5 – 4.0 g/L), salt-induced toxicity was observed in *A. maritima*, manifesting as shoot necrosis (Figs. 1 and 3).

Additionally, at elevated Na^+ concentrations hyperhydricity was observed, particularly in certain TIS regimes. Hyperhydricity is a common physiological disorder in plants cultivated in liquid media and has previously been identified as a challenge that can be mitigated through the use of TIS and optimization of immersion protocols (Etienne & Berthouly, 2002; Lotfi *et al.*, 2020). Two primary approaches have been proposed: frequent short immersion periods or longer, less frequent immersions. For *A. maritima*, the latter strategy demonstrated superior efficacy. Explants subjected to immersion for 15 min every 24 h, in conjunction with 5 min aeration every 2 h, exhibited enhanced quality and diminished symptoms of hyperhydricity compared to explants immersed for 3 min every 3 h followed by 5 min of aeration. While frequent immersion theoretically improves nutrient delivery, repeated contact with liquid medium can lead to excessive tissue hydration. The superior performance of the 24 h immersion regime suggests that limiting the frequency of immersion is more critical than increasing nutrient access for *A. maritima*.

The ability of *A. maritima* to maintain growth under moderate salinity in TIS cultures holds practical significance for large-scale propagation and biomass production. Controlled salinity treatments may potentially be used not only to facilitate the growth of this halophyte but also to modulate the synthesis of bioactive compounds. Consequently, future research should explore the effects of salinity and immersion regimes on secondary metabolite accumulation.

5. CONCLUSIONS

Artemisia maritima rootless proliferating explants exhibited a relatively high tolerance to salinity under *in vitro* conditions. Exposure to relatively low Na^+ concentration (1.0 g/L) did not inhibit growth and, in some cases, stimulated biomass accumulation, thereby confirming the halophytic nature of the species. However, higher Na^+ levels (2.5–4.0 g/L) reduced growth and increased the occurrence of necrosis and hyperhydricity. The cultivation system strongly influenced plant performance. Temporary immersion systems generally promoted greater fresh and dry biomass production compared to agar-solidified medium, although they also increased the risk of physiological disorders, particularly under frequent immersion conditions. Among the tested regimes, Plantform™ TIS regime with immersion for 15 min every 24 h and aeration 5 min every 2 h combined with Na^+ up to 1.0 g/L provided the best combination of biomass accumulation and plant quality. Conversely, immersion every 3 h resulted in the highest hyperhydricity rate. Increasing Na^+ concentration in the culture medium led to progressive Na^+ accumulation and elevated electrical conductivity in plant tissues. Nevertheless, K^+ concentration remained comparatively stable, indicating an effective capacity for ion regulation and maintenance of ionic homeostasis. Overall, the results demonstrate that *A. maritima* rootless explants are well-adapted to moderate salinity and can be successfully cultivated in TIS and agarized medium.

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Date palm artificial seeds: Current results and perspectives

Emna Baklouti[1], Ameni Nasri[1], Ahlem Ben Ahmed, Hazar Akrimi[1], Riadh Drira[1], Saleh Altouwaijri[2], Bart Panis[3], Noureddine Drira[1], Lotfi FKI[1]

[1]Laboratory of Plant Biotechnology, Faculty of Sciences of Sfax- Route Sokra BP 1171, 3000 Sfax, Tunisia, University of Sfax, Tunisia,

[2] Qassim University, Saudi Arabia

[3] Department of Biosystems, KU Leuven, Leuven, Belgium, Bioversity International, Belgian Office, Leuven, Belgium

lotfifki@yahoo.fr

Abstract

Date palm (*Phoenix dactylifera* L.) holds significant socioeconomic value, but its micropropagation is challenging. Artificial seeds offer a promising solution for large-scale commercial regeneration and advancing cryopreservation methods. This study presents an efficient protocol for generating thousands of somatic embryos from juvenile inflorescences. Artificial seeds were successfully developed using 3% (w/v) sodium alginate and 100 mM calcium chloride in MS medium. These concentrations maintained high germination rates, especially when moderate desiccation was applied. Furthermore, encapsulated proembryogenic masses resisted cryotreatments, achieving regeneration rates up to 70%. Despite these advancements, agricultural application remains limited by technical challenges, particularly contamination during soil transfer. To overcome this, integrating artificial intelligence such as computer vision and robotics, offers a pathway to standardize production, optimize formulations, and reduce contamination. This presentation discusses the potential of artificial seeds for date palm propagation and cryopreservation while proposing automation strategies to minimize contamination and facilitate adoption in modern agriculture.

Keywords: *Phoenix dactylifera, somatic embryos, Large scale micropropagation, artificial seeds, cryopreservation*

1. INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is an essential arborescent monocotyledon in arid and semi-arid regions, representing a cornerstone for socio-economic stability, food security, and ecological balance. Traditional propagation methods, such as offshoots, are slow and insufficient to meet the rising commercial demand, while conventional tissue culture faces significant bottlenecks in efficiency and handling. Consequently, micropropagation through somatic embryogenesis has emerged as a powerful alternative. However, the mass distribution of delicate somatic embryos remains limited by their vulnerability to mechanical damage and desiccation (Carlos et Martinez, 1998).

To address these limitations, artificial seed technology offers a promising solution for large-scale commercial regeneration and the optimization of long-term germplasm preservation. Artificial seeds, or synthetic seeds, mimic true seeds by encapsulating somatic embryos or proembryogenic masses (PEMs) within a protective, nutrient-rich hydrogel matrix. This approach provides a scalable system for direct greenhouse or field delivery while serving as an ideal vehicle for cryopreservation. Despite these advantages, widespread agricultural

adoption is hindered by critical technical challenges, most notably micro-organism contamination during the acclimatization and soil transfer phases, alongside a lack of production standardization.

In this study, an efficient protocol was developed to generate thousands of somatic embryos from juvenile inflorescences of *P. dactylifera* L. and encapsulate them using a sodium alginate matrix. The approach systematically evaluated the effects of gel concentrations, chemical complexation agents, and moderate desiccation on germination rates. Furthermore, the protocol assessed the cryotolerance of encapsulated PEMs to enhance long-term storage strategies. Finally, to overcome the persistent challenge of contamination and logistical bottlenecks, this paper explores the integration of automation strategies, including computer vision and robotics, to standardize production and facilitate the adoption of synthetic seeds in modern date palm agriculture.

2. MATERIALS AND METHODS

2.1. Materials

Inflorescences, approximately 15 cm in length, were harvested from healthy adult female date palm (*Phoenix dactylifera* L.) trees of diverse cultivars. These inflorescences were discarded tissues collected during regular agricultural management practices from local plantations. Prior to explant preparation, the spathes enclosing the immature floral tissues were thoroughly washed under running tap water to remove surface dust and debris. The material was then transferred to a laminar air-flow hood for surface sterilization, where the outer sheaths were carefully removed to isolate the inner immature rachis and floral buds under aseptic conditions.

2.2. Methods

Inflorescence disinfection

Prior to explant preparation, the spathes enclosing the immature floral tissues were thoroughly washed under running tap water to remove surface dust and debris. All subsequent surface sterilization procedures were performed strictly under a horizontal laminar air-flow hood. Although the juvenile inflorescences were naturally enclosed and protected within the thick protective spathes, a rigorous disinfection protocol was applied to guarantee complete asepsis. The isolated inflorescences were immersed in a 0.1‰ (w/v) mercuric chloride solution for 45 min and then washed three times with sterile distilled water.

Callus induction

To induce callogenesis and trigger somatic embryogenesis, MS basal media (Murashige and Skoog, 1962) were supplemented with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) as the primary induction agent. This is in accordance with the requirements of many monocotyledons, as reported by Magnaval *et al.* (1997)**.**

Establishment of embryogenic suspension

Embryogenic calli were placed in sterile Petri dishes and chopped using a scalpel. The resulting small fragments were transferred into liquid medium in 250 mL Erlenmeyer flasks. Each flask contained 50 mL of medium and was inoculated with 0.5 g of plant

material. The content of the Erlenmeyer flasks was filtered through a 500 µm sieve, and the collected filtrate was incubated on an orbital shaker (120 rpm) under a 16-hour photoperiod in a culture room maintained at an average temperature of 27°C. The first subculture was performed by dilution (2×) after 20 days of culture, and subsequent subcultures were carried out every 5 days by completely renewing the medium after the sedimentation (decantation) of the calli. Cultures were regularly monitored under an inverted microscope by transferring and analyzing a few milliliters of the cell suspension in a Petri dish.

Artificial seed preparation

Somatic embryos and proembryogenic masses (PEMs) were harvested from active cultures and gently separated for encapsulation. The encapsulation matrix was prepared by dissolving 3% (w/v) sodium alginate directly into MS liquid culture medium. The somatic embryos were thoroughly suspended within this sodium alginate solution.

Artificial seeds were formed by dropping the embryo-gel suspension droplet by droplet into a sterile 100 mM calcium chloride solution, allowing for ionic complexation. This coagulation and bead formation process was performed according to the method described by Lakshmana and Singh (1990). The resulting encapsulated beads were allowed to harden in the complexing solution to achieve optimal rigidity before being rinsed with sterile distilled water to remove excess calcium ions.

Cryopreservation using encapsulation vitrification protocol

For long-term germplasm preservation, an encapsulation-vitrification procedure was applied to the structured tissues. The prepared alginate beads containing somatic embryos and proembryogenic masses (PEMs) were first transferred into a sterile loading solution (LS) (Matsumoto *et al.,* 1994) and incubated for 20 min at room temperature. Following osmoprotection, the LS was decanted and replaced with an ice-cooled plant vitrification solution 2 **(**PVS2**)** (Sakai *et al.,* 1990).

The encapsulated explants were equilibrated in the PVS2 matrix for 60 min strictly maintained at 0°C**.** After dehydration, the alginate beads were transferred into sterile 2 mL cryotubes containing 0.5 mL of fresh, ice-cold PVS2 solution. The cryotubes were then rapidly plunged directly into liquid nitrogen (LN) for ultra-low temperature storage at -196°C**.**

After a storage period of **1** hour in LN, the cryotubes were retrieved and immediately thawed by immersion in a water bath at 40°C for 2 min with gentle agitation. The recovered beads were promptly transferred to a Petri dish filled with a rinsing solution **(**RS**)** (Fki *et al.,* 2017) and held at 25°C for 15 min to remove residual vitrification vectors. For post-thaw recovery, the alginate beads were drained and placed onto two layers of sterile filter paper on top of a preculture treatment medium **(**PTM**)**. The cultures were incubated in absolute darkness at 28°C. After a transitional period of 2 days, the recovering explants were transferred onto a definitive regeneration medium **(**RM**)** (Fki *et al.,* 2017) to evaluate regrowth and development.

2.3. Data analysis

Statistical analyses of data were performed using one-way ANOVA and Duncan's test. *P* values < .05 were considered as statistically significant. Statistical analysis was computed using SPSS 13 software. Experiments were replicated three times. Data expressed in

percentage were transformed by arcsin transformation and then analyzed. Arcsin transformation (y' = arcsin y ½, y = original percentage/100) was undertaken in order to stabilize the variance of data.

3. RESULTS

3.1. Callus induction and cyto-morphological characterization

Callogenesis from juvenile inflorescence explants was strictly dependent on both the hormonal treatment and the plant genotype. The physiological state of the tissues significantly influenced the responsiveness to the 0.5 mg/L 2,4-D initiation shock. A clear variation in callogenesis rates was observed among the tested cultivars. The Barhee cultivar exhibited the highest responsiveness with a 40% callogenesis rate, closely followed by Deglet Nour at 35%. In contrast, the Klasse cultivar was significantly less responsive, yielding a callogenesis rate of only 10%. During culture proliferation, distinct structural types of callus emerged, but only specific phenotypes demonstrated regenerative potential: (i) Embryogenic calli that are characterized by a distinct granular texture composed of small, whitish spherical units corresponding to globular and post-globular stages of somatic embryos (Fig.1). These structures possessed highly active meristematic zones. (ii) Parenchymatous calli that are characterized cytologically by fully differentiated cells, which failed to manifest any regeneration capacity. (iii) Friable calli that are composed of small cells with diffuse nuclei. These calli showed high proliferation rates but lacked the consistent ability to transition into the embryogenic pathway.



Fig. 1. Morphological aspect of high-quality granular embryogenic callus derived from juvenile inflorescence of *Phoenix dactylifera* L. showing globular somatic embryos.

3.2. Optimization of Somatic Embryogenesis in Liquid Medium

The systematic evaluation of hormonal concentrations, mineral strength, and activated charcoal doses revealed specific cultural requirements for inducing secondary somatic embryogenesis in liquid cultures. The physiological state and differentiation kinetics of the somatic embryos were strictly dependent on the dilution rate of the MS mineral solution.

A basal medium consisting of half-strength MS (MS × ½) macro- and micro-elements, supplemented with 1 mg/L 2,4-D and 0.3 g/L activated charcoal, was determined to be the optimal formulation for both embryo initiation and synchronized development. The efficacy of this specific formulation is primarily attributed to the dilution effect of the standard MS mineral core (Fig 2).

Deviations from this optimal ionic strength significantly hindered embryo ontogeny. When a quarter-strength MS (MS × ¼) medium was employed, embryo development was highly unsatisfactory, resulting in a developmental arrest where the majority of embryos remained blocked at the globular stage. Conversely, utilizing an undiluted, full-strength MS medium failed to promote secondary embryogenesis effectively; the total number of induced globular structures remained critically low even after 6 weeks of continuous culture.

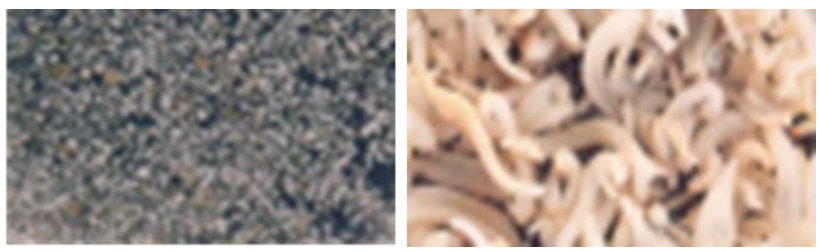


Fig.2. Dare palm somatic embryos: A- Globular stage and B- Mature stage

3.3. Encapsulation and Artificial Seed Production

Somatic embryogenesis and artificial seed production represent prominent plant biotechnologies that offer enhanced crop resilience and productivity, though encapsulation in alginate can delay embryo germination rates. However, tests show that alginate exhibits no detectable cytotoxicity or toxic effects on date palm somatic embryos across developmental stages, maintaining overall viability and growth capability.

Encapsulation trials of structured date palm somatic embryos were conducted to produce artificial seeds (Fig. 3).



Fig. 3. Encapsulated date palm somatic embryos

The study revealed that alginate exhibits no cytotoxicity toward somatic embryos in this species. However, the germination speed of the encapsulated embryos was significantly affected; a 50% germination rate was reached after 31 days of culture for naked somatic embryos, compared to 42 days of culture for the encapsulated ones.

Regarding the encapsulation of embryos at the globular stage (Fig 4), our trials demonstrate that alginate has also no detectable toxic effect at this developmental stage, especially as the embryos resume normal growth within a period not exceeding one month. Furthermore, the high firmness of the beads does not appear to affect embryo viability across the different differentiation stages; thus, they remain perfectly capable of developing after encapsulation using 3% sodium alginate and 100 mM $CaCl_2$.

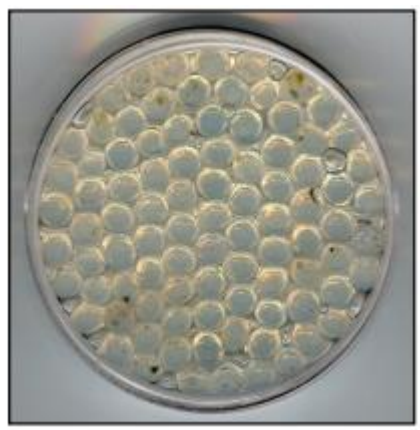


Fig. 4. Encapsulated date palm proembryos

3.4. Cryopreservation of encapsulated somatic embryos

Our experiments demonstrate that only somatic embryos at the globular stage are capable of survival following cryogenic treatment; however, specific measures must be meticulously implemented. Generally, cultures optimal for tolerating freezing at –196°C are those at the end of the lag phase or during the exponential growth phase. A preculture phase on a medium enriched with a high sucrose concentration (180 g L^{-1}) is highly critical to enhance the freezing tolerance of embryogenic cultures. This treatment ensures controlled tissue dehydration and depresses the crystallization temperature of intracellular solutes, thereby preventing lethal ice crystal formation within the cells. In this context, it is noteworthy that dry date palm seeds, with a moisture content not exceeding 15%, germinate without difficulty after storage at –196°C, even in the absence of a cryoprotective solution. This underscores the critical role of proembryos dehydration and suggests that controlled tissue desiccation holds great promise for optimizing future date palm cryopreservation protocols.

The immediate removal of the cryoprotective solution after thawing, followed by tissue transfer to solid or agitated liquid media, often leads to a loss of cell recovery capacity. Successful regrowth is only guaranteed when cells, while still suspended in the cryoprotective solution, are transferred directly onto a solid medium (Fig. 5). This is particularly true when using a non-toxic cryoprotective solution, allowing treated

proembryos to maintain a proliferation rate comparable to that of untreated control calli. Additionally, during the first months of culture, the growth of cryopreserved tissues remains extremely slow, with growth resumption requiring 6 to 8 weeks. This extended lag phase could be attributed to the mortality of a fraction of the cryopreserved cells or to reversible alterations in certain cellular properties. Conversely, the embryogenic capacity of the proembryos remains fully intact, enabling the establishment of highly productive embryogenic suspensions from the cryopreserved proembryos.



Fig.5. Regrowth of encapsulated date palm proembryos after cryopreservation

4. DISCUSSION

The results obtained in this study demonstrate that the micropropagation of date palm (*Phoenix dactylifera* L.) via synthetic seeds is a highly viable alternative for mass clonal propagation, provided that critical physiological and biochemical parameters are controlled. We believe we have determined the key factors ensuring the successful induction and subsequent amplification of embryogenic calli across all studied cultivars. In date palm, the origin of embryogenic calli has been extensively studied (Masmoudi *et al.*, 1999). Histological analysis has revealed two possible origins for neoformed tissues: an internal origin associated with perivascular cells, and a superficial origin involving epidermal cells, which mirrors the patterns observed in oil palm and coconut (Rival *et al.*, 1998).

Quite frequently, structured somatic embryos only achieve satisfactory germination following a partial desiccation phase, during which the moisture content declines from 90% to 80%. This step, which is straightforward to execute, enabled us to reach germination rates exceeding 90%. Furthermore, the encapsulation of somatic embryos in alginate beads for artificial seed production does not impair their germinative capacity. This outcome encouraged us to develop the cryopreservation of somatic embryos utilizing the encapsulation-vitrification technique (Engelmann and Dussert, 2000).

The conservation of plant cells at liquid nitrogen temperature (–196°C) is currently undergoing rapid development, with the pioneering trials conducted in 1968 using flax cell suspensions (Quatrano, 1968). Cryopreservation allows plant cells to be maintained in a defined physiological and phytosanitary state, theoretically indefinitely, without the requirement for regular subculturing onto fresh medium. This technique is cost-effective, requiring only a steady supply of liquid nitrogen.

In this regard, cryopreservation of meristems has been shown to sanitize cultures in certain species. In banana, meristem cryopreservation selectively eliminates differentiated virus-infected cells while preserving the mitotic potential of fully undifferentiated cells, which possess an optimal phytosanitary status (Helliot *et al.*, 2002). Regarding the genetic stability of cryopreserved calli, this treatment does not *a priori* appear to induce somaclonal variations, especially since the morphology of plantlets derived from cryopreserved plant material is strictly comparable to that of control plants.

Regarding the genetic conformity of somatic microplants, the morphological, cytogenetic, and molecular analyses conducted support the high genetic stability of the date palm. This stability is attributed to the small chromosome size and the protracted pace of morphogenetic processes inherent to this species. Currently, the fruiting of these somatic plants remains the ultimate criterion to safely validate the implementation of our protocol as a reliable mass-micropropagation system.

In banana, the clonal fidelity of plantlets produced from cryopreserved embryogenic cell suspensions was evaluated by Côte *et al.* (2000). This study demonstrated that plants regenerated from this type of material exhibit the same agronomic performances as control plants.

The high regeneration rate (70%) observed in encapsulated proembryogenic masses after cryotreatments underscores the dual potential of artificial seed technology as an efficient mass-propagation system and a robust tool for long-term germplasm conservation in date palm. However, despite these promising *in vitro* results, the practical deployment of this technology at the farm level remains heavily bottlenecked by *ex vitro* contamination and non-standardized germination rates during soil transfer.

To overcome these persistent technical barriers, the integration of artificial intelligence (AI) and automated systems represents a paradigm shift. Implementing computer vision algorithms can enable non-destructive, high-throughput selection of morphologically optimal somatic embryos before encapsulation, effectively filtering out non-viable tissues. Furthermore, AI-driven robotics and smart bioreactors can standardize the physical properties of the sodium alginate capsule while minimizing human-handling contaminations. Merging bioactive hydrogel formulations—enriched with antimicrobial agents or biostimulants—with machine learning-optimized desiccation protocols could finally bridge the gap between laboratory micropropagation and large-scale agricultural adoption.

5. CONCLUSIONS

The study we undertook on the date palm successfully led to the development of artificial seeds, which hold great promise not only for industrial-scale production but also for the conservation of plant genetic resources of this species. This plant is currently threatened by lethal diseases and is characterized by a highly protracted natural vegetative propagation rate. This study establishes an efficient, scalable protocol for date palm artificial seed production and cryopreservation. Future research should prioritize integrating artificial intelligence, automated robotics, and bioactive hydrogels to overcome contamination and standardization bottlenecks, thereby scaling the technology for modern industrial agriculture.

