# The 2nd conference of Cost Action CA21157 COPYTREE

## IN VITRO CULTURE OF WOODY CROPS: PROBLEM SOLVING BY NEW APPROACHES

## Book of Proceedings

22 - 24 April, 2024
Bulduri Technical School,
Jūrmala, Latvia

cost
EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

Bulduru Tehnikums

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# Cost Action CA21157 - COPYTREE

# IN VITRO CULTURE OF WOODY CROPS: PROBLEM SOLVING BY NEW APPROACHES

**Organized by**

**Bulduri Technical School, Jūrmala, Latvia**

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**The 2nd Conference of Cost Action CA21157 - COPYTREE:**
***In Vitro* Culture of Woody Crops: Problem Solving by New Approaches**

**Book editing and layout:** Assoc. 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

- **Stefaan Werbrouck –** Ghent University, Belgium; *Chair of CopyTree*
- **Maurizio Lambardi -** National Research Council (CNR), IBE-Institute of BioEconomy, Italy; *Vice-Chair of CopyTree*
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- **Itziar Aurora Montalbán -** NEIKER-BRTA, Spain; *Vice-Leader of WG1*
- **Elif Aylin Ozudogru -** İstinye University Istanbul, Turkey; *Leader of WG2*
- **Claudia Ruta -** University of Bari, Italy; *Vice-Leader of WG2*
- **Nieves Vidal -** Misión Biológica de Galicia (MBG-CSIC), Spain; *Leader of WG3*
- **Liva Purmale -** Bulduri Technical School, Latvia; *Vice-Leader of WG3*
- **Neslihan Yeşim Yalçin Mendi -** Cukurova University, Turkey; *Leader of WG4*
- **Valbona Sota -** University of Tirana, Albania; *Leader of WG5 & Science Communication Coordinator of CopyTree*
- **Branislav Cvjetkovic** - University of Banja Luka, Bosnia and Herzegovina; *Vice-Leader of WG5*
- **Dicle Dönmez** - Cukurova University, Turkey; *Scientific Innovation Network Coordinator of CopyTree.*

## LOCAL ORGANIZING COMMITTEE

**Līva Purmale[1,2]; Rafaels Joffe[2]; Anna Korica[1,2]; Anta Sparinska[1,2]; Kārlis Shvirksts[1,2]; Roberts Krūmiņš[1,2]; Daiga Birzleja[1,2]; Elga Ence[1,2]**

[1]Bulduri Biotechnology center, Latvia

[2]Bulduri Technical School, Latvia

**Contact:**
copytree21157@gmail.comm

# 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 micro-propagation 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 (April, 2024)

Dear colleagues,

Imagine a world where we can easily save and replant threatened tree species *in vitro*, where virus-free orchards flourish and multi-clonal forests thrive sustainably. That's the potential of our work here at the COPYTREE conference, a major milestone in our European COST Action.

This Action addresses a fundamental challenge: how to meet the world's growing demand for trees and woody plants in a sustainable way. They are cornerstones of our ecosystems, providing timber, food, medicine and materials essential to our lives. Micropropagation is a powerful tool, but challenges remain.

That's why we're here. Our action unites us to solve these problems, from overcoming recalcitrant *in vitro* growth to providing clean supplies, streamlining production and building public trust. These efforts will have an impact not only on forestry, but also on global food security and the health of our planet.

This conference promises to be a dynamic exchange of ideas. We'll hear from leading researchers, industry experts and stakeholders. Poster sessions will stimulate discussion, and informal moments - coffee breaks, our conference dinner, and the post-conference excursions - will provide vital space for networking and collaboration. We'll have the chance to forge relationships, arrange short-term scientific missions and plant the seeds for exciting new projects.

I'd like to thank Liva Purmale and her dedicated organizing team, as well as our invaluable core group. Their tireless work has made this event possible. A special thanks goes to our Science Communication Coordinator, Valbona Sota, for maintaining our website and communication platform - a lifeline for our Action.

I'd also like to thank our Grant Holder Scientific Officer, Lucie Fischerova, for her financial expertise, and of course our COST Scientific Officer, Mafalda Quintas, and Administrative Officer, Katchamon Nimprang, for their unwavering support. This conference would not be possible without your contributions.

Most of all, thank you to each and every one of you. Your research, knowledge and passion will drive these next few days. Together, let's plant the seeds for a future where woody plants meet the needs of people and the planet, driven by innovation in micropropagation.

Let the discussions begin!

**Prof. Stefaan WERBROUCK**
Ghent University, Belgium
Chair COPYTREE

# CONFERENCE PROGRAM

| FIRST DAY – April 22, 2024 | |
|---|---|
| **08:30 – 09:15** | Registration of Participants and poster's installation |
| **09:15 – 09:30** | CONFERENCE OPENING '-Welcome Speech |

| WORKING GROUP 1 - RECALCITRANCE<br>Moderators: Sandra Correia, Itziar A. Montalbán | |
|---|---|
| **09:30 – 10:15** | **Keynote Speaker: Jaroslav Nisler**<br>Potential and mode of action of novel CKX inhibitors in *in vitro* cultures |
| **10:15 – 11:00** | **Coffee Break** |
| **11:00 – 11:15** | Strategies to understand and overcome recalcitrance to tissue culture in apple<br>**Giovanni A. L. Broggini, Célia Baroux and Bruno Studer** |
| **11:15 – 11:30** | The project "Wood of value" (Wertholz) — a forest tree breeding story<br>**Anne-Mareen E. Eisold, Cornelia Bäucker, Volker Schneck** |
| **11:30 – 11:45** | The impact of translocation mechanisms and plant architecture on the success or failure of propagation, a thidiazuron case study<br>**Hannes Wilms, Bart Panis** |
| **11:45 – 12:00** | Advancements in *in vitro* culture techniques and genetic transformation for grapevine improvement<br>**Loredana Moffa, Luca Nerva, Ivan Bevilacqua, Anna Narduzzo, Irene Perrone, Chiara Pagliarani, Riccardo Velasco, Giorgio Gambino, Walter Chitarra** |
| **12:00 – 12:45** | **Poster session** |
| **12:45 – 14:15** | **Lunch Break** |
| **14:15 – 14:30** | Does infrared spectrometry help in the study of somatic embryos maturation?<br>**Parisa Savane, Nassim Belmokhtar, Armelle Delile, Nathalie Boizot, Céline Ridel, Marie-Anne Lelu-Walter, Caroline Teyssier** |
| **14:30 – 14:45** | The effect of ethylene modulation on Solanum betaceum Cav. *in vitro* regeneration: from meristem propagation to somatic embryogenesis<br>**Mariana Neves, Sandra Correia and Jorge Canhoto** |
| **14:45 – 15:00** | Improvement of *in vitro* regeneration in *Passiflora quadrangularis* - a recalcitrant species<br>**Paula Oros, Corina Catana** |

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|---|---|
| **15:00 – 15:15** | Topolin cytokinins enhanced shoot proliferation, reduced hyperhydricity and altered cytokinin metabolism in *Pistacia vera* L. seedling explants<br>**Dhekra Abdouli, Lenka