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Artificial intelligence for large-scale and data-driven optimization of micropropagation: Towards open-access AI platforms

Pedro Pablo Gallego, Tomás A. Arteta

AgroBioTech for Health, Plant Biology and Soil Science Department, Biology Faculty, University of Vigo, 36310, Vigo, Spain

pgallego@uvigo.es

Abstract

For more than fifteen years, our group has combined artificial neural networks (ANNs) with fuzzy logic (neurofuzzy logic) and with genetic algorithms (ANN-GA) to mine historical and purpose-designed datasets and to optimize *in vitro* culture media across seven micropropagated species: grapevine (*Vitis vinifera*), kiwifruit (*Actinidia chinensis* var. *deliciosa*), apricot (*Prunus armeniaca*), a *Prunus* rootstock (GF677, *Prunus persica* × *P. amygdalus*), pistachio (*Pistacia vera*), kiwiberry (*Actinidia arguta*) and medicinal genus *Bryophyllum*. This work synthesises the validated results obtained across these species with a two-step workflow, neurofuzzy rule extraction followed by artificial neural network + genetic algorithm (ANN–GA) optimization, that consistently identified the critical culture-media factors and generated genotype-specific protocols matching or outperforming manually developed media. Because the commercial software underlying this workflow is no longer supported, ADAM (Advanced Design and AI-Driven Modeling), an open-access, browser-based platform, was co-developed to reproduce and extend the same design of experiments, data quality control, multi-algorithm modeling and evolutionary optimization workflow; its models have matched or exceeded the predictive performance of the original, manually optimized approaches. Looking forward, scaling up and automating micropropagation is expected to generate two complementary data streams that require different artificial-intelligence strategies: high-dimensional, largely unlabeled digital-phenotyping images, which call for unsupervised or self-supervised methods, and structured, labeled data on media composition and plant responses, which remain the domain of the supervised optimization implemented in ADAM. We argue that integrating both data types within open, reproducible platforms, together with rigorous and pre-defined model validation criteria, is a key step towards automatable, large-scale micropropagation of elite genotypes.

Keywords: *Design of experiments; Digital phenotyping; Evolutionary algorithms; Machine learning; Scaling up and automation.*

1. INTRODUCTION

Plant tissue culture (PTC) is now a mature technology, and the volume and diversity of the underlying literature have grown accordingly: a recent bibliometric analysis of the Scopus database covering ninety years of research (1933–2023) identified 2,498 papers on the subject and documented a steady rise in annual output (Negi *et al.*, 2024). Despite this expansion, protocol development for micropropagation media remains, in most laboratories, an empirical, trial-and-error process. The most widely used basal medium in the history of the field, MS (Murashige & Skoog, 1962), was itself built following a one-factor-at-a-time (OFAT) strategy in which each mineral component was varied independently against a fixed background, the only design strategy available at the time. OFAT and related classical approaches (triangulation, serial single-element testing) return partial, per-factor optima, ignore interactions between factors, and require a rapidly growing number of experimental

units as the number of factors increases (Bethge *et al.*, 2026a). More than sixty years later, many recalcitrant woody and non-model species are consequently still cultured on MS media that were never designed for them.

Response-surface and mixture-design approaches were applied to plant tissue culture media, independently of neurofuzzy logic and artificial neural networks, well before our own studies began. Niedz and Evens (2006) showed that mineral nutrition media formulated from salts, rather than from ions, routinely confound the ion of interest with its associated co-ion, and proposed a linear-programming method to calculate salt mixtures that hold every ion independent of the others; a decade later, the same authors placed this practice within the broader framework of design of experiments (DOE) for *in vitro* culture, contrasting it with the one-factor-at-a-time tradition (Niedz & Evens, 2016). Building on this framework, Reed and coworkers used five-dimensional, computer-generated response-surface designs to identify the mineral factors responsible for growth and physiological disorders in diverse *Pyrus* germplasm (Reed *et al.*, 2013a, 2013b), and Poothong and Reed extended the same mixture-design logic to the nitrogen and mesos components of red raspberry and other *Rubus* media (Poothong & Reed, 2015, 2016). Although these studies rely on classical experimental-design and response-surface statistics rather than machine learning, they establish, independently of our own group, that plant tissue culture media are multifactorial systems whose components must be varied simultaneously, and at the level of the ion rather than the salt, if their individual effects are to be correctly attributed.

Since 2009, our group has explored whether artificial intelligence (AI) tools originally developed for pharmaceutical formulation could be transferred to plant tissue culture, treating culture media design as a problem structurally analogous to it: both consist of quantifiable inputs (media components or excipients) and processing conditions from which a reproducible product-quality outcome must be obtained (Landín *et al.*, 2009; Gago *et al.*, 2010a). Using historical or purpose-designed datasets, we combined artificial neural networks (ANNs) with fuzzy logic (neurofuzzy logic) and with genetic algorithms (GA) to extract interpretable IF-THEN rules, identify the media components that significantly affect growth and quality parameters, and generate optimized, genotype-specific formulations. This paper (i) synthesises the validated results obtained with this approach across seven woody and medicinal micropropagated species over more than a decade, and (ii) reports on the transition of this workflow from proprietary, now-discontinued software to an open-access AI platform, and on the strategy required to extend AI-based optimization to the two complementary data streams, structured media/response data and high-dimensional digital-phenotyping data, that scaling up and automation are expected to generate.

2. MATERIALS AND METHODS

2.1. Materials

The datasets analyzed and referenced in this synthesis correspond to *in vitro* proliferation, rhizogenesis and acclimatization trials in seven micropropagated species, presented here in the chronological order in which our group first applied these tools to each of them: kiwifruit (*Actinidia chinensis* var. *deliciosa* (A.Chev.) A.Chev.; Gago *et al.*, 2010a, 2014), grapevine (*Vitis vinifera* L. cvs. Albariño and Mencía; Gago *et al.*, 2010b), apricot (*Prunus armeniaca* L., five cultivars: Helena, Lorna, Búlida, Canino and Currot; Gago *et al.*, 2011),

a *Prunus* rootstock (GF677 hybrid rootstock, *Prunus persica* (L.) Batsch × *P. amygdalus* Batsch; Nezami-Alanagh *et al.*, 2014), pistachio (*Pistacia vera* L. cv. Ghazvini; Nezami-Alanagh *et al.*, 2017), the medicinal genus *Bryophyllum* (*B. daigremontianum* Raym.-Hamet et Perr., *B. tubiflorum* Harv. and *B.* × *houghtonii* D.B.Ward [*B. daigremontianum* × *tubiflorum*]; García-Pérez *et al.*, 2020), and the kiwiberry (*Actinidia arguta* (Siebold & Zucc.) Planch. ex Miq.; Hameg *et al.*, 2020).

Depending on the study, inputs corresponded to mineral ions, plant growth regulators, vitamins, cultivar and processing variables, and outputs corresponded to shoot number, shoot length, fresh weight, rooting and acclimatization percentages, physiological disorders (hyperhydricity, shoot-tip necrosis) and, for *Bryophyllum*, phenolic and flavonoid content.

2.2. Methods

The core analytical workflow comprises up to two steps, and Table 1 specifies which step, and which specific software, was used in each case. First, neurofuzzy logic (FormRules®, Intelligensys Ltd.) is applied to the dataset to generate a set of IF-THEN rules; this step identifies the subset of input factors that significantly affect each output and removes non-informative factors, which is the principal safeguard against overfitting. Second, an ANN combined with a GA (INForm®, Intelligensys Ltd.) is trained on the same, or a purpose-designed, dataset to predict the response surface for each output and to search that surface for the input combination that optimizes one or several outputs simultaneously. The earliest studies (kiwifruit, grapevine) applied only the second step, using ANNs to benchmark against classical statistics before neurofuzzy rule extraction was incorporated; several subsequent studies (apricot, the *Prunus* rootstock, *Bryophyllum*) applied only the first step, for data mining and rule extraction, without a further optimization stage; the full two-step workflow was used only once both components were mature enough to be combined (pistachio, kiwiberry). As described by Gago *et al.* (2010a), model quality was assessed from the train-set coefficient of determination (R^2) and by comparing, through analysis of variance (ANOVA), the experimental and model-predicted values; model predictions were, where a new medium was designed, subsequently tested in the laboratory against the original, manually developed media.

Since 2025, this workflow has been reproduced and extended in ADAM (Advanced Design and AI-Driven Modeling for Plant Tissue Culture Media Optimization), an open-access, browser-based platform co-developed through the COPYTREE network (Bethge *et al.*, 2026a). ADAM implements the same overall logic, design of experiments, automated data quality control, model building and evolutionary optimization, through five integrated modules, offering nine machine learning algorithms with automated selection and four evolutionary algorithms (genetic algorithm, particle swarm optimization, NSGA-II and SMS-EMOA) for single- and multi-objective optimization, without requiring specialised hardware, software licences or programming skills.

2.3. Model validation criteria

Across the studies synthesised here, a train-set R^2 between 70% and 99.9% was taken as an acceptable indication of model robustness (Colbourn & Rowe, 2007); values above 99.9% were treated as a warning sign of overfitting rather than as a target, and an ANOVA F-ratio above the critical value, computed between experimental and model-predicted values, was required as a complementary robustness check (Gago *et al.*, 2010a; Nezami-Alanagh *et al.*,

2017). Because the interpretation of model-reported accuracy metrics is not yet standardised across the plant biotechnology literature, we favor explicit reporting of data splits and of the specific metrics used, together with laboratory validation of the biological plausibility of each optimized solution, in line with the community-wide DOME (Data, Optimization, Model, Evaluation) recommendations for supervised machine learning validation (Walsh *et al.*, 2021).

3. RESULTS

Table 1 summarises the validated outcomes obtained with the neurofuzzy/ANN–GA workflow across the seven species considered. In every case, the workflow identified a reduced subset of the tested factors as biologically relevant and used it to generate a medium or protocol that matched or outperformed the manually developed reference.

Table 1. Summary of validated neurofuzzy/ANN–GA-optimized protocols across seven micropropagated species

Species	AI approach (software)	Key validated outcome	Model performance (R^2)	Reference
1. Kiwifruit (*Actinidia chinensis* var. *deliciosa* (A.Chev.) A.Chev.)	ANN via INForm® (2010a); neurofuzzy logic via FormRules® (2014)	97.2% of explants proliferating at the predicted optimum (2.5 shoots/explant) (2010a); optimal acclimatization at 2.3% sucrose and 122–130 µmol m^{-2} s^{-1} light (2014)	Train 91.4–98.1%; validation 93.1–98.0% (2010a)	Gago *et al.* (2010a, 2014)
2. Grapevine (*Vitis vinifera* L. cvs. Albariño, Mencía)	ANN via INForm® (no neurofuzzy step)	93–100% rooting; acclimatization above the 65–98% range previously reported for these cultivars	Root number: train 95.6%; test 78.5%; validation 97.0%	Gago *et al.* (2010b)
3. Apricot (*Prunus armeniaca* L., 5 cvs.: Helena, Lorna, Búlida, Canino, Currot; data mined)	Neurofuzzy logic via FormRules® (rule extraction only, no INForm/GA step)	5 of 10 input factors retained (cultivar, BA, NO_3^-, NH_4^+, Mg_2^+); shoot length poorly predicted	Train >70% (all outputs); validation >74% (shoot number, productivity); 45% for shoot length	Gago *et al.* (2011)
4. *Prunus* rootstock, GF677 (*Prunus persica* (L.) Batsch × *P. amygdalus* Batsch)	Neurofuzzy logic via FormRules® (rule extraction only, no INForm®/GA step)	NO_3^- and its interactions ($\times Ca^{2+}$, $\times Ca^{2+} \times K^+$) key to all growth parameters; reduced hyperhydricity/chlorosis	Correlation ratio: 77.5% (total shoots); 91.8% (healthy shoots); 90.9% (bud number)	Nezami-Alanagh *et al.* (2014)
5. Pistachio (*Pistacia vera* L. cv. Ghazvini)	FormRules® (neurofuzzy) + INForm® (ANN–GA) — full two-	3.73 vs 1.64 new shoots/explant, POM (Pistachio Optimal Medium) vs MS-derived control	Train 86.0–95.4%; test 71.2–81.7%	Nezami-Alanagh et al. (2017)

	step workflow			
6. *Bryophyllum* spp. (*B. daigremontianum*, *B. tubiflorum*, *B.* × *houghtonii*)	Neurofuzzy logic via FormRules® (rule extraction only, no INForm®/GA step)	Maximum phenolic content under ½MS macronutrients and NH_4^+ below 15 mM	TPC: 75.8%; FC: 98.1%	García-Pérez *et al.* (2020)
7. Kiwiberry (*Actinidia arguta* (Siebold & Zucc.) Planch. ex Miq.)	DOE + FormRules® (neurofuzzy) + INForm® (ANN–GA) — full two-step workflow	11 of 18 tested ions retained; optimized medium increased shoot quality, reduced basal callus/hyperhydricity	Train >70%	Hameg *et al.* (2020)

For apricot, the model itself indicated its own limits: shoot number and productivity were well predicted (train-set R^2 above 70%, validation above 74%), whereas average shoot length was not (R^2 = 45%), a result reported rather than concealed and used as a criterion for trusting the remaining predictions. For the GF677 *Prunus* rootstock, the same neurofuzzy approach, applied to eight macronutrient ions rather than to plant growth regulators, pinpointed NO_3^- and its interactions with Ca^{2+} and K^+ as the dominant factors behind the hyperhydricity and chlorosis that had made this economically important peach × almond hybrid rootstock difficult to propagate.

For pistachio, the ANN–GA-designed medium more than doubled proliferation relative to a Murashige-and-Skoog-derived control (3.73 vs 1.64 new shoots per explant), at the cost of a modest reduction in shoot length, illustrating the multi-objective character of media optimization.

The pistachio study also documented a methodological limitation intrinsic to mineral nutrition datasets built from salt-based media: when an ion has a single salt source across the dataset, its correlation with the corresponding counterion approaches 1, and no algorithm, however well trained, can separate their individual effects. In that case, the ANN–GA model proposed a Na_2EDTA concentration that was manually overridden to preserve the Fe:EDTA ratio of the original MS formulation, since chemistry, not the model, set that constraint. The kiwiberry study addressed this limitation prospectively, using a design of experiments in which eighteen mineral ions were varied so that eleven of them could be causally interpreted; neurofuzzy logic retained these eleven ions as jointly explaining shoot quality, basal callus and hyperhydricity, and the resulting optimized medium significantly improved all three outcomes relative to standard media.

Validated across two independent plant tissue culture applications, ADAM's machine-learning models matched or exceeded the predictive performance of the manually optimized approaches reported in the original studies, while requiring neither specialised hardware nor a software licence. Approximately 80% of published AI-based optimization studies in this field are estimated to still rely on licensed software, underlining the practical barrier that this open-access approach is intended to remove.

4. DISCUSSION

Taken together, these results indicate that a two-step neurofuzzy/ANN–GA workflow is not merely an alternative to classical statistics but a complementary tool that is particularly well suited to the multifactorial, non-linear and genotype-dependent nature of micropropagation media design. Unlike the OFAT and triangulation strategies that produced most historical media, including MS (Murashige & Skoog, 1962), this workflow explicitly models interactions among factors and, when properly validated, allows optimization across several, sometimes conflicting, outputs simultaneously, as illustrated by the pistachio proliferation *vs.* length trade-off (Nezami-Alanagh *et al.*, 2017). The GF677 *Prunus* rootstock case adds a further dimension: applying the same approach to eight ion macronutrients, rather than to a single limiting factor, revealed NO_3^- and its two- and three-way interactions with Ca^{2+} and K^+ as the drivers of a physiological disorder, hyperhydricity and chlorosis, that classical statistics had not resolved, with correlation ratios between experimental and predicted values of 77–92% across the three growth parameters modelled (Nezami-Alanagh *et al.*, 2014). The apricot case illustrates a further methodological point we consider as important as any single optimization result: a model that reports where it fails, rather than only where it succeeds, is more trustworthy than one that does not (Gago *et al.*, 2011).

The ion-collinearity finding in the pistachio study is a caution that extends beyond that single case, and one that Niedz and Evens (2006) had already identified in a broader experimental-biology context: any design built from salt-level, rather than ion-level, treatments risks confounding ions that share a single salt source, and no downstream algorithm can recover information the design did not generate (Nezami-Alanagh *et al.*, 2017). The kiwiberry work shows that this limitation can be addressed at the design stage, by choosing salt combinations that vary the ions of interest independently (Hameg *et al.*, 2020), the same logic that Reed and coworkers had already applied, through classical response-surface designs rather than machine learning, to pear and *Rubus* mineral nutrition (Reed *et al.*, 2013a, 2013b; Poothong & Reed, 2015, 2016). We consider this design-level safeguard, rather than any specific algorithm, to be the more general lesson for future media optimization studies, including those built on ADAM.

A second, practical lesson from this trajectory concerns reproducibility of the tools themselves. Because the commercial neurofuzzy and ANN–GA software used in the studies summarised in Table 1 is no longer commercially supported, every result obtained with it, however well validated, was in a strict sense produced with a tool that others could not obtain, license or verify independently. Co-developing ADAM as an open-access, browser-based reproduction and extension of the same workflow (Bethge *et al.*, 2026a) directly addresses this dependency and is, in our view, as significant a contribution as any individual optimized medium reported here: an estimated 80% of published AI-based optimization studies in this field still rely on licensed software (Bethge *et al.*, 2026a), so full reproducibility of the kind reported in Table 1 remains the exception rather than the rule.

Looking forward, scaling up and automating micropropagation is expected to generate two data streams that are structurally different from those analyzed in this synthesis. Optical digital-phenotyping systems, RGB, hyperspectral, multispectral, fluorescence and 2.5D/3D imaging, generate high-dimensional, largely unlabeled data at a rate that manual annotation cannot match, and are more naturally suited to unsupervised or self-supervised learning than

to the supervised workflow described here (Bethge *et al.,* 2026b). Structured data on media composition, plant growth regulators and morpho-physiological responses, by contrast, remain squarely within the supervised, labeled-data paradigm implemented in ADAM, and are likely to benefit further from the chemical and formulation innovations, cytokinin-homeostasis modulators, novel auxin and cytokinin analogues, and controlled-release delivery systems, reviewed by Taheri *et al.,* (2026). Because supervised and unsupervised methods answer different questions from different kinds of data, we consider that scaling up micropropagation will require deliberately combining both within the same open platform, rather than treating one as a substitute for the other, and doing so under explicit, pre-registered validation criteria such as those proposed by the DOME recommendations (Walsh *et al.,* 2021).

This synthesis has the limitations inherent to any retrospective account of a research program: the datasets summarised in Table 1 differ in size, in whether they were purpose-designed or mined from pre-existing records, and in the extent to which their optimized media were subsequently validated outside the original laboratory. Generalising any single result to a new genotype or species therefore still requires case-by-case data collection and model validation; what we consider transferable is the workflow, and the validation discipline around it, rather than any specific optimized formulation.

5. CONCLUSIONS

Across seven micropropagated species and more than fifteen years, a two-step neurofuzzy/ANN–GA workflow has consistently transformed an empirical, one-factor-at-a-time process into a data-driven one, generating genotype-specific media that matched or outperformed manually developed controls, while also identifying its own predictive limits. The field's dependence on now-discontinued commercial software (FormRules® and INForm®) for this workflow has been addressed through ADAM, an open-access platform whose models have matched or exceeded the performance of the original manually optimized approaches. We conclude that the next challenge, scaling up and automating micropropagation, requires deliberately combining supervised optimization of structured media and response data with unsupervised or self-supervised extraction of features from high-dimensional digital-phenotyping data, within open, reproducible platforms and under explicit validation criteria, as the key step towards an intelligent, genotype-specific and reproducible alternative to the sixty-year-old Murashige and Skoog paradigm.

Author Contributions

P.P.G. conceived and coordinated the fifteen-year research program synthesised in this paper, supervised the doctoral and postdoctoral work underlying the studies summarised in Table 1, and drafted and revised the manuscript. T.A.A. contributed to the design of experiments and data curation underlying the most recent of these studies and to the co-development of the ADAM platform discussed here. Both authors read and approved the submitted version.

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WORKING GROUP 4

Technological risk assessment, public acceptance, legislation, and commercialization

Micropropagation of *Alocasia* "Bambino" via tissue culture

Soner Yağ[1], Bora Onur Hallaç[2], Cansu Ece Sari[2], Yeşim Yalçin Mendi[1]

[1]*Çukurova University, Faculty of Agriculture, Department of Horticulture, Adana*

[2]*Bolu Abant İzzet Baysal University, Postgraduate Education Seed Science and Technology, Bolu*

soneryag@gmail.com

Abstract

*Alocasia "*Bambino" (Araceae) is a tropical ornamental plant valued for its compact growth habit and decorative foliage. Conventional vegetative propagation may provide limited numbers of uniform plantlets, whereas tissue culture can support rapid multiplication under controlled conditions. The objective of this study was to establish a sequential *in vitro* micropropagation protocol for *Alocasia "*Bambino". Plantlets 8-10 cm in length were used as explants. Following 30 min of rinsing under running tap water, 1,500 explants were divided equally among 5, 10, and 20 min surface-sterilization treatments using 0.1% mercury(II) chloride and 1 mL L^{-1} polysorbate 20. The 5-min treatment resulted in the highest contamination-free explant recovery (60%). For shoot induction, 600 sterile explants were distributed among MS medium containing 30 g L^{-1} sucrose, 6 g L^{-1} agar, and 0.1, 0.5, or 1.0 mg L^{-1} 6-benzylaminopurine (BAP). The 0.5 mg L^{-1} BAP treatment showed the highest induction frequency (90%) and produced 3.0 shoots per responding explant. For multiplication, 20 shoots from each BAP treatment were selected, and mean multiplication coefficients were 5.2, 6.5, and 4.8 shoots per starting shoot at 0.1, 0.5, and 1.0 mg L^{-1} BAP, respectively. For rooting, 20 shoots were assigned to each NAA treatments (0, 0.1, 0.5, and 1.0 mg L^{-1}), and all treatments produced 100% rooting. The results indicate that 0.5 mg L^{-1} BAP was the most responsive concentration for shoot induction and multiplication, while the evaluated NAA treatments all supported successful rooting.

Keywords: *Alocasia "*Bambino*", micropropagation, tissue culture, BAP, NAA*

1. INTRODUCTION

*Alocasia "*Bambino" is a compact ornamental plant valued for its distinctive foliage, contrasting venation, and suitability for indoor cultivation. It is a dwarf cultivar of *Alocasia amazonica*, itself a hybrid of *A. sanderiana* and *A. longiloba* 'Watsoniana' . Recent morphological work on "Bambino" has documented characteristic leaf and growth traits and highlighted its ornamental value, while also indicating that its vegetative growth pattern differs from other *Alocasia* taxa (Krisantini *et al.*, 2024). The compact architecture and distinctive foliage of "Bambino" make the production of uniform, healthy, and commercially consistent planting material an important consideration in its cultivation.

For ornamental crops conventional vegetative propagation may be insufficient when a large number of uniform plants are required within a relatively short period. *In vitro* culture provides an alternative approach because a defined quantity of starting material can be multiplied under controlled environmental and nutritional conditions. The practical value of this approach is particularly relevant to "Bambino", for which consistent shoot production is important for maintaining uniform plant size, leaf development, and marketable quality. Recent production research using tissue-cultured "Bambino" plants has also shown that post-culture environmental conditions, including substrate water status and nutrient supply,

can substantially influence subsequent growth and photosynthetic performance (Hsieh & Yeh, 2025). These findings underline the importance of establishing a reliable *in vitro* production stage before plants are transferred to *ex vitro* production systems.

A successful micropropagation protocol requires sequential optimization of culture establishment, shoot induction, shoot multiplication, and rooting. These stages are biologically related but cannot necessarily be optimized by applying a single culture condition throughout the protocol. Responses may change according to explant physiological status, medium composition, cytokinin concentration, auxin concentration, and the duration of exposure to sterilizing agents. Previous tissue-culture studies in *Alocasia* have demonstrated that these factors can alter regeneration and proliferation responses (Jo *et al.,* 2008; Chan & Chong, 2010; Raju *et al.,* 2022). For "Bambino", therefore, each stage should be evaluated according to the response parameter that is most relevant to that stage rather than by relying on a single overall propagation measure.

Plant material obtained from actively growing conditions carry microorganisms on the external surface, whereas excessive exposure to disinfectants can damage tissues and reduce their capacity to establish *in vitro.* An effective sterilization treatment must therefore provide sufficient microbial control while maintaining tissue viability. Previous *Alocasia* micropropagation studies have used different combinations of disinfectants and exposure periods, illustrating that sterilization conditions require adjustment according to the explant material and culture system (Chan & Chong, 2010; Siregar *et al.,* 2025).

Once aseptic cultures are established, shoot induction becomes the principal determinant of the number of propagules that can enter the subsequent multiplication stage. Cytokinins, particularly 6-benzylaminopurine (BAP), are commonly used to stimulate axillary shoot formation and shoot proliferation in ornamental tissue culture. However, the response is concentration dependent, and excessive or insufficient cytokinin levels may reduce the desired morphogenic response. Studies in *Alocasia* have demonstrated that cytokinin concentration and the balance between cytokinins and auxins can substantially influence shoot initiation and multiplication (Jo *et al.,* 2008; Chan & Chong, 2010; Raju *et al.,* 2022). Consequently, identifying a suitable BAP concentration is necessary to obtain both a high proportion of responding explants and an adequate number of shoots per responding explant.

Shoot induction and multiplication should also be considered as separate stages. Induction refers to the initial formation of shoots from the explant, whereas multiplication evaluates the capacity of established shoots to produce additional shoots during subsequent culture. A BAP concentration that is effective for initial induction may not necessarily produce the same multiplication response. The use of a defined number of starting shoots for the multiplication experiment therefore allows multiplication efficiency to be assessed independently of the total number of shoots generated during induction. This distinction is particularly important when propagation outputs are used to describe a practical protocol.

The rooting stage determines whether the shoots produced during multiplication can develop into plantlets suitable for transfer to *ex vitro* conditions. Auxins such as α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA) have been used in *Alocasia* tissue culture for the induction and development of roots, but the magnitude of the response depends on genotype, shoot condition, and medium composition (Jo *et al.,* 2008; Raju *et al.,* 2022; Siregar *et al.,* 2025). A rooting treatment

should therefore be evaluated using more than rooting percentage alone, because the number and length of roots can also affect subsequent plantlet establishment. For *Alocasia "*Bambino*"*, examining a hormone-free control together with several NAA concentrations provides an opportunity to determine whether exogenous auxin is required to obtain adequate rooting under the culture conditions used.

The biological characteristics of *Alocasia* "Bambino" also make the quality of regenerated material an important consideration beyond the number of shoots produced. Uniform development, stable leaf formation, and successful transition from *in vitro* to *ex vitro* conditions are all relevant to the practical value of a micropropagation protocol. Recent Bambino-specific studies have focused on vegetative growth, leaf morphology, and post-tissue-culture production conditions, showing that growth and physiological performance can vary with environmental management after culture (Krisantini *et al.*, 2024; Hsieh & Yeh, 2025). This supports the need for an *in vitro* protocol that produces a sufficiently large and morphologically suitable supply of rooted shoots for subsequent acclimatization and cultivation.

Despite the increasing horticultural use of this variety, the literature identified for this study provides more information on its morphology and subsequent production performance than on a complete, sequential *in vitro* protocol under a single experimental framework. Existing *Alocasia* tissue-culture studies provide useful methodological evidence, but their results were obtained under different explant types and culture conditions and therefore cannot be assumed to represent the optimum requirements of "Bambino" (Jo *et al.*, 2008; Chan & Chong, 2010; Raju *et al.*, 2022; Siregar *et al.*, 2025). A cultivar-specific evaluation is consequently needed to define suitable conditions for reproducible propagation of this ornamental plant.

Accordingly, the present study aimed to establish a sequential micropropagation protocol for *Alocasia* "Bambino" by evaluating surface-sterilization duration, BAP concentration during shoot induction and multiplication, and NAA concentration during rooting.

2. MATERIALS AND METHODS

2.1. Materials

Plantlets of *Alocasia* "Bambino" measuring 8–10 cm in length were used as explant material. The plantlets were obtained from healthy plants grown under greenhouse conditions at 25 °C.

2.2 Methods

Surface sterilization

A total of 1,500 explants were equally assigned to three surface-sterilization treatments of 5, 10, and 20 min, with 500 explants allocated to each treatment. Prior to chemical sterilization, the explants were rinsed under running tap water for 30 min. The explants were then immersed in a solution containing 0.1% mercury(II) chloride ($HgCl_2$) and 1 mL L^{-1} polysorbate 20 for the assigned treatment duration; polysorbate 20 was incorporated into the surface-sterilization solution to improve contact between the sterilizing agent and the explant surface. After surface sterilization, the explants were rinsed three times with sterile distilled water to remove residual mercury(II) chloride. The explants were then transferred

to *in vitro* culture, and contamination status was recorded for each treatment. The use of mercury(II) chloride and the evaluation of different exposure periods were based on previously reported *Alocasia* micropropagation procedures, with adjustments to the explant material and experimental conditions.

Culture medium preparation

MS basal medium was prepared according to the formulation of Murashige and Skoog (1962) and supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar. BAP was incorporated into the medium during the shoot induction and multiplication stages at 0.1, 0.5, and 1.0 mg L^{-1}. During rooting, NAA was incorporated at 0, 0.1, 0.5, and 1.0 mg L^{-1}, with the hormone-free medium serving as the control. The selection of MS medium and the plant growth regulator treatments was based on previous tissue-culture studies of *Alocasia* species (Jo *et al.,* 2008; Chan & Chong, 2010; Raju *et al.,* 2022; Siregar *et al.,* 2025).

Culture growth condition

All cultures were maintained in a growth chamber at 25 ± 2 °C under a 16 h light / 8 h dark photoperiod, with a light intensity of 2,000 lux. These conditions were maintained throughout the culture stages.

Shoot induction

For shoot induction, the experiment was arranged with 200 explants assigned to each of three BAP treatments. Explants were cultured on MS medium supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar containing 0.1, 0.5, or 1.0 mg L^{-1} BAP. The response of explants was evaluated by recording shoot induction and the number of shoots produced. Induction frequency was calculated as the proportion of explants showing a shoot response.

Shoot multiplication

Twenty shoots from each BAP treatment were selected from the induction stage and transferred to fresh MS medium containing the corresponding BAP concentration used during induction. Thus, the 0.1, 0.5, and 1.0 mg L^{-1} BAP treatments were maintained during the multiplication stage. Multiplication performance was evaluated using the multiplication coefficient, calculated as the number of shoots produced relative to the number of shoots initially transferred to each treatment. Maintaining the corresponding BAP concentration between successive culture stages was used to evaluate the continued response of the shoots to the respective cytokinin treatment.

In vitro rooting

For *in vitro* rooting, 20 shoots were assigned to each of four rooting treatments: hormone-free MS medium containing 0 mg L^{-1} NAA and MS medium supplemented with 0.1, 0.5, or 1.0 mg L^{-1} NAA. Rooting percentage was calculated as the number of rooted shoots divided by the total number of shoots introduced into the respective treatment and expressed as a percentage. Rooting response was evaluated based on rooting percentage, mean number of roots per rooted shoot, and mean root length. The selection of NAA and inclusion of hormone-free control were based on previous *Alocasia* tissue-culture studies evaluating auxin-mediated rooting under different culture conditions (Jo *et al.*, 2008; Raju *et al.,* 2022; Siregar *et al.*, 2025).

2.2.1 Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the effect of BAP concentration on shoot production per responding explant during shoot induction and the effects of NAA concentration on mean root number and mean root length during rooting. Tukey's honestly significant difference (HSD) test was used for post-hoc comparisons following significant ANOVA results. For the multiplication coefficient, where treatment variances were heterogeneous, Welch's ANOVA followed by Games–Howell multiple comparisons were used. Differences were considered significant at $P \leq 0.05$. Chi-square tests were used to evaluate differences in explant sterility among sterilization treatments and shoot induction frequency among BAP treatments.

3. RESULTS

3.1. Surface sterilization

Among the three exposure periods evaluated, the 5-min treatment provided the highest sterilization success, with 300 of the 500 treated explants remaining sterile, corresponding to 60.00%. The 10-min treatment resulted in 200 sterile explants and a sterilization success of 40.00%, whereas the 20-min treatment resulted in only 100 sterile explants, corresponding to 20.00%. Thus, sterilization efficiency decreased progressively as the exposure period to $HgCl_2$ was extended from 5 to 20 min. Across the three treatments, a total of 600 sterile explants were obtained from 1,500 initial explants. The results indicate that the shortest exposure period tested provided the highest proportion of sterile explants under the conditions of the present study. Surface sterilization response varies with treatment duration (Table 1). Overall, 600 of the 1,500 explants were retained as sterile material for the subsequent induction stage. A chi-square test revealed a significant association between sterilization duration and explant sterility ($\chi^2(2) = 166.67$, $P < 0.001$) (Table 1).

Table 1. Surface sterilization results for *Alocasia* "Bambino".

Sterilization time (min)	Initial explants	Sterile explants	Contaminated explants	Sterility (%)
5	500	300	200	60.00
10	500	200	300	40.00
20	500	100	400	20.00
Total	1500	600	900	40.00

3.2. Shoot induction

Shoot induction responses differed among the BAP concentrations evaluated. The highest induction frequency was obtained with 0.5 mg L^{-1} BAP, where 180 of 200 explants responded and produced shoots, corresponding to an induction frequency of 90.00%. This treatment also produced the highest shoot production per responding explant, with a mean of 3.00 shoots per responding explant and a total of 540 shoots.

The 0.1 mg L^{-1} BAP treatment resulted in 150 responding explants out of 200, giving an induction frequency of 75.00%, with a mean of 2.60 shoots per responding explant and a total production of 390 shoots. At 1.0 mg L^{-1} BAP, 140 of the 200 explants responded, corresponding to an induction frequency of 70.00%, while the mean shoot production was 2.40 shoots per responding explant, resulting in 336 shoots. Therefore, both induction frequency and shoot production showed their highest values at 0.5 mg L^{-1} BAP within the tested concentration range.

Overall, 470 of the 600 sterile explants responded to shoot induction, corresponding to 78.33% of the explants introduced into the induction treatments. A total of 1,266 shoots were produced across the three BAP concentrations. From these induced shoots, a defined subset was subsequently selected for the multiplication experiment.

Statistical analysis showed that BAP concentration significantly affected the number of shoots produced per responding explant (one-way ANOVA, $F(2, 467) = 16.31$, $P < 0.001$). Tukey's HSD test indicated that the 0.5 mg L^{-1} BAP treatment produced significantly more shoots per responding explant than the 0.1 and 1.0 mg L^{-1} treatments, whereas no significant difference was detected between the 0.1 and 1.0 mg L^{-1} treatments ($P > 0.05$). The induction frequencies also differed significantly among BAP treatments ($\chi^2(2) = 25.53$, $P < 0.001$) (Table 2; Figure 1).

Table 2. Shoot induction response at different BAP concentrations.

BAP (mg L^{-1})	Explants	Responding explants	Induction (%)	Total shoots	Shoots/responding explant
0.1	200	150	75.00	390	2.60 ± 1.28^{b}
0.5	200	180	90.00	540	3.00 ± 0.00^{a}
1.0	200	140	70.00	336	2.40 ± 1.17^{b}
Total	600	470	78.33	1266	-

Note: Values are presented as mean ± standard deviation (SD). Means followed by different superscript letters are significantly different according to Tukey's HSD test ($p < 0.05$).

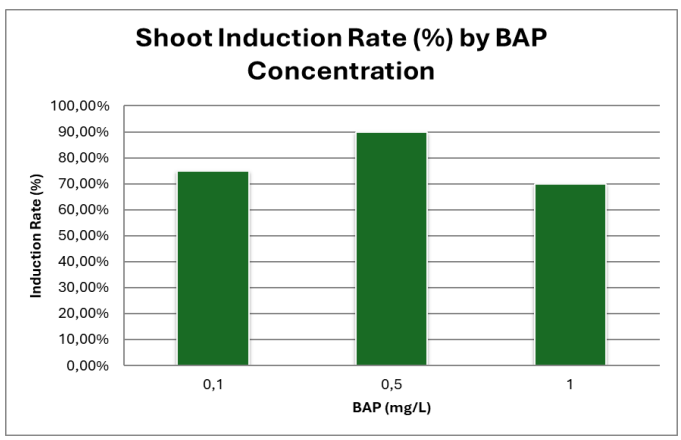


Fig. 1. Shoot induction frequency (%) of Alocasia "Bambino" on MS medium supplemented with different BAP concentrations (0.1, 0.5, and 1.0 mg L^{-1}).

3.3. Shoot multiplication

Twenty starting shoots from each BAP concentration were transferred to the corresponding multiplication treatment. The multiplication coefficient varied among the three BAP concentrations.

The highest multiplication response was obtained with 0.5 mg L^{-1} BAP, with a mean multiplication coefficient of 6.5 shoots per starting shoot. The corresponding mean multiplication coefficients were 5.2 and 4.8 shoots per starting shoot for 0.1 and 1.0 mg L^{-1} BAP, respectively. Based on the 20 starting shoots used for each treatment, the calculated multiplication outputs were 104, 130, and 96 shoots, respectively, resulting in a total calculated output of 330 shoots.

Statistical analysis indicated a significant effect of BAP concentration on multiplication coefficient (Welch's ANOVA, $F(2, 35.07) = 72.06$, $P < 0.001$). Because the treatment variances were heterogeneous, Games–Howell multiple comparisons were used. The 0.5 mg L^{-1} BAP treatment differed significantly from both 0.1 and 1.0 mg L^{-1} BAP, whereas the difference between 0.1 and 1.0 mg L^{-1} BAP was not significant (Table 3; Figure 2).

Table 3. Shoot multiplication response of Alocasia "Bambino" under different BAP concentrations

BAP (mg L^{-1})	Starting shoots	Mean multiplication coefficient	Calculated shoots
0.1	20	5.2 ± 0.22^{b}	104
0.5	20	6.5 ± 0.51^{a}	130
1.0	20	4.8 ± 0.31^{b}	96
Total	60	-	330

Values are presented as mean ± SD. Different superscript letters within the column indicate significant differences according to Games–Howell multiple comparisons at $P \leq 0.05$.



Fig. 2. Axillary shoot proliferation of Alocasia "Bambino" in culture vessel on MS medium supplemented with 0.5 mg L^{-1} BAP.

Figure 3 illustrates the relationship between BAP concentration, shoot induction frequency, and multiplication coefficient in Alocasia "Bambino". Both response parameters showed a similar concentration-dependent pattern, with the highest values recorded at 0.5 mg L^{-1} BAP. At 0.1 and 1.0 mg L^{-1} BAP, both shoot induction frequency and multiplication coefficient were lower. This pattern provides a visual comparison of the responses observed during the shoot induction and multiplication stages across the three BAP treatments.

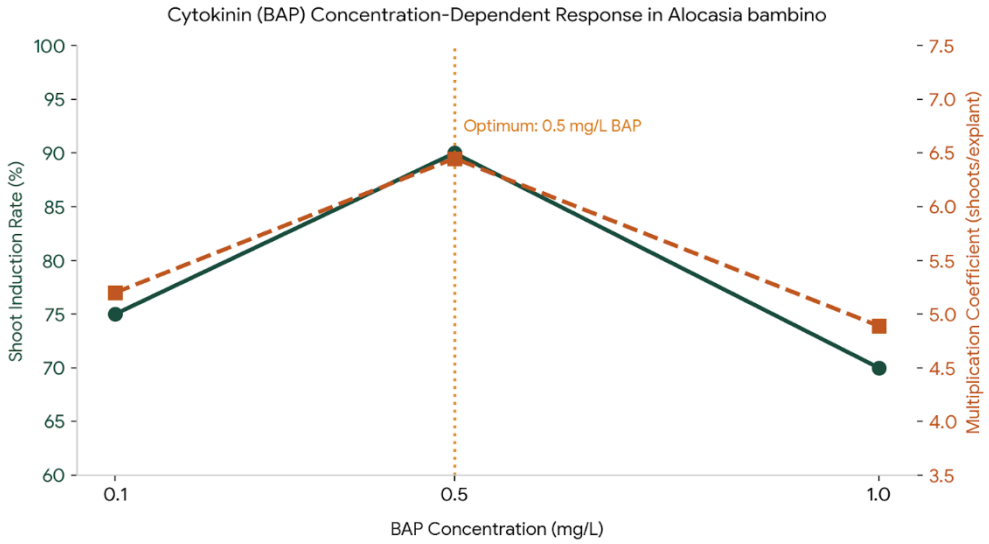


Fig. 3. Concentration-dependent response of Alocasia "Bambino" to BAP supplementation, showing shoot induction rate (%) on the primary axis and multiplication coefficient on the secondary axis across 0.1, 0.5, and 1.0 mg L^{-1} BAP.

3.4. In vitro rooting

Root formation was evaluated using shoots transferred to hormone-free MS medium and MS medium supplemented with NAA at 0.1, 0.5, and 1.0 mg L^{-1}. Twenty shoots were introduced into each rooting treatment.

All shoots developed roots under the tested conditions, resulting in a rooting frequency of 100.00% for the hormone-free control as well as for all three NAA concentrations. Accordingly, no difference was observed in rooting percentage among the treatments. The complete rooting response obtained in both hormone-free and NAA-supplemented media indicates that exogenous NAA was not required to achieve root formation under experimental conditions.

Although rooting percentage was identical among treatments, differences in root development were assessed using the mean number of roots per shoot and mean root length. One-way ANOVA revealed no significant effect of NAA concentration on mean root number ($F(3, 76) = 0.54$, $P = 0.654$). Similarly, mean root length did not differ significantly among the treatments ($F(3, 76) = 0.88$, $P = 0.454$). Thus, none of the tested NAA concentrations produced a statistically significant difference in the quantitative rooting characteristics evaluated (Table 4; Figures 4 and 5).

Table 4. *In vitro* rooting response of Alocasia "Bambino" under different NAA concentrations.

NAA (mg L^{-1})	Shoots introduced	Rooting (%)	Mean roots/shoot	Mean root length (cm)
0	20	100.00	4.30	5.25
0.1	20	100.00	4.55	4.90
0.5	20	100.00	4.35	5.27
1.0	20	100.00	4.65	5.07
Total	80	100.00	-	-
ANOVA (P-value)	-	-	0.654	0.454

ns: Not significant at P > 0.05.

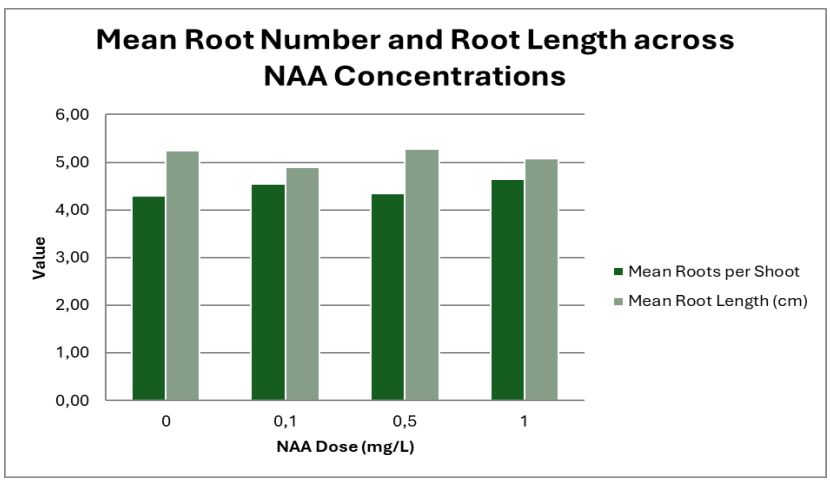


Fig. 4. *In vitro* rooting characteristics of Alocasia "Bambino", showing mean root number per shoot and mean root length (cm) across different NAA concentrations (0, 0.1, 0.5, and 1.0 mg L^{-1}).



Fig. 5. Aseptic harvest and root morphological evaluation of an individual Alocasia "Bambino" plantlet inside a laminar air flow cabinet, demonstrating elongated adventitious roots and intact leaf morphology alongside a culture vessel.

3.5. Overall Propagation Response

The overall propagation process consisted of sequential sterilization, shoot induction, shoot multiplication, and *in vitro* rooting stages. Among the sterilization treatments, the 5-min treatment retained the highest proportion of sterile explants (60.00%), providing 300 sterile explants from 500 initial explants. Following sterilization, 600 sterile explants were transferred to the shoot induction stage.

During shoot induction, the highest response was obtained with 0.5 mg L^{-1} BAP, which resulted in a 90.00% induction frequency and a mean of 3.00 shoots per responding explant. A total of 1,266 shoots were produced across the three BAP treatments. For the subsequent multiplication stage, 20 shoots from each BAP treatment were used. The multiplication coefficients were 5.2, 6.5, and 4.8 shoots per starting shoot for 0.1, 0.5, and 1.0 mg L^{-1} BAP, respectively, corresponding to calculated outputs of 104, 130, and 96 shoots.

All shoots transferred to the rooting stage developed roots, resulting in a rooting frequency of 100.00% under all NAA treatments. Overall, the sequential culture system demonstrated successful progression from surface sterilization through shoot induction, multiplication, and rooting, with the strongest shoot induction and multiplication responses observed at 0.5 mg L^{-1} BAP. (Table 5; Figure 6).

Table 5. Overall material flow through the micropropagation protocol

Stage	**Treatment/group**	**Input**	**Output**
Sterilization	5 min	500	300 sterile
Sterilization	10 min	500	200 sterile
Sterilization	20 min	500	100 sterile
Induction	0.1 mg L^{-1} BAP	200	390 shoots
Induction	0.5 mg L^{-1} BAP	200	540 shoots
Induction	1.0 mg L^{-1} BAP	200	336 shoots
Multiplication	0.1 mg L^{-1} BAP	20	104 calculated shoots
Multiplication	0.5 mg L^{-1} BAP	20	130 calculated shoots
Multiplication	1.0 mg L^{-1} BAP	20	96 calculated shoots
Rooting	Control	20	100% rooted
Rooting	0.1 mg L^{-1} NAA	20	100% rooted
Rooting	0.5 mg L^{-1} NAA	20	100% rooted
Rooting	1.0 mg L^{-1} NAA	20	100% rooted

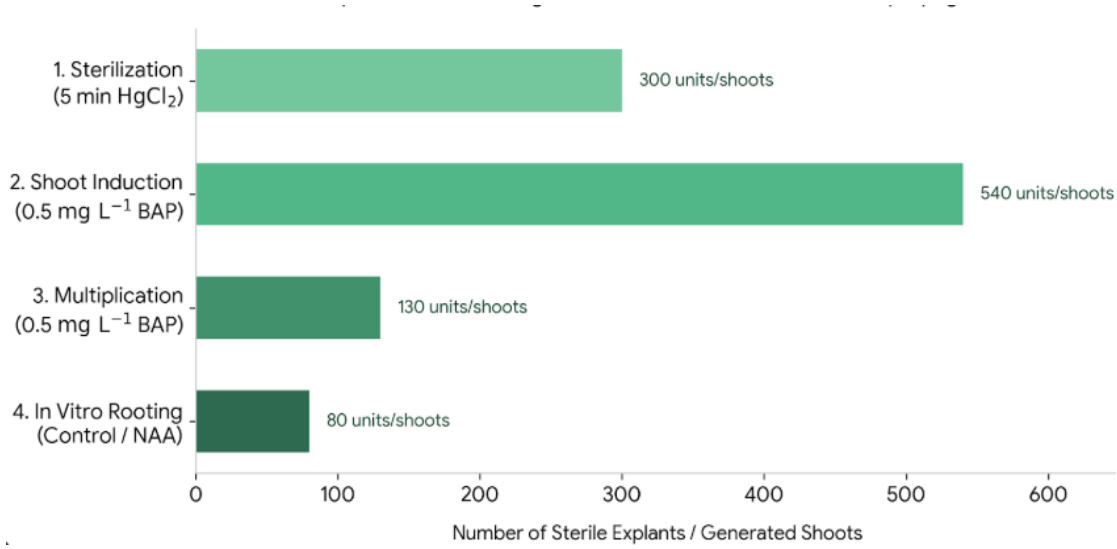


Fig. 6. Representative outputs recorded at the successive stages of the Alocasia "Bambino" micropropagation experiment.

4. DISCUSSION

The results demonstrated that the response of Alocasia "Bambino" to *in vitro* culture varied according to the stage of micropropagation and the treatment applied. The strongest treatment-dependent differences were observed during surface sterilization, shoot induction, and shoot multiplication, whereas rooting percentage was uniform across all rooting treatments. This stage-specific response indicates that establishment and shoot proliferation were the principal steps requiring optimization under the conditions evaluated in the present study.

Surface sterilization showed a clear response to treatment duration. The 5-min exposure to 0.1% $HgCl_2$ resulted in the highest sterile explant recovery (60%), whereas extending the treatment to 10 and 20 min reduced the percentage of sterile explants to 40 and 20%, respectively. The progressive decline in sterile explant recovery with increasing exposure time suggests that prolonged chemical treatment was not advantageous under the conditions of the present experiment. Although longer exposure may increase the opportunity to eliminate surface contaminants, the lower recovery observed at 10 and 20 min indicates that sterilization duration must be balanced against the ability of explants to remain suitable for subsequent culture. A significant association between sterilization duration and explant sterility was detected in the present study ($\chi^2(2) = 166.67$, $P < 0.001$), supporting the observed treatment-dependent response. Similar consideration of sterilization conditions has been reported in Alocasia tissue-culture studies, where successful establishment depended on the interaction between sterilizing treatment and explant response (Jo *et al.*, 2008; Bhatt *et al.*, 2013). In the present study, therefore, the 5-min treatment provided the most favorable establishment response within the sterilization periods tested.

Shoot induction was strongly affected by BAP concentration. The 0.5 mg L^{-1} treatment produced the highest induction frequency (90%) and the highest average shoot production per responding explant (3.0), whereas both lower and higher concentrations resulted in

lower responses. This pattern indicates that A. "Bambino" responded most effectively to an intermediate BAP concentration within the range evaluated. Cytokinin concentration is an important determinant of shoot initiation and proliferation in ornamental tissue culture, but the optimum concentration can differ substantially according to species, genotype, explant type, and culture conditions (Mehbub *et al.,* 2022). The present findings are consistent with this concentration-dependent response. Bhatt *et al*. (2013), for example, reported effective shoot proliferation of several Alocasia species at higher BA concentrations, while Abdulhafiz *et al.* (2020) observed the highest shoot production in Alocasia longiloba at 3 mg L^{-1} BAP. The lower optimum concentration observed for A. "Bambino" in the present experiment therefore reinforces the importance of developing species- or genotype-specific regeneration protocols rather than directly transferring a hormone regime established for another Alocasia species.

Statistical analysis confirmed a significant effect of BAP concentration on shoot production per responding explant ($F(2, 467) = 16.31$, $P < 0.001$). Tukey's HSD comparison further showed that the 0.5 mg L^{-1} treatment produced significantly more shoots per responding explant than the 0.1 and 1.0 mg L^{-1} treatments, while the latter two treatments did not differ significantly. The significant difference in induction frequency among BAP treatments ($\chi^2(2) = 25.53$, $P < 0.001$) further supports the concentration-dependent response observed in this experiment.

An additional consideration is the culture system used for shoot multiplication. The present experiment employed agar-solidified MS medium, which provides a relatively simple and operationally accessible system for routine propagation. However, previous studies have shown that the physical culture system can influence proliferation in Alocasia. Jo *et al.* (2008) compared semisolid and liquid culture systems in A. amazonica and demonstrated that culture format affected micropropagation performance. Similarly, Bhatt *et al.* (2013) reported that liquid culture enhanced shoot proliferation in several Alocasia species compared with conventional agar-gelled culture. These findings suggest that the multiplication response obtained here could potentially be improved further by evaluating liquid culture, temporary immersion, or other high-efficiency culture systems. Nevertheless, the response obtained with the present agar-based system demonstrates that an effective multiplication stage can be achieved without changing the basic culture format.

Rooting produced a different response pattern from that observed during shoot induction and multiplication. All treatments, including hormone-free MS medium and the three NAA concentrations, resulted in 100% rooting. The absence of a treatment difference in rooting frequency indicates that exogenous NAA was not necessary for root initiation under the experimental conditions. Quantitative analysis also showed no significant effect of NAA concentration on mean root number ($F(3, 76) = 0.54$, $P = 0.654$) or mean root length ($F(3, 76) = 0.88$, $P = 0.454$). This response may be practically important because successful rooting on hormone-free medium can simplify the propagation protocol by reducing the number of plant growth regulator manipulations required during the final *in vitro* stage. Previous Alocasia tissue-culture studies have demonstrated that rooting responses can vary depending on genotype, auxin concentration, culture conditions, and the developmental state of the shoots, indicating that rooting requirements should be established experimentally for each propagation system. The complete rooting response observed in the present study therefore represents a favorable characteristic of the A. "Bambino" protocol,

although rooting percentage alone does not provide information on subsequent root quality or *ex vitro* establishment.

Taken together, the results indicate that the main optimization points in the A. "Bambino" micropropagation protocol were explant establishment and shoot proliferation rather than root initiation.

Overall, the findings are consistent with the broader observation that Alocasia micropropagation is strongly influenced by species-specific responses to growth regulators and culture conditions (Jo *et al.*, 2008; Bhatt *et al.*, 2013; Abdulhafiz *et al.*, 2020; Mehbub *et al.*, 2022). The response of A. "Bambino" in the present study was characterized by a distinct optimum at 0.5 mg L^{-1} BAP for both shoot induction and multiplication, together with complete rooting across the tested treatments. These characteristics provide a useful basis for establishing a reproducible micropropagation protocol for this ornamental species. Further optimization could focus on culture-system comparisons, shoot quality, root architecture, and acclimatization performance so that the *in vitro* response can be linked more directly to successful *ex vitro* plant establishment.

5. CONCLUSIONS

The present study established a sequential *in vitro* micropropagation protocol for Alocasia "Bambino" based on the most favorable conditions identified for surface sterilization, shoot induction, shoot multiplication, and rooting. Surface sterilization was strongly influenced by treatment duration, with the 5-min exposure providing the highest sterile explant recovery among the treatments evaluated. Shoot induction and multiplication were significantly affected by BAP concentration, and 0.5 mg L^{-1} BAP provided the most favorable response during both stages. In contrast, all tested rooting treatments resulted in 100% rooting, while NAA concentration did not significantly affect mean root number or mean root length.

These findings demonstrate that the response of A. "Bambino" is stage-dependent and that optimization of the establishment and shoot proliferation phases is particularly important for efficient micropropagation. The protocol developed in the present study provides a practical basis for the *in vitro* propagation of A. "Bambino" and may facilitate the production of uniform plant material for ornamental horticulture and further propagation studies. Future research should evaluate the performance of the established shoots during *ex vitro* acclimatization and investigate alternative culture systems, shoot quality, and root development to further improve propagation efficiency.

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Micropropagation of *Caladium* 'Sunfire' via tissue culture

Bora Onur Hallaç[1], Soner Yağ[2], Cansu Ece Sari[1], Yeşim Yalçin Mendi[2]

[1]*Bolu Abant İzzet Baysal University, Postgraduate Education Seed Science and Technology, Bolu*
[2]*Çukurova University, Faculty of Agriculture, Department of Horticulture, Adana*
bora@fsbbiotech.com

Abstract

Caladium bicolor 'Sunfire' (Araceae) is a high-value ornamental cultivar valued for its decorative foliage, which combines bright red, pink, and green hues. Conventional propagation is comparatively slow and may be insufficient for the rapid production of uniform planting material. Plant tissue culture provides a means of producing large numbers of plants from limited starting material under controlled conditions. The objective of this study was to establish an efficient *in vitro* micropropagation protocol for *Caladium* 'Sunfire'. Young shoots 6-8 cm in length, grown under greenhouse conditions, were used as explants. After rinsing under running tap water for 40 min, explants were surface sterilized for 5, 10, and 15 min in a solution containing 0.1% mercury (II) chloride and 1 mL L^{-1} polysorbate 20. The 10-min treatment produced the highest sterility percentage (72%). For shoot induction, explants were cultured on Murashige and Skoog (MS) medium containing 30 g L^{-1} sucrose and 7 g L^{-1} agar and supplemented with 0.25, 0.75, or 1.25 mg L^{-1} 6-benzylaminopurine (BAP). The 0.75 mg L^{-1} BAP treatment gave the highest shoot induction frequency (94.24%) and 4.2 shoots per responding explant. During multiplication, the same BAP concentrations were evaluated, with 0.75 mg L^{-1} producing 8.1 shoots per starting shoot. Rooting was evaluated on MS media containing 0, 0.25, 0.75, or 1.25 mg L^{-1} α-naphthaleneacetic acid (NAA). The highest rooting percentage was 98% at 0.25 mg L^{-1} NAA, accompanied by the strongest root development among the treatments evaluated. The results support the use of 0.75 mg L^{-1} BAP for shoot induction and multiplication and 0.25 mg L^{-1} NAA for rooting in a micropropagation protocol for *Caladium* 'Sunfire'.

Keywords: *Caladium 'Sunfire', micropropagation, tissue culture, BAP, NAA*

1. INTRODUCTION

Caladium bicolor 'Sunfire' is an ornamental cultivar of the family Araceae valued for its attractive foliage, characterized by a combination of red, pink, and green color. The distinctive leaf color and morphology of *Caladium* cultivars are important components of their ornamental and commercial value, making the production of uniform and healthy planting material an important consideration in commercial cultivation. Conventional vegetative propagation of *Caladium* is mainly based on tuber division and may provide a relatively limited number of propagules from a single mother plant. *In vitro* culture offers an alternative propagation approach by enabling the production of large numbers of plants from a limited amount of starting material under controlled environmental and nutritional conditions (Zhu *et al.*, 1984; Ahmed *et al.*, 2002). For a commercially valuable cultivar such as 'Sunfire', the establishment of a reproducible micropropagation system may therefore contribute to the efficient production of uniform planting material.

The development of an effective micropropagation protocol for *Caladium* 'Sunfire' requires the optimization of several successive stages rather than the application of a single culture condition. Culture establishment, shoot induction, shoot multiplication, and rooting are physiologically interconnected stages, but the conditions that favor one stage may not necessarily provide the best response at another stage. Explant type, physiological age, culture conditions, and plant growth regulator concentration can all influence the morphogenic response of *Caladium* tissues. Ahmed *et al.* (2002) reported that explant age influenced the incidence of variants in *Caladium bicolor* and that young tissues were associated with a low incidence of variation. Similarly, Ahmed *et al.* (2007) demonstrated that the source of young green leaf tissue influenced the production of true-to-type *Caladium bicolor* plants. These findings emphasize the importance of selecting appropriate and physiologically suitable explant material when developing a cultivar-specific micropropagation protocol.

Surface sterilization represents a critical first step in the establishment of aseptic cultures. Explants obtained from greenhouse-grown plants carry microorganisms on their external surfaces, which can interfere with culture establishment and subsequent shoot development. At the same time, prolonged exposure to chemical sterilizing agents may cause tissue injury and reduce explant viability. Therefore, an effective sterilization treatment must provide adequate microbial control while maintaining the physiological capacity of the explant to survive and respond under *in vitro* conditions. Previous studies have evaluated different sterilization approaches in *Caladium*, including chlorine-based disinfectants and mercuric chloride treatments, demonstrating that sterilization efficiency depends on the disinfectant, exposure period, and characteristics of the plant material (Ali *et al.,* 2022). Because 'Sunfire' was not specifically evaluated in these previous studies, determining an appropriate sterilization duration is an important initial step in establishing an efficient protocol for this cultivar.

Following successful culture establishment, shoot induction and multiplication constitute the principal stages determining the propagation efficiency of an *in vitro* system. Cytokinins, particularly 6-benzylaminopurine (BAP), have been widely used to stimulate shoot initiation and proliferation in *Caladium*. Ali *et al.* (2007) reported successful shoot initiation from apical meristem explants of *Caladium bicolor* using BAP-supplemented Murashige and Skoog (MS) medium. Other studies have also demonstrated that the concentration and combination of plant growth regulators can substantially affect shoot regeneration and multiplication responses in *Caladium* (Rattana *et al.,* 2017; Thepsithar *et al.,* 2010; Ortiz-Sánchez *et al.*, 2021). Ahmed *et al.* (2023) further demonstrated that *in vitro* regeneration responses can vary according to the culture conditions used. These studies indicate that BAP concentration should be evaluated specifically for each cultivar because a concentration producing a positive response in one genotype may not necessarily provide the same response in another.

The response to BAP is also relevant when shoot induction and multiplication are considered as successive stages. The treatment that promotes the initiation of shoots may not necessarily maintain the same level of proliferation during subsequent subculture. Therefore, evaluating shoot production during both induction and multiplication provides a more complete assessment of the effectiveness of a cytokinin treatment. This distinction is particularly relevant for the development of a practical micropropagation system for 'Sunfire', where the

objective is not only to initiate shoots but also to obtain sufficient numbers of shoots for subsequent rooting and plant establishment. Accordingly, cultivar-specific evaluation of BAP concentration is necessary to determine whether the same or different cytokinin conditions provide favorable responses during successive stages of shoot production.

Rooting represents another critical stage because successful shoot multiplication must ultimately result in plantlets capable of establishment outside the *in vitro* environment. Several auxins, including α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA), have been evaluated for rooting in *Caladium* under different experimental conditions (Ali *et al.*, 2007; Ali *et al.*, 2022; Ahmed *et al.*, 2023). However, rooting responses can vary according to genotype, shoot physiological status, auxin type, and concentration. In addition to rooting percentage, the number of roots produced per shoot and root length are important characteristics because they provide information about the development of the root system.

The maintenance of cultivar characteristics is particularly important in the micropropagation of *Caladium* because leaf color, pattern, and morphology are among the primary traits determining ornamental quality. Although tissue culture enables rapid multiplication, regenerated plants may exhibit phenotypic or genetic variation under certain culture conditions. Ahmed *et al.* (2004a) reported that auxin treatment could influence the occurrence of leaf-color variants in *Caladium* regenerated from leaf explants, while Ahmed *et al.* (2004b) demonstrated differences in leaf-color stability among micropropagated *Caladium* cultivars. Somkanae *et al.* (2007) and Thepsithar *et al.* (2010) also reported somaclonal variation in micropropagated *Caladium*, including variation in leaf color and other morphological characteristics. Cao *et al.* (2016) provided morphological, cytological, and molecular evidence of somaclonal variation in 'Red Flash', while Chen *et al.* (2021) associated long-term subculture with the development of leaf-color variants. These findings demonstrate that the development of a propagation protocol should be considered together with the need to maintain the characteristic phenotype of the cultivar.

The use of young shoot material and direct shoot proliferation may be particularly relevant in this context because regeneration through direct shoot development may reduce the risk of somaclonal variation. Previous studies have demonstrated that explant age, regeneration pathway, plant growth regulator treatment, and culture history can influence the occurrence of variation in *Caladium* (Ahmed *et al.*, 2002; Ahmed *et al.*, 2004a, 2004b; Cao *et al.*, 2016; Chen *et al.*, 2021). However, the genetic and phenotypic stability of regenerated 'Sunfire' plants remains to be evaluated separately. Therefore, the primary objective at the protocol-development stage is to establish culture conditions that provide efficient shoot production and rooting while recognizing that subsequent stability assessment is necessary for complete validation of the propagation system.

Although previous studies have provided substantial information on tissue culture and micropropagation of *Caladium bicolor* and several other *Caladium* cultivars, including 'Thep Songsil', 'Red Flash', and 'Fancy', responses cannot necessarily be extrapolated directly to 'Sunfire' because genotype and explant characteristics influence *in vitro* responses (Ahmed *et al.*, 2002; Thepsithar *et al.*, 2010; Cao *et al.*, 2016; Syeed *et al.*, 2022). Based on the literature reviewed for the present study, no peer-reviewed study was identified that specifically evaluated the complete sequence of surface sterilization, BAP-mediated shoot induction, BAP-mediated multiplication, and NAA-based rooting in

Caladium 'Sunfire'. This cultivar-specific gap indicates the need for a sequential evaluation of the conditions required for efficient propagation of 'Sunfire'.

Accordingly, the present study aimed to establish a sequential *in vitro* micropropagation protocol for *Caladium* 'Sunfire'. The effects of surface sterilization duration on culture establishment, different BAP concentrations on shoot induction and multiplication, and different NAA concentrations on rooting were evaluated. Shoot induction, multiplication coefficient, rooting percentage, root number, and root length were assessed at the respective stages to identify the most responsive treatments under the experimental conditions. The resulting protocol provides cultivar-specific information for the *in vitro* propagation of *Caladium* 'Sunfire' and establishes a basis for subsequent studies on acclimatization, phenotypic stability, and larger-scale production.

2. MATERIALS AND METHODS

2.1. Materials

Young *Caladium bicolor* 'Sunfire' shoots measuring 6-8 cm and maintained under greenhouse conditions were used as the explant source.

2.2. Methods

2.2.1. Surface sterilization

The initial explant material consisted of 1,500 young shoots, which were equally distributed among three surface sterilization treatments of 5, 10, and 15 min, with 500 explants assigned to each treatment. For shoot multiplication, shoot production per starting shoot was analyzed using the individual starting shoots as experimental observations, and the multiplication coefficient was calculated as the treatment mean. Prior to chemical sterilization, the explants were thoroughly rinsed under running tap water for 40 min to remove adhering soil particles and surface contaminants. The explants were then transferred to a sterilizing solution containing 0.1% $HgCl_2$ and 1 mL L^{-1} polysorbate 20 and exposed for the assigned treatment duration. Following sterilization, the explants were rinsed three times with sterile distilled water to remove residual $HgCl_2$ and were subsequently transferred to the *in vitro* culture medium. The number of sterile explants obtained from each treatment was recorded to determine sterilization efficiency. The use of mercuric chloride as a surface sterilizing agent has previously been reported in Caladium micropropagation studies (Ali *et al.,* 2022).

2.2.2. Shoot induction

The surviving explants were transferred to Murashige and Skoog (MS) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar. For shoot induction, three concentrations of 6-benzylaminopurine (BAP), namely 0.25, 0.75, and 1.25 mg L^{-1}, were evaluated. The experiment included 191, 191, and 190 explants in the 0.25, 0.75, and 1.25 mg L^{-1} BAP treatments, respectively. Shoot induction was assessed by recording the number of explants showing shoot emergence, and induction frequency was calculated based on the proportion of responding explants in each treatment. The total number of shoots produced was also recorded to quantify shoot production during the induction stage.

2.2.3. Shoot multiplication

Twenty shoots from each BAP treatment were selected from the induction stage and transferred to fresh MS medium. Accordingly, the 0.25, 0.75, and 1.25 mg L^{-1} BAP treatments were maintained at their respective concentrations during the multiplication stage. This approach was used to evaluate the multiplication response of each BAP treatment across the successive stages of shoot production. Multiplication performance was evaluated using the multiplication coefficient, calculated as the number of shoots produced relative to the number of shoots initially transferred to each treatment.

2.2.4. In vitro rooting

The regenerated shoots were transferred to rooting media consisting of MS medium supplemented with three different NAA treatments containing 0.25, 0.75, or 1.25 mg L^{-1} NAA compared to a hormone-free MS medium**.** Twenty shoots were assigned to each rooting treatment. Rooting percentage was recorded for each treatment. In addition to rooting percentage, mean root number per rooted shoot and mean root length were recorded for each treatment.

2.2.5. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) to determine the effects of sterilization duration, BAP concentration, and NAA concentration on the measured response variables at the respective stages of the micropropagation protocol. When significant differences were detected, means were compared using Tukey's honestly significant difference (HSD) test at the 5% significance level ($p < 0.05$). For shoot induction, the number of shoots per responding explant was analyzed using the individual responding explants as experimental observations. Each sterilization treatment consisted of five replicate groups of 100 explants. Root number and root length were analyzed using rooted shoots. Data are presented as mean ± standard error (SE). Statistical analyses were performed separately for each experimental stage.

3. RESULTS

3.1. Surface Sterilization

A total of 1,500 *Caladium* 'Sunfire' explants were subjected to three surface sterilization durations: 5, 10, and 15 min. The sterilization response differed markedly among the treatments (Table 1). The 5-min treatment resulted in 120 sterile explants out of 500, corresponding to a sterility percentage of 24.00%. Increasing the treatment duration to 10 min substantially increased the number of sterile explants, with 360 of 500 explants remaining sterile and a sterility percentage of 72.00%. In contrast, extending the treatment to 15 min resulted in 92 sterile explants and a sterility percentage of 18.40%. Thus, among the tested durations, the 10-min treatment yielded the highest proportion of sterile explants. Overall, 572 of the 1,500 explants were retained as sterile material for the subsequent stages of the experiment. One-way ANOVA revealed a significant effect of sterilization duration on sterility percentage ($F(2,12) = 158.21$, $p < 0.001$). According to Tukey's HSD test, the 10-min treatment resulted in a significantly higher sterilization percentage than both the 5- and 15-min treatments, whereas the latter two treatments did not differ significantly from each other (Table 1).

Table 1. Surface sterilization results for *Caladium* 'Sunfire'

Sterilization time (min)	Initial explants	Sterile explants	Contaminated explants	Sterility (%)
5	500	120	380	24.00 ± 2.26^{b}
10	500	360	140	72.00 ± 2.72^{a}
15	500	92	408	18.40 ± 1.99^{b}
Total	1500	572	928	38.13

Note: Values are presented as mean ± standard error (SE). Means followed by different superscript letters are significantly different according to Tukey's HSD test ($p < 0.05$).

3.2. Shoot induction

Shoot induction responses varied among the tested BAP concentrations (Table 2). The 0.25 mg L^{-1} BAP treatment resulted in 145 responding explants from 191 cultured explants, corresponding to an induction frequency of 75.92%. A total of 493 shoots were produced in this treatment, with an average of 3.40 shoots per responding explant. The 0.75 mg L^{-1} BAP treatment produced the highest induction frequency, with 180 of 191 explants responding (94.24%). This treatment also produced the highest shoot production per responding explant, with 4.20 shoots and a total of 756 shoots. At 1.25 mg L^{-1} BAP, 142 of 190 explants responded, corresponding to an induction frequency of 74.74%, while 454 shoots were produced at an average of 3.20 shoots per responding explant.

The 0.75 mg L^{-1} BAP treatment therefore produced the highest response across the induction parameters evaluated in the present study. Across all three BAP treatments, 467 explants responded to culture and a total of 1,703 shoots were produced. A subset of 20 shoots from each BAP treatment was subsequently selected for the multiplication experiment. BAP concentration significantly affected shoot production per responding explant ($F(2,464) = 29.27$, $p < 0.001$). The highest shoot production was obtained with 0.75 mg L^{-1} BAP (4.20 ± 0.05 shoots/responding explant), which was significantly higher than those obtained with 0.25 mg L^{-1} (3.40 ± 0.12) and 1.25 mg L^{-1} BAP (3.20 ± 0.13). No significant difference was detected between the 0.25 and 1.25 mg L^{-1} BAP treatments according to Tukey's HSD test ($p < 0.05$) (Table 2).

Table 2. Shoot induction response at different BAP concentrations.

BAP (mg L^{-1})	Explants	Responding explants	Induction (%)	Total shoots	Shoots/responding explant
0.25	191	145	75.92	493	3.40 ± 0.12^{b}
0.75	191	180	94.24	756	4.20 ± 0.05^{a}
1.25	190	142	74.74	454	3.20 ± 0.13^{b}
Total	572	467	81.64	1703	-

Note: Values are presented as mean ± standard error (SE). Means followed by different superscript letters are significantly different according to Tukey's HSD test ($p < 0.05$).

3.3. Shoot multiplication

Shoot multiplication was evaluated using 20 shoots for each BAP treatment. The same BAP concentrations used during shoot induction were maintained during the multiplication stage. The mean multiplication coefficients were 5.6, 8.1, and 6.4 shoots per starting shoot for 0.25, 0.75, and 1.25 mg L^{-1} BAP, respectively. The highest mean multiplication coefficient was obtained with 0.75 mg L^{-1} BAP. Based on the respective multiplication coefficients, the calculated shoot outputs were 112, 162, and 128 shoots, resulting in a total calculated output of 402 shoots across the three BAP treatments. BAP concentration significantly affected shoot production per starting shoot ($F(2,57) = 166.39$, $p < 0.001$). The highest multiplication coefficient was recorded at 0.75 mg L^{-1} BAP (8.10 ± 0.07), followed by 1.25 mg L^{-1} (6.40 ± 0.11) and 0.25 mg L^{-1} BAP (5.60 ± 0.11). Tukey's HSD test indicated that all three BAP treatments differed significantly from each other ($p < 0.05$) (Table 3) (Figure 1, 2).

Table 3. Shoot multiplication output obtained from induced shoots

BAP (mg L^{-1})	Starting shoots	Mean multiplication coefficient	Calculated shoots
0.25	20	5.6 ± 0.11^{c}	112
0.75	20	8.1 ± 0.07^{a}	162
1.25	20	6.4 ± 0.11^{b}	128
Total	60	-	402

Note: Values are presented as mean ± standard error (SE). Means followed by different superscript letters are significantly different according to Tukey's HSD test ($p < 0.05$).

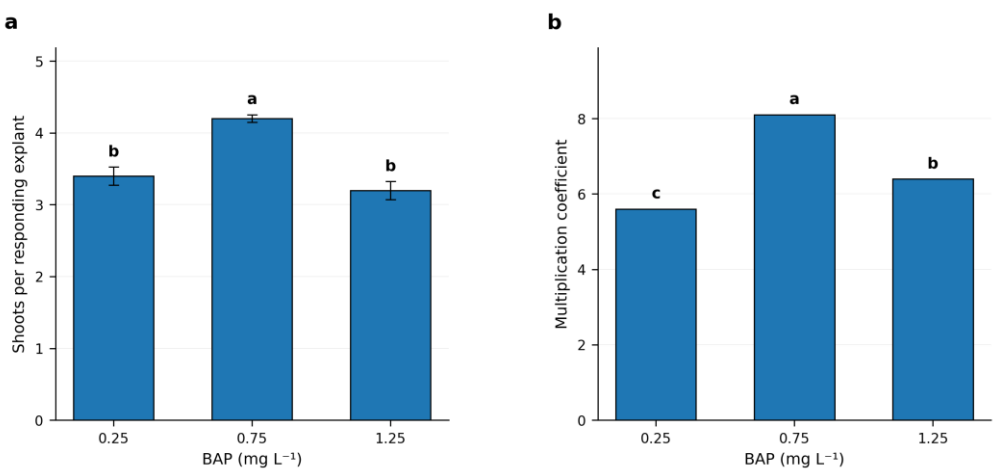


Fig. 1. Shoot response of *Caladium* 'Sunfire' to BAP concentration. (a) Shoot induction; (b) multiplication coefficient. Induction values are mean ± SE calculated from responding explants; multiplication values are means calculated from individual starting shoots. Different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).



Fig. 2. Shoot proliferation of *Caladium* 'Sunfire'

3.4. In vitro rooting

In vitro rooting was evaluated using four treatments, including hormone-free MS medium (0 mg L^{-1} NAA) and MS medium supplemented with 0.25, 0.75, or 1.25 mg L^{-1} NAA. Twenty shoots were assigned to each treatment. The highest rooting response was obtained with 0.25 mg L^{-1} NAA, which showed a rooting percentage of 98.00%, whereas the hormone-free control showed 85.00% rooting. The 0.75 and 1.25 mg L^{-1} NAA treatments resulted in rooting percentages of 88.74% and 78.08%, respectively. The mean number of roots per rooted shoot was 3.76, 5.89, 4.67, and 4.06 for the control, 0.25, 0.75, and 1.25 mg L^{-1} NAA treatments, respectively. Mean root lengths were 4.39, 6.31, 5.35, and 4.51 cm, respectively. The 0.25 mg L^{-1} NAA treatment showed the highest rooting percentage as well as the highest mean root number and mean root length among the treatments evaluated. NAA concentration significantly affected both the number of roots per shoot ($F(3,66) = 48.76$, $p < 0.001$) and root length ($F(3,66) = 741.15$, $p < 0.001$). For both parameters, the 0.25 mg L^{-1} NAA treatment produced the highest values, with 5.89 ± 0.13 roots per shoot and a mean root length of 6.31 ± 0.03 cm. The 0.75 mg L^{-1} treatment showed intermediate values, whereas the control and 1.25 mg L^{-1} NAA treatments did not differ significantly from each other for either parameter (Tukey's HSD, $p < 0.05$) (Table 4) (Figure 3).

Table 4. *In vitro* rooting response to NAA treatments.

NAA (mg L^{-1})	Shoots introduced	Rooting (%)	Mean roots/shoot	Mean root length (cm)
0	20	85.00	3.76 ± 0.16^{c}	4.39 ± 0.04^{c}
0.25	20	98.00	5.89 ± 0.13^{a}	6.31 ± 0.03^{a}
0.75	20	88.74	4.67 ± 0.11^{b}	5.35 ± 0.03^{b}
1.25	20	78.08	4.06 ± 0.14^{c}	4.51 ± 0.03^{c}

Note: Values are presented as mean ± standard error (SE). Means followed by different superscript letters within a column are significantly different according to Tukey's HSD test ($p < 0.05$).

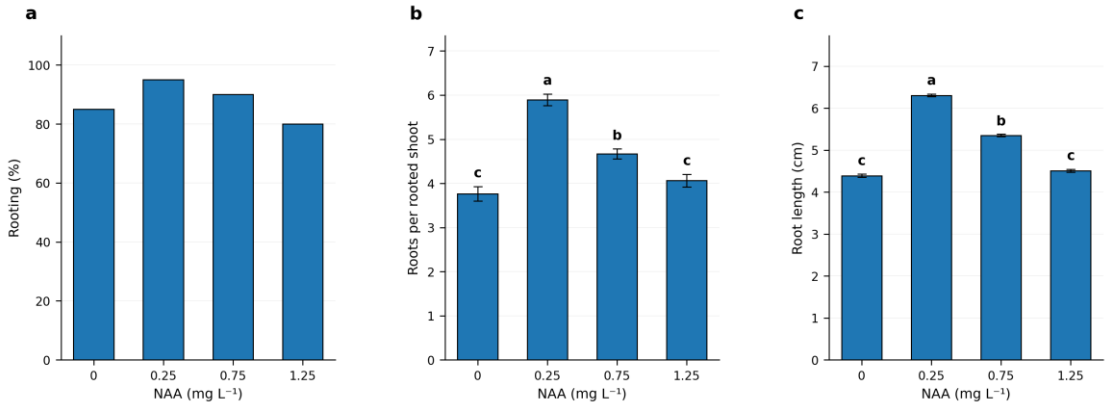


Fig. 3. Rooting response of *Caladium* 'Sunfire' to NAA concentration. (a) Rooting percentage; (b) mean number of roots per rooted shoot; (c) mean root length. Root number and root length are presented as mean ± SE.

3.5. Overall material flow

The sequential material flow of the *Caladium* 'Sunfire' micropropagation protocol is summarized in Table 5. Following surface sterilization, 572 of the initial 1,500 explants were retained as sterile material. During shoot induction, a total of 1,703 shoots were produced across the three BAP treatments. For the subsequent multiplication experiment, 20 shoots were selected from each BAP treatment, resulting in 60 starting shoots. Based on the mean multiplication coefficients of 5.6, 8.1, and 6.4, the calculated shoot outputs were 112, 162, and 128, respectively, giving a total calculated output of 402 shoots. For the rooting experiment, 20 shoots were assigned to each of the four NAA treatments, resulting in 80 shoots evaluated for rooting response (Table 5).

Table 5. Overall material flow through the micropropagation protocol.

Stage	Treatment/group	Input	Output
Sterilization	5 min	500	120 sterile
Sterilization	10 min	500	360 sterile
Sterilization	15 min	500	92 sterile
Induction	0.25 mg L^{-1} BAP	191	493 shoots
Induction	0.75 mg L^{-1} BAP	191	756 shoots
Induction	1.25 mg L^{-1} BAP	190	454 shoots
Multiplication	0.25 mg L^{-1} BAP	20	112 shoots
Multiplication	0.75 mg L^{-1} BAP	20	162 shoots
Multiplication	1.25 mg L^{-1} BAP	20	128 shoots
Rooting	0 mg L^{-1} NAA	20	85.00% rooted

Stage	Treatment/group	Input	Output
Rooting	0.25 mg L^{-1} NAA	20	98.00% rooted
Rooting	0.75 mg L^{-1} NAA	20	88.74% rooted
Rooting	1.25 mg L^{-1} NAA	20	78.08% rooted

4. DISCUSSION

The present study demonstrated that the response of *Caladium bicolor* 'Sunfire' to *in vitro* culture varied according to sterilization duration and plant growth regulator concentration. Surface sterilization, shoot induction, multiplication, and rooting each showed distinct treatment responses, indicating that successful micropropagation of this cultivar requires optimization of successive culture

Surface sterilization was an important determinant of culture establishment Among the three sterilization durations evaluated, the 10-min treatment resulted in the highest sterility percentage (72.00%), whereas lower sterility percentages were observed after 5 and 15 min. The reduced response obtained at 15 min indicates that extending the exposure period did not provide an additional benefit under the conditions tested. Previous work on *Caladium* has similarly demonstrated that sterilization procedures should be adapted to the characteristics and physiological condition of the explant material and to the disinfectant and exposure period used (Ali *et al.,* 2022). Statistical analysis further confirmed a significant effect of sterilization duration on sterilization percentage ($F(2,12) = 158.21$, $p < 0.001$). The 10-min treatment resulted in a significantly higher sterilization percentage than both the 5- and 15-min treatments, whereas no significant difference was detected between the 5- and 15-min treatments according to Tukey's HSD test ($p < 0.05$).

Shoot induction was clearly influenced by BAP concentration. Among the concentrations evaluated, 0.75 mg L^{-1} BAP produced the highest induction frequency (94.24%) and the highest mean number of shoots per responding explant (4.20). This concentration-dependent response is consistent with the general importance of cytokinins in shoot initiation and multiplication in ornamental tissue culture, although the optimum concentration may vary according to genotype, explant type, and culture conditions (Mehbub *et al.*, 2022). Previous *Caladium* studies have likewise demonstrated concentration-dependent responses to BAP. Ali *et al.* (2007) reported successful shoot initiation in *Caladium bicolor* using BAP-containing MS medium, whereas Ortiz-Sánchez *et al.* (2021) also demonstrated that the balance and concentration of growth regulators affected *in vitro* responses. The higher response obtained with 0.75 mg L^{-1} BAP in the present study therefore suggests that this concentration was particularly suitable for shoot induction of 'Sunfire' under the conditions tested. The statistical analysis confirmed that BAP concentration significantly affected shoot production per responding explant ($F(2,464) = 29.27$, $p < 0.001$). The 0.75 mg L^{-1} BAP treatment produced significantly more shoots per responding explant than the 0.25 and 1.25 mg L^{-1} treatments, whereas the latter two treatments did not differ significantly from each other according to Tukey's HSD test ($p < 0.05$).

The multiplication results followed a similar response pattern. The mean multiplication coefficients were 5.6, 8.1, and 6.4 shoots per starting shoot at 0.25, 0.75, and 1.25 mg L^{-1}

BAP, respectively, with the highest coefficient obtained at 0.75 mg L^{-1} BAP. Based on the 20 starting shoots used for each treatment, the corresponding calculated outputs were 112, 162, and 128 shoots. The consistency of the response between induction and multiplication indicates that 0.75 mg L^{-1} BAP maintained a favorable response when the same concentration was used during the successive multiplication stage. However, the response of *Caladium* to culture conditions is not determined by cytokinin concentration alone. Daquinta *et al.* (2007) demonstrated that culture-system design could also influence shoot proliferation, with temporary immersion providing an alternative approach to conventional culture. Thus, the 0.75 mg L^{-1} BAP treatment identified in the present study may provide a useful starting point for future comparisons between semi-solid culture and liquid or temporary immersion systems. Statistical analysis demonstrated a significant effect of BAP concentration on shoot production per starting shoot ($F(2,57) = 166.39$, $p < 0.001$). The multiplication coefficient obtained with 0.75 mg L^{-1} BAP was significantly higher than those obtained with 0.25 and 1.25 mg L^{-1} BAP, and all three treatments differed significantly from each other according to Tukey's HSD test ($p < 0.05$).

Rooting responses differed from the shoot multiplication response and indicated that the most responsive NAA concentration for root development was lower than the concentrations evaluated during shoot proliferation. The 0.25 mg L^{-1} NAA treatment produced the highest rooting percentage among the treatments evaluated (98.00%), together with the highest mean number of roots per rooted shoot (5.89) and the greatest mean root length (6.31 cm). The hormone-free control also showed a comparatively high rooting response (85.00%), whereas the 0.75 and 1.25 mg L^{-1} NAA treatments produced lower rooting responses and reduced mean root development. Previous studies have demonstrated that auxins, including NAA and other auxin types, can influence rooting in *Caladium*, but responses vary among genotypes and experimental conditions (Ali *et al.,* 2007; Ahmed *et al.,* 2023). The present findings therefore reinforce the cultivar-specific nature of auxin responses and suggest that increasing NAA concentration beyond the favorable range did not further improve rooting of 'Sunfire'. The statistical analysis revealed significant effects of NAA concentration on both the number of roots per shoot ($F(3,66) = 48.76$, $p < 0.001$) and root length ($F(3,66) = 741.15$, $p < 0.001$). For both parameters, 0.25 mg L^{-1} NAA produced significantly higher values than the other treatments. The 0.75 mg L^{-1} treatment showed intermediate values, while the control and 1.25 mg L^{-1} NAA treatments did not differ significantly from each other according to Tukey's HSD test ($p < 0.05$).

The results should also be considered in relation to cultivar stability. *Caladium* is valued primarily for its distinctive leaf color and morphology, and therefore multiplication efficiency must be considered together with the maintenance of desirable cultivar characteristics. Ahmed *et al.* (2002) reported that explant age influenced the incidence of variants, while Ahmed *et al.* (2004a, 2004b) demonstrated that regeneration conditions and auxin treatment could affect leaf-color variation and stability. Additional studies have documented somaclonal variation in several *Caladium* cultivars, including 'Thep Songsil' and 'Red Flash' (Thepsithar *et al.*, 2010; Cao *et al.*, 2016), and long-term subculture has also been associated with the formation of leaf-color variants (Chen *et al.,* 2021). The use of young shoot material and direct shoot proliferation in the present study may therefore be advantageous for cultivar maintenance; however, genetic and phenotypic stability was not directly assessed and should be examined in future work.

The sequential nature of the present protocol also highlights the importance of evaluating each stage independently. Although the 10-min sterilization treatment provided the highest sterilization response, all sterile materials were retained within the working material-flow calculations. Likewise, only a defined subset of induced shoots was selected for the multiplication experiment, and 20 shoots were subsequently assigned to each rooting treatment. Therefore, the calculated multiplication outputs represent the selected experimental material rather than the theoretical production capacity of all 1,703 shoots obtained during induction. This distinction is important when interpreting the results and when translating the experimental protocol into a larger-scale production system.

Overall, the present findings indicate that 0.75 mg L^{-1} BAP provided the most favorable response for shoot induction and multiplication, whereas 0.25 mg L^{-1} NAA provided the strongest rooting response under the conditions evaluated. The results contribute cultivar-specific information for *Caladium* 'Sunfire', for which a complete sequential evaluation of sterilization, BAP-mediated shoot induction and multiplication, and NAA-mediated rooting was not identified in the literature reviewed for this study.

5. CONCLUSIONS

The present study developed a sequential *in vitro* micropropagation framework for *Caladium* 'Sunfire' covering explant surface sterilization, shoot induction, shoot multiplication, and *in vitro* rooting. Among the sterilization treatments evaluated, the 10-min exposure to 0.1% mercury(II) chloride resulted in the highest sterility response (72.00%), indicating that this treatment provided the most favorable balance for culture establishment under the conditions tested. During shoot induction, 0.75 mg L^{-1} BAP produced the highest induction frequency (94.24%) and the greatest mean number of shoots per responding explant (4.20). The same BAP concentration also resulted in the highest mean multiplication coefficient (8.1 shoots per starting shoot), compared with 5.6 and 6.4 shoots per starting shoot at 0.25 and 1.25 mg L^{-1} BAP, respectively. These findings indicate that the response of *Caladium* 'Sunfire' was strongly dependent on cytokinin concentration and that 0.75 mg L^{-1} BAP provided the most favorable shoot proliferation response among the concentrations evaluated.

Rooting responses further demonstrated the importance of optimizing plant growth regulator concentration for the final stage of micropropagation. The 0.25 mg L^{-1} NAA treatment showed the highest rooting response among the treatments evaluated (98.00%) and was also associated with the highest mean number of roots per rooted shoot (5.89) and the greatest mean root length (6.31 cm). The hormone-free control, as well as the higher NAA concentrations, produced lower rooting responses and reduced root development. The observed differences in shoot production per responding explant, multiplication coefficient, root number, and root length were statistically significant, supporting the treatment-dependent responses observed across the successive stages of the protocol. Taken together, the results provide cultivar-specific information for establishing a micropropagation protocol for *Caladium* 'Sunfire', with 10-min surface sterilization, 0.75 mg L^{-1} BAP for shoot induction and multiplication, and 0.25 mg L^{-1} NAA for rooting representing the most responsive conditions within the evaluated treatments.

Because phenotypic or genetic stability and acclimatization were not directly assessed in the present study, further experiments should focus on these parameters to validate the reproducibility and practical applicability of the proposed protocol.

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WORKING GROUP 5

Communication, dissemination, and technology transfer

Public attitude toward new technologies in FRM production – Case study Bosnia and Herzegovina

Branislav Cvjetković

University of Banja Luka, Faculty of Forestry, Bulevar vojvode Petra Bojovića 1A, 78000, Banja Luka, Bosnia and Herzegovina

branislav.cvjetkovic@sf.unibl.org

Abstract

In the era of climate change, forestry faces increasing challenges in ensuring sufficient quantities of forest reproductive material. Current trends indicate use of a wide range of forest tree species, mainly propagated from seeds. To assess the level of awareness among forestry practitioners, a survey involving 30 forestry engineers engaged in silvicultural activities in Bosnia and Herzegovina was conducted. The questionnaire included 26 questions related to reforestation needs, types of planting material, and familiarity with specific vegetative seedling production technologies. Approximately 25% of respondents were familiar with these technologies. About 41% stated they would use seedlings produced in this way, while 33.3% were uncertain. All respondents emphasized the need for additional education, primarily through professional seminars. Most participants (72.7%) identified direct contact – seminars - as the most effective form of knowledge transfer, while others mentioned the internet and professional literature. All respondents indicated willingness to use such planting material if its advantages were adequately presented. Innovative technologies in forest reproductive material production are expected to gain increasing importance under climate change conditions. Effective knowledge transfer to forestry practitioners is therefore essential for wider implementation of advanced production technologies in forestry practice.

Keywords: forestry practitioners, new propagation technologies

1. INTRODUCTION

The establishment of new forests requires a stable and reliable production of forest reproductive material (abbreviation "FRM"). According to FAO (2025), although the global rate of deforestation has declined over the last decade, forest loss remains significant. At the same time, projections of future economic development indicate a growing demand for wood as a renewable and environmentally friendly raw material suitable for cascading use. Global wood consumption is expected to exceed 5 billion m^3 in the coming decades (Peng *et al.*, 2023; FAO, 2025). In addition to their economic importance, forests contribute substantially to rural employment opportunities (Renner, 2017) and provide a wide range of ecosystem services, the value of which is often underestimated (Grammatikopoulou & Vačkářová, 2021), although these services may be less pronounced in planted forests than in natural forest ecosystems (Pérez-Silos *et al.*, 2021).

Large-scale afforestation and reforestation initiatives frequently overlook the actual capacity of FRM production systems, creating significant challenges for long-term planning and implementation (Haase & Davis, 2017). Achieving global restoration targets

will require a several-fold increase in seedling production worldwide (Fargione *et al.,* 2021). Forest establishment has considerable potential for climate change mitigation, with estimates suggesting a mitigation capacity of 4.9 Gt CO_2 $year^{-1}$ at a carbon price of 200 US$ t^{-1} CO_2 by 2050, resulting in the cumulative sequestration of approximately 410 Gt CO_2 by 2100 under the SSP2 scenario aimed at limiting global warming to 2°C (Doelman *et al.,* 2020). Such efforts may substantially reduce the overall costs of climate change mitigation while simultaneously generating positive socio-economic effects for local communities (Gibertini *et al.,* 2026) and contributing to erosion control across extensive areas (Zhe *et al.,* 2017). According to Stanturf *et al.* (2019), forest ecosystem restoration is a complex process that cannot be approached through universal solutions. Instead, restoration programs require site-specific strategies, particularly in large-scale projects. In smaller countries such as Bosnia and Herzegovina, afforestation has a long tradition; however, efforts have often been constrained by insufficient planting intensity and relatively low plantation success rates.

The first recorded afforestation activities in Bosnia and Herzegovina date back to 1884 (Ballian & Božič, 2017). Initially, afforestation progressed slowly and was often difficult to implement effectively. Throughout the twentieth century, numerous strategic and technical documents were developed to improve afforestation practices; however, their impact on operational forestry remained limited (Knežević, 1966). In subsequent decades, greater investments were directed toward forest establishment, with particular emphasis placed on improving nursery production technologies, planting methods, and the economic efficiency of afforestation activities (Radovanović, 1982). Intensive plantations of fast-growing species were established to meet industrial demands (Nedović, 1972), while the use of a broader range of tree species, including numerous introduced species, was proposed within afforestation plans extending to the year 2000 (Pintarić, 1977). Afforestation activities increased until the early 1990s, when political and socio-economic instability interrupted many forestry programs.

Over the last 5 years, the area afforested annually in Bosnia and Herzegovina has shown a changing trend (Republic of Srpska Institute of Statistics, 2025, Institute for Statistics of Federation of Bosnia and Herzegovina, 2025). Climate change has emerged as an additional challenge, significantly affecting afforestation success and plantation establishment (Cvjetković & Mataruga, 2020) decreasing available seed and affecting its quality for specific species (Cvjetković *et al.,* 2013; Mataruga *et al.,* 2025). Consequently, current research focuses on improving species-site matching and selecting species better adapted to future environmental conditions (Mataruga *et al.,* 2020a; Mataruga *et al.,* 2020b; Mataruga *et al.,* 2021). Existing strategic documents emphasize the potential use of approximately 50 tree species for afforestation and highlight the need to produce planting material adapted to both current site conditions and anticipated future challenges.

Despite these efforts, none of the existing strategic documents explicitly recommends the operational implementation of advanced vegetative propagation technologies, as suggested by Sota *et al.* (2025a, 2025b). Similarly, limited attention has been paid to the application of innovative nursery technologies such as mycorrhization (Brenko *et al.,* 2025) or the use of physiological quality assessment methods for planting stock (Mataruga *et al.,* 2023). *In vitro* production of FRM has received particularly little attention, largely because forest seed production has traditionally been considered stable and sufficient for operational

needs. Some small *in vitro* activities were carried out only for very specific species such as yellow beech (Tošič, 2006) However, climate change has increasingly affected seed production in many forest tree species, leading to reduced seed yields, lower seed quality, and a growing reliance on reproductive material of uncertain origin (Jalonen *et al.*, 2018).

At the same time, forestry professionals are increasingly encouraged to adopt the concept of Target Seedling Production (Landis, 2011; Haase & Davis, 2017) and to bridge the gap between laboratory-based innovations and operational forestry practice (Aronen *et al.*, 2025). Such advances are considered essential for improving forest health and enhancing the economic, ecological, and social benefits derived from forest ecosystems.

In Southeast Europe, FRM production remains predominantly based on generative propagation from seed (Mataruga *et al.*, 2023), while the application of *in vitro* technologies is still in its early stages of development (Sota *et al.*, 2025a, 2025b). Although the importance of high-quality FRM is widely recognized and the benefits of vegetative propagation have been repeatedly emphasized (Tikkinen *et al.*, 2021), it remains unclear whether forestry professionals possess sufficient knowledge, motivation, and willingness to adopt these advanced technologies in practice having in mind the knowledge gap with FRM in general (Konnert *et al.*, 2015).

Therefore, the aim of this study was to assess the knowledge, attitudes, and level of interest of forestry professionals involved in silviculture and forest regeneration regarding modern vegetative propagation technologies. Particular attention was given to a specific ecological region of Bosnia and Herzegovina, where future afforestation and forest restoration efforts may increasingly depend on the successful implementation of innovative propagation techniques.

2. MATERIALS AND METHODS

The study was conducted using a questionnaire survey administered to 30 forestry practitioners directly involved in afforestation and reforestation planning in Bosnia and Herzegovina. All respondents were employed by the public forest enterprise responsible for managing approximately 80% of the country's state-owned and private forests.

Data were collected through a structured questionnaire consisting of 26 closed-ended questions. The questionnaire was designed to assess respondents' knowledge, experience, and attitudes regarding FRM, afforestation practices, and the application of modern vegetative propagation technologies in forestry.

The questionnaire was organized into seven thematic sections:

1. Demographic and professional characteristics – respondents' age and years of professional experience in the forestry sector;
2. Knowledge of planted forests – respondents' awareness of the current status and importance of planted forests at both global and national levels, with a particular focus on Bosnia and Herzegovina.
3. Afforestation and reforestation activities – information regarding the types of sites afforested or reforested and the annual area managed through these activities;

4. Use of FRM – tree species used in afforestation programs and the types of FRM commonly employed in operational forestry practice;
5. Knowledge of vegetative propagation technologies – respondents' familiarity with vegetative propagation methods, species propagated using these techniques, and their perceived advantages compared to conventional seed-based production;
6. Knowledge transfer and capacity building – respondents who indicated limited or no knowledge of tissue culture, micropropagation, or related propagation techniques were asked to identify the most appropriate mechanisms for acquiring such knowledge and skills;
7. Acceptance of advanced propagation technologies – respondents' awareness of existing FRM producers utilizing vegetative propagation technologies and their willingness to use planting stock produced through tissue culture, micropropagation, or other advanced propagation methods.

The survey was conducted anonymously to encourage unbiased responses and to ensure the confidentiality of participants. The collected data were subsequently analyzed to evaluate the current level of awareness and acceptance of modern vegetative propagation technologies among forestry practitioners in Bosnia and Herzegovina.

3. RESULTS

The results are presented according to the structure of the questionnaire, beginning with respondents' demographic characteristics and ending with their preferred approaches for knowledge transfer and their acceptance of advanced propagation technologies.

3.1. Demographic and professional characteristics

The surveyed forestry practitioners represented a relatively experienced professional group. More than half of the respondents (54%) were between 35 and 45 years of age, while 30% were aged between 25 and 35 years. The remaining respondents were older than 45 years. In terms of professional experience, 70% of respondents reported between 10 and 20 years of employment in the forestry sector, suggesting that the survey captured the opinions of professionals actively engaged in operational forestry and forest management.

3.2. Knowledge of planted forests

Respondents demonstrated a relatively high awareness of global forest trends. The majority (81.48%) stated that forest area at the global level is decreasing, while 14.81% were uncertain about current trends. Perceptions differed at the national level, where 62.96% of respondents believed that forest area in Bosnia and Herzegovina is increasing. In contrast, 18.52% considered forest area to be decreasing, whereas 14.81% perceived no significant changes. Only a small proportion of respondents expressed uncertainty regarding national forest area trends.

These findings suggest that forestry practitioners are generally familiar with broad global and national developments in the forestry sector. However, the variation in responses concerning national forest area dynamics may indicate differences in access to information, interpretation of available statistics, or regional differences in forest management conditions.

3.3. Afforestation and Reforestation Practices

Afforestation and reforestation activities were found to be common silvicultural practices among all surveyed respondents. Every participant reported conducting planting activities on barren or non-forested land, confirming that afforestation remains an important management activity within forest management units in Bosnia and Herzegovina.

Planting was also widely used as a supplementary measure to support natural regeneration. A total of 85% of respondents indicated that planting activities are carried out in high-value forest stands to enhance regeneration success (Fig. 1).

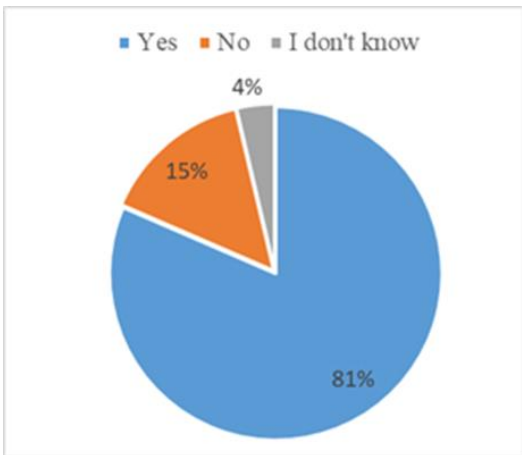


Fig 1. Planting in high valuable forests

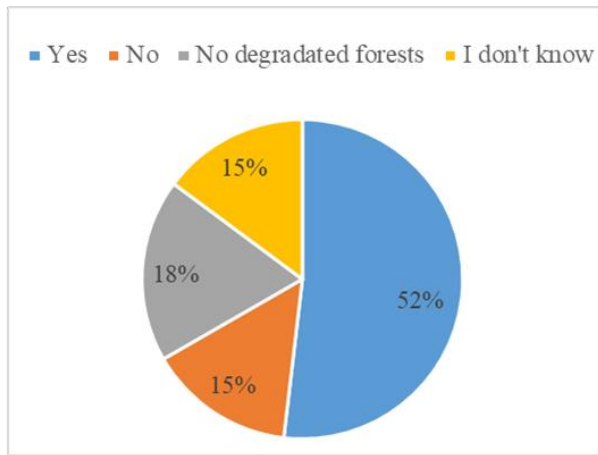


Fig 2. Planting in degraded forests

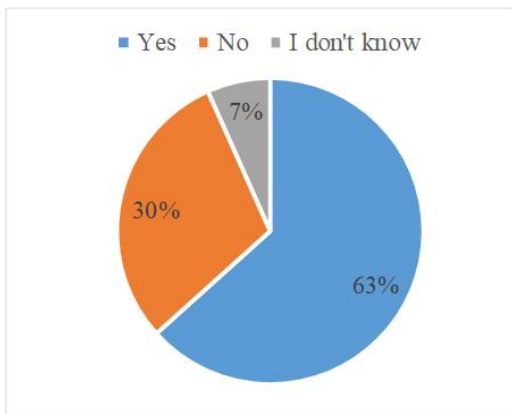


Fig. 3. Planting in coppice forests

Degraded forest ecosystems represented another frequent target for planting activities. More than half of the respondents (52%) reported implementing planting measures in degraded forests. However, responses also indicated considerable variability among management units, with some respondents reporting the absence of degraded forests or the lack of such activities within their areas of responsibility (Fig. 2).

The conversion of coppice forests into more productive and ecologically valuable high forests remains an important silvicultural objective. Accordingly, 63% of respondents stated

that planting is regularly used in coppice stands, while 30% reported that such practices are not implemented within their management units (Fig. 3).

Overall, the results demonstrate that forestry practitioners are engaged in a broad range of forest establishment and restoration activities, including afforestation of non-forested land, rehabilitation of degraded forest ecosystems, support of natural regeneration, and conversion of coppice forests.

3.4. Use of FRM

A considerable diversity of tree species is used in afforestation and reforestation activities. Respondents identified twelve commonly planted tree species, while some provided broader answers such as "conifers". Considering the current nursery production structure in Bosnia and Herzegovina, this category mainly refers to Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), Austrian pine (*Pinus nigra*), and silver fir (*Abies alba*).

The species most frequently mentioned by respondents was black poplar (*Populus nigra*), reflecting the fact that many respondents work in forest management units located in the northern part of Bosnia and Herzegovina, where large river systems dominate the landscape. Norway spruce and Scots pine together accounted for 31% of all responses, while broadleaved species such as maples (*Acer* spp.), oaks (*Quercus* spp.), and European beech (*Fagus sylvatica*) also represented an important component of afforestation programs (Fig. 4).

Several respondents highlighted species currently facing significant threats. Particular attention was given to narrow-leaved ash (*Fraxinus angustifolia*), which is increasingly affected by ash dieback caused by *Hymenoscyphus fraxineus* and by root diseases. Respondents expressed concern that this species may experience a decline similar to that observed in elm populations affected by Dutch elm disease caused by *Ophiostoma novo-ulmi*.

Regarding the type of FRM used in practice, most respondents (63%) reported using both bare-root and containerized seedlings. Only 22% relied exclusively on containerized planting stock, while 15% used only bare-root seedlings (Fig. 5). When asked about their understanding of nursery production processes, most respondents indicated that they were only partially familiar with the methods used for producing FRM (Fig. 6).

3.5. Knowledge transfer and capacity building

Vegetative propagation represents an important method for producing FRM and is indispensable for certain tree species and breeding objectives. Respondents were therefore asked about their familiarity with different vegetative propagation techniques.

The results revealed that forestry practitioners were most familiar with propagation by cuttings (54%), followed by grafting (22%). In contrast, only 5% of respondents reported familiarity with modern *in vitro* propagation techniques, including tissue culture and micropropagation (Fig. 7).

Nevertheless, when asked whether they had heard of the term "*in vitro*", 73.33% responded positively. Familiarity with the term "micropropagation" was substantially lower. More than half of respondents (53.33%) had never heard the term, while 81.50% reported that they did not know its exact meaning or practical application.

Respondents were also asked to identify tree species that can be propagated vegetatively. Multiple responses were permitted. Black poplar (*Populus nigra*) was the most frequently

mentioned species (33.3%), followed by willow (*Salix* spp.) (13.3%). Other species included forest fruit trees, black locust (*Robinia pseudoacacia*), and Norway spruce (*Picea abies*) (Fig. 8).

When asked about the advantages of vegetative propagation technologies, responses indicated a generally low level of knowledge. Approximately 37% of respondents believed that such methods offer specific advantages, whereas 30% stated that they were unfamiliar with potential benefits. A further 33% were uncertain. Despite this limited knowledge, respondents demonstrated a relatively positive attitude toward the use of planting stock produced through advanced propagation techniques. Approximately 40% stated that they would use such seedlings in operational forestry, while one quarter indicated that they would not. The remaining one-third of respondents were uncertain.

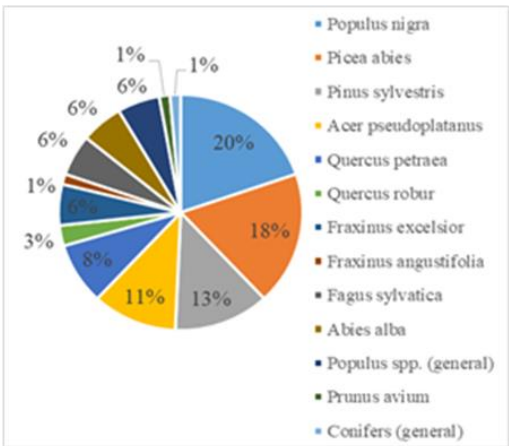


Fig 4. Share of species which FE used for planting

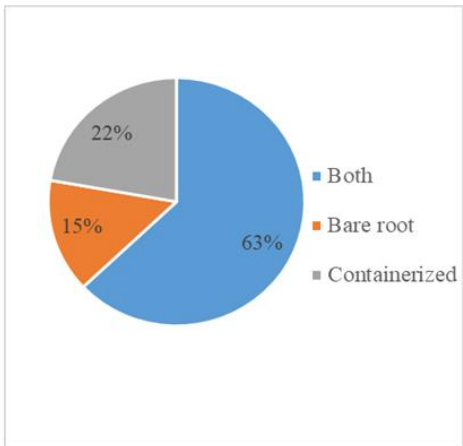


Fig. 5. Share of type of FRM

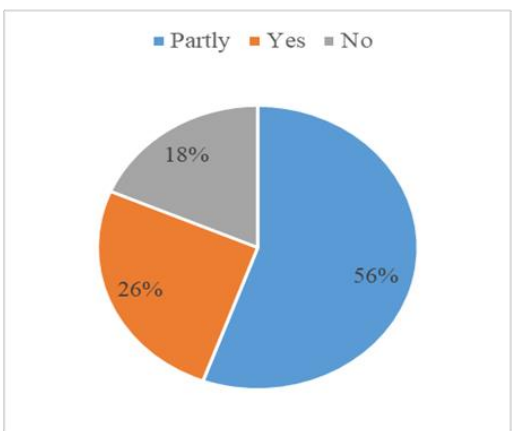


Fig. 6. Familiarity with FRM production

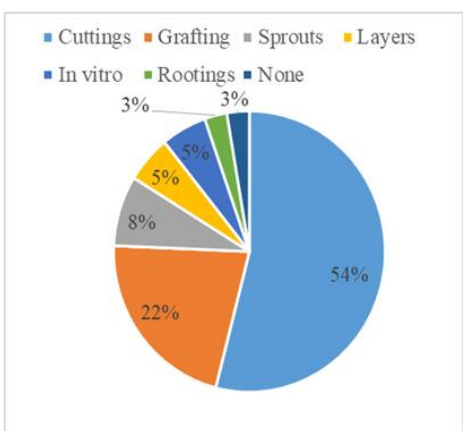


Fig. 7. Type for FRM familiar to forest practicioners

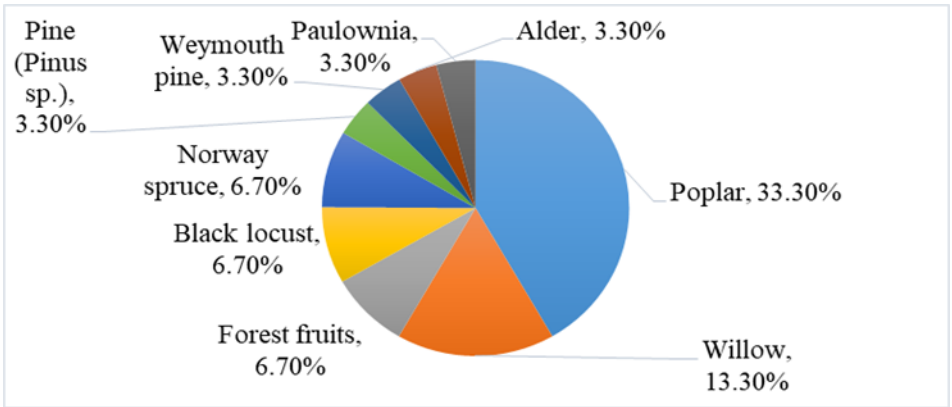


Fig. 8. The species which forest practitioners find possible to be reproduced using specific

Concerning the possible advantages of aforementioned methods of FMR production, forest practitioners in be almost same percentage stated that they are (37%), they are not familiar with the advantages which this kind of reproduction of FRM could provide (30%) or they are not sure (33%). If they would have an opportunity to plant seedlings produced in some techniques which are mentioned, 40% of forest practitioners would use those seedlings, one quarter of respondents would not use those seedlings. The high percentage is also not sure, one third of surveyed forest practitioners.

3.6. Knowledge of vegetative propagation technologies

Given the limited familiarity with advanced FRM production technologies, respondents were asked to identify the most appropriate methods for acquiring relevant knowledge and skills. Multiple answers were permitted. Seminars were identified as the preferred knowledge-transfer mechanism by 69% of respondents. Internet-based resources and professional literature were each selected by 14% of respondents (Fig. 9). Regarding online learning resources, 75% expressed a preference for specialized websites, while 25% favored video-based educational content (Fig. 10). In terms of face-to-face education, practical training sessions involving direct handling and evaluation of planting stock were preferred by 67% of respondents (Fig. 11). Among respondents who selected literature as a learning tool, two-thirds (66.7%) preferred textbooks and professional manuals, whereas one-third preferred scientific publications (Fig. 12). None of the surveyed practitioners reported being aware of television programs or similar media sources that provide information on advanced FRM production technologies.

3.7. Acceptance of advanced propagation technologies

The final section of the survey examined respondents' familiarity with producers using advanced propagation technologies and their willingness to utilize such planting material in practice.

None of the respondents reported being familiar with a producer of FRM generated through *in vitro* or other advanced propagation techniques. However, despite this lack of awareness, all respondents (100%) expressed an interest in testing and evaluating planting stock produced through modern propagation technologies in future afforestation and reforestation activities.

This result indicates a high level of openness among forestry practitioners toward technological innovations in FRM production, despite their currently limited knowledge and practical experience with these methods.

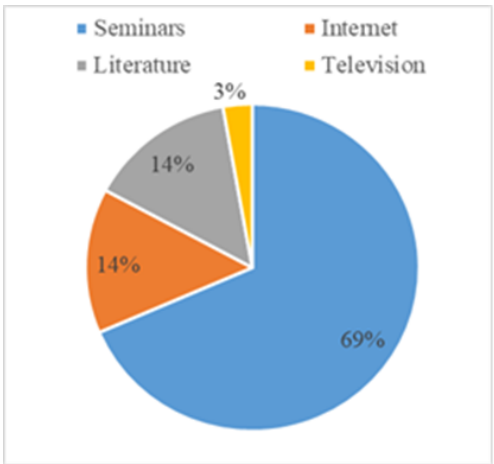


Fig. 9. The options for knowledge transfer to forest practitioners

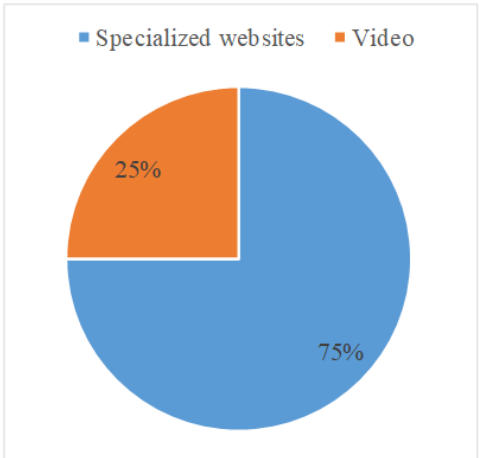


Fig. 10. The internet transfer knowledge share

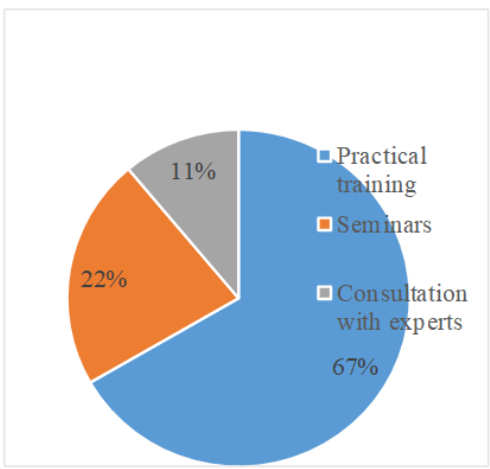


Fig. 11. Transfer of knowledge in person

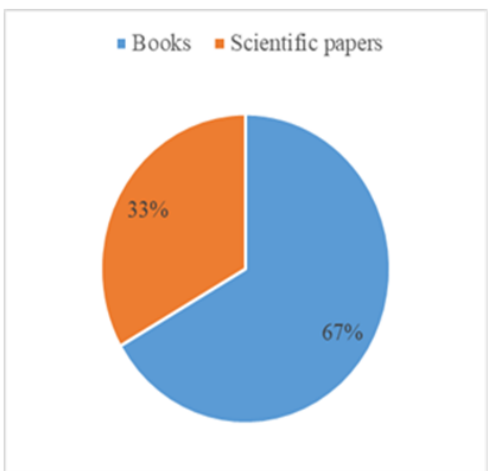


Fig. 12. Transfer of knowledge through literature

4. DISCUSSION

The successful implementation of afforestation and forest restoration programs depends not only on the availability of FRM, but also on the capacity of forestry professionals to adopt and utilize innovative production technologies. The results of this study indicate that forestry practitioners in Bosnia and Herzegovina are actively involved in a wide range of afforestation and reforestation activities, while their knowledge of modern vegetative

propagation technologies remains limited. At the same time, their willingness to adopt such technologies appears to be remarkably high.

The surveyed practitioners demonstrated a good understanding of global forest trends, particularly regarding the ongoing decline of forest area worldwide. This finding is consistent with recent international reports highlighting the continued loss of forest ecosystems (FAO, 2025). However, perceptions of forest area trends in Bosnia and Herzegovina were more heterogeneous. Such differences may reflect regional variability in forest management conditions.

The results further confirm that afforestation and reforestation remain important silvicultural activities in Bosnia and Herzegovina. Planting is not limited to afforestation of barren land but is also widely applied in degraded forests, coppice forests, and as a supplementary measure supporting natural regeneration. These findings support the observations of Stanturf *et al.* (2019), who emphasized that forest restoration encompasses a broad range of interventions adapted to specific ecological and management objectives. The diversity of planting situations identified in this study also suggests that future restoration efforts will require a broad spectrum of FRM types and production technologies.

A particularly important finding is the diversity of tree species currently used in afforestation programs. The predominance of species such as Norway spruce, Scots pine, black poplar, oaks, maples, and beech reflects both traditional silvicultural approaches and the ecological diversity of Bosnia and Herzegovina. However, respondents also highlighted concerns regarding species affected by pests and diseases, especially narrow-leaved ash (*Fraxinus angustifolia*). The increasing impact of ash dieback caused by *Hymenoscyphus fraxineus* and associated decline processes demonstrates the importance of developing alternative strategies for the conservation and propagation of valuable genetic resources. Similar challenges have been documented throughout Europe, where forest managers increasingly seek resistant genotypes and alternative propagation approaches for threatened tree species (Tikkinen *et al.*, 2021). One of factors which influence the decision of forest practitioners in afforestation/reforestation efforts is biodiversity loss when using clonal forestry which decrease biodiversity but increase genetic gain (Ivetic *et al.,* 2016). Nevertheless, severe impact of species such as e.g. *Picea omorika* or, lately species which tends to be very endangered *Fraxinus angustifolia* species where the reduction of seed production and quality is in stake, can promote willingness to use novel vegetative technologies to save species.

Despite the importance of high-quality planting stock, the results revealed that forestry practitioners possess only limited knowledge of vegetative propagation technologies. Most respondents were familiar with traditional methods such as cuttings and grafting, while only a very small proportion reported familiarity with *in vitro* propagation techniques. This finding is not surprising, considering that FRM production in Southeast Europe remains predominantly based on seed propagation (Mataruga *et al.,* 2023), whereas tissue culture and micropropagation technologies are still at an early stage of operational application (Sota *et al.,* 2025a; Sota *et al.,* 2025b). Nevertheless, the observed gap between awareness of the term "*in vitro*" and understanding of its practical applications suggests that knowledge transfer from research institutions to forestry practitioners remains insufficient.

The concept of overcoming the gap between scientific innovation and operational forestry has been emphasized in recent years (Aronen *et al.*, 2025). The present study provides evidence supporting this need. Although most respondents were unable to identify specific applications or advantages of advanced propagation technologies, a substantial proportion expressed interest in using planting material produced through such methods. More importantly, all respondents indicated that they would be willing to test FRM produced through *in vitro* propagation techniques in afforestation and reforestation projects. This finding represents one of the most significant outcomes of the study. It suggests that the primary barrier to the adoption of advanced propagation technologies is not resistance among practitioners but rather the lack of accessible information, practical experience, and technology transfer mechanisms.

The survey also provides insights into preferred approaches for professional education. Forestry practitioners clearly favored seminars and practical training over passive learning methods. Similar conclusions have been reported in studies addressing innovation adoption in forestry, where direct interaction with experts and hands-on experience are considered essential for building confidence in new technologies. The strong preference for practical demonstrations indicates that future technology-transfer programs should focus on field-based training, nursery visits, demonstration plots, and workshops that enable practitioners to directly evaluate the performance of planting stock produced using advanced propagation techniques.

The complete absence of awareness regarding producers of *in vitro*-derived FRM further illustrates the limited integration of modern propagation technologies into operational forestry in Bosnia and Herzegovina. This situation contrasts with developments in several European countries, where micropropagation is increasingly applied for the conservation of endangered species, clonal forestry, and the production of genetically superior planting stock (Tikkinen *et al.*, 2021). Given the increasing challenges posed by climate change, pests, diseases, and irregular seed production, reliance solely on conventional seed-based propagation may prove insufficient in the future.

The findings of this study should also be considered in the context of the Target Seedling Production concept (Landis, 2011; Haase & Davis, 2017), which emphasizes the production of planting stock tailored to specific site conditions and management objectives. Advanced propagation technologies, including micropropagation, somatic embryogenesis, and other biotechnological approaches, may significantly contribute to this objective by enabling the mass production of selected genotypes with desirable adaptive characteristics. Such technologies could become particularly important in regions facing increasing environmental uncertainty and the need for climate-resilient forest ecosystems.

Although the study was based on a relatively small sample size and focused on a specific group of forestry professionals, it provides one of the first insights into practitioners' perceptions of vegetative propagation technologies in Bosnia and Herzegovina. Future studies should include a broader range of stakeholders, including nursery managers, private forest owners, policy makers, and researchers, in order to obtain a more comprehensive understanding of opportunities and barriers associated with the adoption of advanced FRM production technologies.

Overall, the results indicate that while operational forestry in Bosnia and Herzegovina remains strongly oriented toward conventional seed-based production systems, there is considerable openness among practitioners toward innovative propagation methods. Strengthening cooperation between research institutions, forest nurseries, and forest management organizations may therefore represent a crucial step toward improving the availability, quality, and adaptability of FRM required for future afforestation and forest restoration efforts.

5. CONCLUSIONS

This study assessed the knowledge, attitudes, and willingness of forestry practitioners in Bosnia and Herzegovina to adopt modern vegetative propagation technologies for the production of forest reproductive material (FRM). The results provide several important conclusions.

Forestry practitioners are actively involved in a wide range of afforestation and reforestation activities, including the establishment of forests on non-forested land, rehabilitation of degraded forests, support of natural regeneration, and conversion of coppice stands. These activities require a continuous supply of high-quality FRM adapted to increasingly challenging environmental conditions. The surveyed practitioners demonstrated a good understanding of the importance of afforestation and global forest trends. However, their knowledge of advanced vegetative propagation technologies, particularly *in vitro* propagation and micropropagation, was generally limited. Traditional methods such as propagation by cuttings and grafting were considerably better known and more widely recognized.

Despite this limited knowledge, respondents expressed a highly positive attitude toward technological innovation in FRM production. Although none of the surveyed practitioners were familiar with producers of FRM generated through advanced propagation technologies, all respondents indicated their willingness to test and use such planting material in future afforestation and reforestation programs. The findings also revealed a strong demand for professional training and technology-transfer activities. Seminars, practical demonstrations, and field-based training were identified as the most effective approaches for improving knowledge and facilitating the adoption of innovative propagation methods.

Given the increasing impacts of climate change, irregular seed production, emerging pests and diseases, and the growing need for climate-resilient forests, advanced vegetative propagation technologies may become an important complement to conventional seed-based production systems. Strengthening cooperation among research institutions, forest nurseries, and forest management organizations will be essential for transferring scientific advances into operational forestry practice.

Overall, the study demonstrates that the main obstacle to the wider application of advanced propagation technologies in Bosnia and Herzegovina is not the reluctance of forestry practitioners, but rather the limited availability of information, training opportunities, and practical experience. Addressing these gaps could significantly enhance the production of high-quality FRM and support future afforestation and forest restoration initiatives.

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Mapping the CopyTree ecosystem: A complexity science approach to knowledge sharing in woody plant biotechnology

Buhara Yücesan

Department of Seed Science and Technology, Faculty of Agriculture, Bolu Abant Izzet Baysal University, 14030 Bolu, Türkiye

buhara@ibu.edu.tr

Abstract

Woody plant biotechnology is a distributed research field in which knowledge is produced by many small groups working on different species, methods and objectives. Mapping how these efforts connect is difficult with citation-based bibliometrics, because much of the activity appears first in conference abstracts rather than in indexed journals. This study applies network analysis to the abstract books of three consecutive conferences of COST Action CA21157 (CopyTree), held in Latvia (2024), Portugal (2025) and Albania (2026). In total, 247 records (19 + 91 + 137) were classified in Microsoft Excel by year, country, institution, plant species, research method, research objective, application area, plant group, commercial level and working group. Power Query was used to convert the record-based table into relational node and edge datasets, which were then analyzed in Gephi 0.10.1. Edge weights represent the number of records in which two entities occurred together within a given year. The combined network contained 490 nodes connected by 3,098 directed edges. Graph density was low (0.013), but the average path length was short (2.24) and the diameter was six, describing a sparse yet efficiently connected structure. Micropropagation, somatic embryogenesis and protocol optimization emerged as the recurrent attractors around which the network organizes, while *Castanea sativa*, *Picea abies*, *Vitis vinifera* and *Prunus* species carried the strongest weighted relationships. The division between forest trees and fruit crops reappeared at every level of the network, from institutional specialization to commercial level, showing a self-similar pattern. Reading an Action's own output as a network offers a practical way to see where its knowledge concentrates and where it dissolves.

Keywords: *Network analysis, Complexity science, Knowledge sharing, Woody plants, Gephi*

1. INTRODUCTION

Woody plants supply timber, fiber, fruit and a wide range of high-value products, and their propagation through *in vitro* culture has grown into a research field of its own. Progress in this field is slow and uneven. Species differ widely in how readily they regenerate, protocols rarely transfer between laboratories without modification, and the cost of scaling up remains high (Correia *et al.*, 2026; Sota *et al.*, 2025). COST Action CA21157, known as CopyTree (European Network for Innovative Woody Plant Cloning, 2022-2026), was established to reduce this fragmentation across Europe by harmonizing protocols, sharing knowledge and connecting academic groups with industry (Yücesan *et al.*, 2026). Over four years the Action brought together several hundred contributions in its annual conferences, yet what that body of work looks like as a whole has not been examined.

The usual way to describe a research field quantitatively is bibliometrics, a term introduced by Pritchard (1969) for the statistical study of publications. Co-citation analysis (Small 1973) and, later, mapping software such as VOSviewer (van Eck and Waltman 2010) made

it possible to describe large literatures through keyword co-occurrence, co-authorship and citation frequency. Plant tissue culture has been described in this way several times recently: Negi *et al.* (2024) covered ninety years of the field, Regni *et al.* (2025) focused on blackberry micropropagation, Poenaru *et al.* (2025) on automation and artificial intelligence in horticulture, and Negi *et al.* (2025) on hairy root culture. These studies give a reliable picture of publication growth, leading authors and dominant countries. What they do not show is how the parts of a field hold together: which topics act as bridges, which groups sit at the edge, and how the structure changes from one year to the next.

Network analysis answers a different kind of question, because it treats relationships rather than documents as the unit of analysis. Its origins go back to the sociograms of Moreno (1934) and to the centrality measures formalized by Freeman (1977). Systems thinking after 1950 provided a framework for studying patterns and interactions inside complex structures (McCarthy *et al.,* 2000), and work on cybernetics, fractal geometry, self-organizing systems, non-linear dynamics and chaos theory from the 1970s onward laid the ground for modern network science (Lorenz 1963; Waldrop 1992). The field acquired its present shape with the small-world (Watts and Strogatz 1998) and scale-free (Barabási and Albert 1999) models, which showed that very different real systems share the same structural signatures: short paths between any two elements, and a small number of highly connected hubs. Complexity science now informs research in health, policy and several interdisciplinary areas (Hossain *et al.,* 2020), and it offers a vocabulary of attractors, self-organization and self-similarity that becomes useful once a system is too large to follow case by case. Meta-analysis, introduced by Glass (1976), developed in parallel as a way of synthesizing results across many individual studies. All three approaches describe a field from above, but only network analysis keeps the relational detail of who works with whom, on what, and by which method.

Applying this to a funded research network raises a practical difficulty. Conference abstracts are not indexed and carry no citations, so citation-based tools do not apply to them. They do, however, describe what a community is working on at the moment of reporting rather than two or three years later, and for an Action with a fixed lifetime they are the most complete record available. A recent citation-network study of the plant tissue culture literature showed that treating keywords as thematic attractors reveals structure that conventional bibliometric mapping misses (Yücesan 2026). The present study takes the same approach one level down, to the output of a single research network. The aim was to map the CopyTree ecosystem across its three conference years as one weighted network, using the seven bilateral relationships listed in Table 1, and to read its structure through the lens of complexity science.

2. MATERIALS AND METHODS

2.1. Data collection and classification of scientific records

The dataset was built from the abstract books of three CopyTree conferences: Jūrmala, Latvia (2024), Coimbra, Portugal (2025) and Tirana, Albania (2026) (CopyTree 2024, 2025, 2026). Each record was read individually and classified in a structured framework developed in Microsoft Excel 2024, using eleven variables: year, country, institution, plant species, research method, research objective, application area, plant group, commercial level, working

group and institutional collaboration. The 247 contributions published across the three books (19 from Jūrmala, Latvia; 91 from Coimbra, Portugal; and 137 from Tirana, Albania) were screened for inclusion. Records from which no plant species could be identified, mainly keynote lectures, software and business-model presentations and communication posters, were left out, since a species-centered network cannot represent them.

Each variable described a different aspect of the reported work. “Plant Species” identified the main taxon investigated and “Plant Group” its broader biological or production category. “Research Method” recorded the principal methodological approach and “Research Objective” the research focus. “Application Area” placed the study in a wider context such as forestry, fruit production or plant propagation, while “Commercial Level” distinguished the reported level of practical relevance as academic, potential or commercial. “Working Group” recorded the relevant CopyTree working group, and “Collaboration” the joint institutional authorship. Where a record was relevant to more than one category, multiple assignments were preferred over forcing it into a single class, which preserves both the interdisciplinary character of the contributions and the relational detail needed for network construction. Terminology was standardized during data entry, so that names differing only in spelling or abbreviation would resolve to a single node.

2.2. Data transformation and construction of the network datasets

Microsoft Power Query was used to convert the record-based table into relational datasets. The workflow imported the source data, standardized headers and data types, cleaned text values, separated multiple categorical entries, and produced a separate relational table for each analytical category. Nine such tables were generated: University-Plant Species, Plant Species-Method, Plant Species-Objective, Plant Species-Application, Plant Species-Plant Group, Plant Species-Commercial Level, University-Country, University-Working Group and institution-institution collaboration.

The network was then built from two complementary datasets. The node dataset held the unique entities identified across all categories, including institutions, plant species, methods, objectives, application areas, plant groups, countries, working groups and commercial levels. The edge dataset held the relationships between them, each edge consisting of a source, a target, an edge type recording its analytical category, a year and a weight. The weight is the number of records in which a given relationship occurred within a given year. For example, the weight of a University-Plant Species edge is the number of records in which the same university and the same plant species appeared together in that year, and the remaining combinations were treated in the same way (Table 1).

2.3. Network analysis and visualization using Gephi

Both datasets were exported as comma-separated values (CSV) files and imported into Gephi 0.10.1 (Bastian *et al.,* 2009) to construct a directed and weighted network. ForceAtlas 2 (Jacomy *et al.,* 2014) was used as the main layout, with alternative layouts applied to individual subnetworks where they gave a clearer reading. Subnetworks for each relationship type and each conference year were obtained with the attribute filters available in Gephi, and the network statistics were recalculated for each filtered graph. Community structure was assessed with the modularity algorithm implemented in Gephi (Blondel *et al.,* 2008), with edge weights taken into account and at the default resolution of 1.0. The metrics obtained for the combined network are reported with their interpretation in Table 2.

3. RESULTS

3.1. Overall network structure: three-year static analysis

The 2026 Albania layer dominates the combined network, accounting for 57.5% of all elements, while the Latvia and Portugal meetings cover the remainder. Roughly six out of every ten elements in the network therefore entered it in the final conference year. Taken together, the three years produced 490 nodes connected by 3,098 directed edges. Although the graph density was low (0.013), the short average path length (2.24) and the network diameter of six indicate that the network maintains an efficient structural connectivity. The average weighted degree (9.94) was higher than the average degree (6.32), which points to recurrent and stronger associations among specific entities (Table 2 and Figure 1).

Table 1. Investigated edge relationships and the strongest observed pair in each of them

Edge relationship	Strongest observed pair (edge weight)[a]
University → Plant Species	Misión Biológica de Galicia - *Castanea sativa* (13)
Plant Species → Method	*Picea abies* - Somatic embryogenesis (11)
Plant Species → Objective	*Castanea sativa* - Protocol optimization (7)
Plant Species → Application	*Picea abies* - Forestry (11)
Plant Species → Plant Group	*Castanea sativa* - Forest tree (17)
Plant Species → Commercial Level	*Castanea sativa* - Potential (16)
University → Collaboration	Misión Biológica de Galicia - Universidade de Santiago (5)

[a] *Edge weight is the number of records in which the two entities occurred together within a single conference year.*

Table 2. Network-level metrics of the combined 2024-2026 network

Network metric	Value	Brief interpretation
Number of nodes	**490**	Unique entities across all analytical categories
Number of directed edges	**3,098**	Directional associations derived from the classified records
Average degree	**6.3**	Each node is connected to about six others
Average weighted degree	**9.9**	Connection strength rises to about ten once repeated relationships are counted
Network diameter	**6**	Longest shortest path between two connected nodes
Graph density	**0.013**	Only 1.3% of possible connections are present; sparse but structured
Average path length	**2.2**	Two connected nodes are about two steps apart on average
Connected components	**3**	One dominant structure plus a small number of isolated entries

Average clustering coefficient	**0.09**	Limited local clustering; nodes connect across categories rather than within them
Modularity	**0.404**	Moderate community structure; the network separates into recognizable groups that remain interlinked
Number of communities	**50**	Fine-grained partition, with a mean community size of about ten nodes

3.2. Weighted relationships among research entities

University - Plant Species

After the leading pair given in Table 1, the CEBAS-CSIC - *Prunus armeniaca* relationship followed with a weight of 7. Other notable connections, with weights of 5 to 6, included Misión Biológica de Galicia-*Quercus ilex*, Institute of Experimental Botany-*Picea abies*, University of Coimbra-*Solanum betaceum*, NEIKER-*Pinus radiata*, Natural Resources Institute Finland-*Picea abies*, and Humboldt-Universität zu Berlin-*Larix* × *eurolepis* (Figure 1).

Plant Species - Method

Vitis vinifera - Micropropagation and *Castanea sativa* - Somatic embryogenesis followed the leading pair, each with a weight of 7. Other relatively high-weight relationships included *Prunus domestica*-Temporary immersion system (6), *Larix* × *eurolepis*-Somatic embryogenesis (6) and *Vitis vinifera*-Monitoring (6). Micropropagation and somatic embryogenesis were the most recurrent methodological categories and were associated with several woody and fruit species. The distribution of edge weights showed that particular methods were repeatedly associated with specific species, while some species were represented by multiple methodological approaches (Figure 2).

Plant Species - Application

Prunus domestica - Fruit production and *Vitis vinifera* - Fruit production both reached a weight of 9, followed by *Prunus armeniaca* - Fruit production with 8. Forestry-related connections were also observed for *Pinus radiata* (7), *Quercus ilex* (6) and *Larix* × *eurolepis* (6). *Prunus domestica* was connected to both fruit production (9) and plant propagation (3), while *Myrtus communis* and *Solanum betaceum* each showed plant propagation relationships with weights of 4. Forestry and fruit production were therefore the most frequent application categories among the higher-weight relationships (Figure 2).

Plant Species - Objective

Picea abies - Protocol optimization, *Prunus domestica* - Protocol optimization and *Vitis vinifera* - Genetic characterization each carried a weight of 6, and *Quercus ilex* - Protocol optimization and *Castanea sativa* - Stress tolerance was represented by 5 records each. Several species were associated with multiple objectives. In *Picea abies* these included hormone metabolism, mass propagation and protocol optimization, while *Castanea sativa* was associated with clonal production, gene discovery, genome editing, stress tolerance and protocol optimization (Figure 3).

Plant Species - Plant Group

Vitis vinifera - Fruit crop, *Picea abies* - Forest tree and *Prunus domestica* - Fruit crop each carried a weight of 11, *Prunus armeniaca* - Fruit crop a weight of 9, and *Pinus radiata* -

Forest tree 7 records. This layer was dominated by two plant groups. *Castanea sativa*, *Picea abies*, *Pinus radiata*, *Quercus ilex* and *Larix* × *eurolepis* were predominantly associated with Forest tree, whereas *Vitis vinifera*, *Prunus domestica*, *Prunus armeniaca*, *Malus domestica* and *Pistacia vera* fell mainly within Fruit crop (Figure 3).

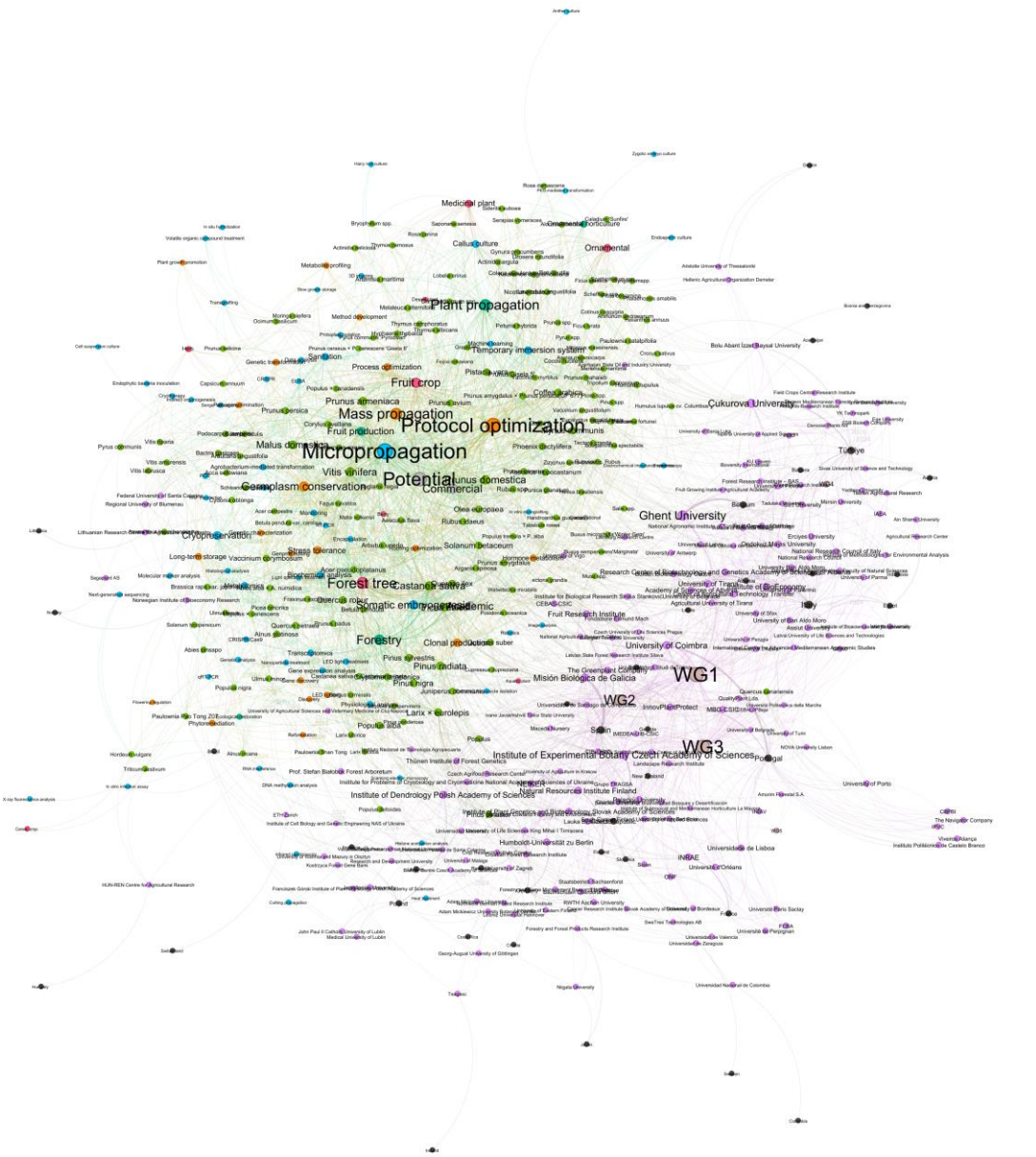


Figure 1. The combined CopyTree network for 2024-2026, drawn with the ForceAtlas 2 layout in Gephi®. Nodes are the unique entities recorded across all analytical categories and edges are their co-occurrences within a conference year, weighted by the number of records. Node size reflects weighted degree and color distinguishes the analytical categories.

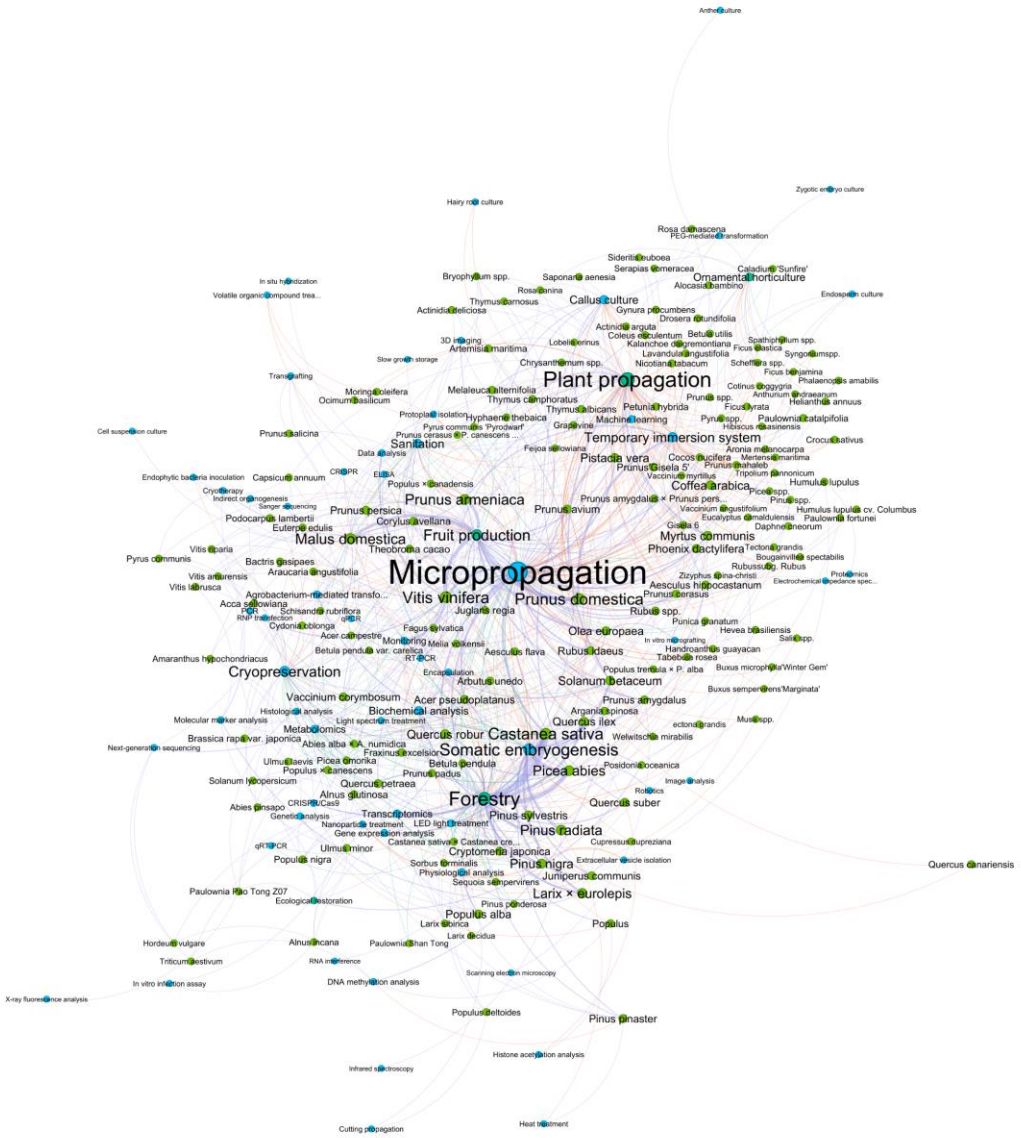


Figure 2. The Plant Species-Method--Application layers of the network. Edge weight is the number of records in which a species occurred together with a given method or application category within a conference year with edges (2024 in green, 2025 in orange and 2026 in violet). Micropropagation is the most widely shared method and connects to species from every plant group, whereas somatic embryogenesis concentrates on conifers and on species propagated for mass clonal production. Temporary immersion systems and cryopreservation appear as smaller and more specialized hubs.

University - Collaboration

Three institutional pairs shared the highest weight of 5: Misión Biológica de Galicia-Universidade de Santiago, Misión Biológica de Galicia-The Greenplant Company, and Research Center of Biotechnology-University of Tirana. Weights of 4 were recorded for

Czech Agrifood Research Center-Institute for Problems of Cryobiology and for Lauka Biotech Ltd-Natural Resources Institute Finland. Further collaborations with a weight of 3 included Assiut University - University of Bari Aldo Moro, Institute of BioEconomy - Research Center of Biotechnology, Bolu Abant İzzet Baysal University-Çukurova University, and Ghent University-National Agronomic Institute. Most collaboration weights ranged between 2 and 5, with only a limited number of pairs recurring across multiple records (Figure 4).

Plant Species-Commercial Level

Prunus domestica - Potential (12), *Prunus armeniaca* - Potential (11) and *Picea abies* - Potential (10) followed the leading pair, and *Castanea sativa* - Academic reached a weight of 9. Potential - level relationships were also observed for *Olea europaea* (7), *Vitis vinifera* (6) and *Pistacia vera* (6). Commercial - level relationships were recorded for *Picea abies* (6), *Vitis vinifera* (6), *Pinus radiata* (5) and *Prunus cerasus* (5), while the Academic category carried relatively high weights for *Castanea sativa* (9), *Solanum betaceum* (8), *Pinus nigra* (4) and *Betula pendula* (4). Potential was the most frequent commercial-level category among the higher - weight relationships, although both Academic and Commercial were represented for several species such as *Castanea sativa*, indicating variation in the recorded development level across the dataset (Figure 5).

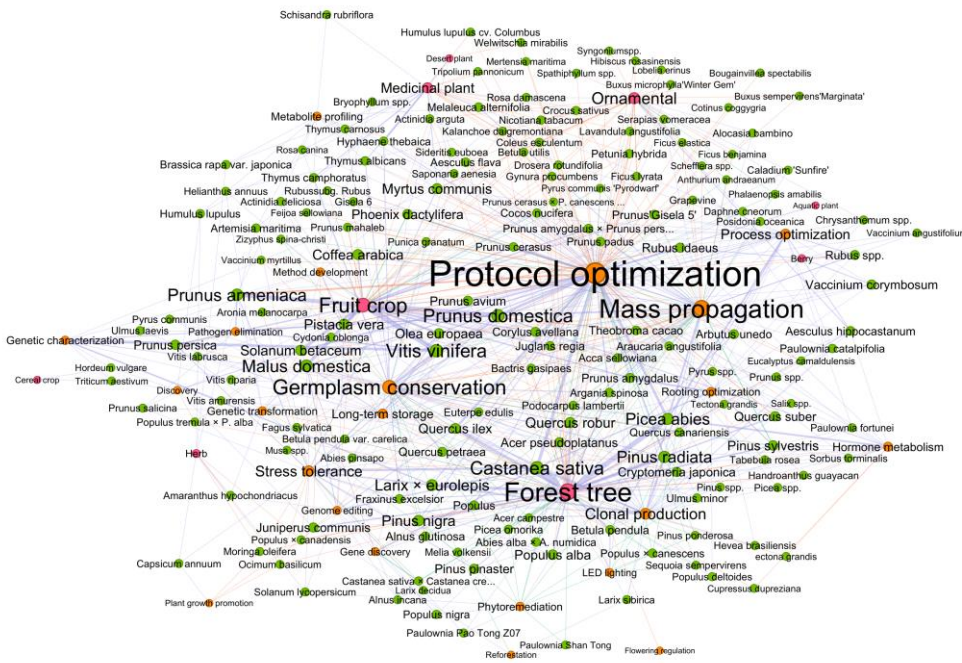


Figure 3. The Plant Species-Objective-Plant Group layers. Protocol optimization is the dominant research objective across the three years and is the strongest objective for *Castanea sativa*, *Picea abies* and *Prunus domestica* alike, followed by mass propagation and germplasm conservation. The two plant-group nodes, Forest tree and Fruit crop, divide the species into the two blocks that reappear at every other level of the network (edges of 2024 in green, 2025 in orange and 2026 in violet).



Figure 4. The University-Collaboration layer, the only one-mode part of the network, in which both source and target are institutions. Edge weight is the number of records representing the same institutional partnership within a conference year (edges of 2024 in green, 2025 in orange and 2026 in violet). Most pairs are connected by two to five shared records; Misión Biológica de Galicia, Ghent University and the Institute of Experimental Botany of the Czech Academy of Sciences occupy the denser part of the structure, and commercial partners appear alongside research institutes.



Figure 5. The Plant Species-Commercial Level layer, showing how species distribute across the three recorded levels of practical relevance (edges of 2024 in green, 2025 in orange and 2026 in violet). Potential is the most frequent category throughout, well ahead of Commercial and Academic, and the distance between the Potential and Commercial nodes indicates how much of the reported work has not yet reached the market.

4. DISCUSSION

The combined network is sparse, with only 1.3% of the theoretically possible connections present. Taken alone, that figure would suggest a loosely connected field, but the other metrics indicate otherwise. An average path length of 2.24 and a diameter of six mean that almost any two entities are separated by two or three steps. This is the signature of a small-world structure (Watts and Strogatz 1998), and it appears here for a structural reason: the design places University and Plant Species as the two hubs to which the remaining categories attach, so any method, objective or application is reachable through a species, and any species through an institution. The low clustering coefficient (0.09) should be read in the same light. In a multi-partite network of this kind, triangles can only form where categories of different types happen to intersect, so a low value is expected and is not in itself a sign of fragmentation. The more informative figure is the gap between the average degree (6.32) and the average weighted degree (9.94): roughly a third of the connection strength comes from repetition, meaning that a limited set of pairs is revisited year after year. In the vocabulary of complexity science these recurring pairs behave as attractors, since the system keeps returning to them (Yücesan 2026).

The University - Plant Species layer shows that institutional identity in this field is tied to species rather than to technique. Groups with high weighted degrees work on a narrow set of species over several years, which is consistent with the way woody plant protocols develop. Regeneration competence is strongly genotype-dependent, and moving a working protocol to a new species usually means starting the optimization cycle again (Correia *et al.*, 2026). The same pattern explains why the harmonization objective of the Action is difficult to reach through protocol transfer alone (Sota *et al.*, 2025).

In the Plant Species - Method layer, micropropagation and somatic embryogenesis are the two recurrent methodological categories, but they behave differently within the network. Micropropagation is spread thinly across many species, whereas somatic embryogenesis concentrates on conifers and on species where mass clonal production is the goal (Aronen *et al.,* 2026). Temporary immersion systems appear with lower weights and on fewer species, which is what would be expected of a technique still moving from research into production (Vidal *et al.,* 2026). A comparable three-way division between a mature general technique, a specialized high-value technique and an emerging one was reported for the indexed literature on plant tissue culture (Yücesan 2026). Finding it again in a conference corpus that shares almost no records with that dataset suggests the pattern belongs to the field itself rather than to a particular database.

Protocol optimization is the dominant research objective, carrying the highest weight for *Castanea sativa*, *Picea abies* and *Prunus domestica* alike, with genetic characterization following mainly through *Vitis vinifera*. This distribution deserves attention. After several decades of woody plant tissue culture, a large share of current work is still concerned with making protocols function rather than with applying them. Recalcitrance remains the practical bottleneck (Correia *et al.,* 2026), and objectives further along the translation path, such as long-term storage, stress tolerance and hormone metabolism, carry lower weights. The picture is not one of a field advancing uniformly toward application, but one in which each new species restarts the protocol cycle.

The Application and Plant Group layers give the clearest structural result of the study. Across both, the network divides into two blocks: forest trees studied for forestry, and fruit crops studied for fruit production. The same division reappears in the Commercial Level layer and, through institutional specialization, in the University-Plant Species layer. A pattern that repeats at several levels of description is what complexity science calls self-similarity, and it has a straightforward reading here. CopyTree is not one community but two overlapping ones, held together by shared methods rather than by shared species. *Castanea sativa* is the clearest exception to the division, since it carries high weights in both the academic and the potential categories and is one of the few species where forestry and fruit interests meet.

Modularity adds a second scale to this reading. The network resolves into 50 communities with a modularity of 0.404, indicating real but moderate community structure. These communities are small, with a mean size of about ten nodes, the size to be expected of an institution together with the species and methods attached to it. The forest tree and fruit crop division is therefore not what the algorithm detects; it becomes visible only once those small modules are aggregated by application and plant group. Structure appearing at two scales at once is the self-similarity that complexity science describes, and it is a more informative outcome than a clean partition into two halves would have been. A citation network of the indexed plant tissue culture literature returned a modularity of 0.49 over a much smaller node set (Yücesan 2026).

The collaboration layer is the only one-mode part of the network, and its weights are lower than elsewhere, with most institutional pairs connected by two to five shared records. Misión Biológica de Galicia appears in several of the strongest pairs, including one with a commercial partner. The presence of company nodes such as The Greenplant Company and Lauka Biotech Ltd alongside research institutes is a small but meaningful signal, because

the distance between academic output and industrial uptake is the recurring difficulty in this sector (Sota *et al.*, 2025; Kreimech and Tikkinen 2026). The Research Center of Biotechnology - University of Tirana pair, and the several Albanian institutions entering the network in 2026, also show the effect a conference venue has on who takes part. This is a limitation of abstract-based data and, at the same time, a finding: hosting an event brings local groups into a network in which they were not previously visible. Weak ties of this kind are the usual route by which new information enters a research community (Granovetter 1973).

The commercial-level layer is where the translation gap shows most clearly. Potential is the dominant category throughout, led by *Castanea sativa*, *Prunus domestica*, *Prunus armeniaca* and *Picea abies*, while commercial weights remain between five and six. Recorded commercial activity in European woody micropropagation is concentrated in Italy, Spain, Türkiye and Germany (Sota *et al.*, 2025), and the broader regional picture shows the same imbalance between research capacity and industrial infrastructure (Yücesan *et al.*, 2026). The network therefore describes intent rather than production: it shows where the community believes commercial relevance lies, not where plants are actually being sold. Read that way, Potential is the most honest label in the dataset, and the distance between Potential and Commercial is a measure of how much of the Action's work has not yet reached the market.

Compared with citation-based bibliometrics, this approach loses impact weighting and gains immediacy. No abstract carries a citation count, so nothing reported here measures influence. What the method captures instead is the state of a research community at the moment of reporting, two to three years before the same work reaches indexed journals, which for an Action with a fixed lifetime matters more than citation weight. It also produces something conventional bibliometric mapping does not, namely a relational structure in which the analytical categories are themselves nodes, so that a method, an objective and a commercial level can be compared within a single graph (Newman 2001).

Several limitations should be stated. Abstracts are self-reported and uneven in detail, and the classification depends on the judgement of the reader. Contributions without an identifiable plant species, such as keynote lectures, software presentations, business-model analyses and communication posters, cannot enter a species-centered network, which leaves the working groups dealing with legislation, commercialization and public perception under-represented. The 2024 and 2025 layers also carry duplicated edges inherited from the original data files, which slightly inflates the cumulative weights without altering their rank order. Finally, the digital visibility dimension proposed in the original abstract of this contribution, that is the resonance of this work outside academic channels, was not quantified here. Combining the present structural map with a visibility measure drawn from repositories, professional platforms and the Action's own digital outputs would be the natural next step, and would allow the same network to be read for reach as well as for content.

5. CONCLUSIONS

Reading three years of CopyTree conference abstracts as a single weighted network gives a picture that neither the abstract books nor a conventional bibliometric count would provide. The output of the Action is sparse but well connected, with short paths between almost any

two entities and a clear set of recurring pairs that hold the structure together. Micropropagation, somatic embryogenesis and protocol optimization act as the attractors of this system, while *Castanea sativa*, *Picea abies*, *Vitis vinifera* and the *Prunus* species carry the strongest relationships. The division between forest trees and fruit crops repeats at every level examined, from institutional specialization to commercial level, which points to two communities working with a shared methodological toolbox rather than to one uniform field.

Two open questions follow. The first is whether the concentration of effort on protocol optimization will shift once the Action ends, or whether each new species will continue to restart the cycle. The second is how far the Potential category can be moved toward Commercial, which depends less on laboratory results than on infrastructure, certification and cost. Mapping the same network again after the Action closes would answer part of both.

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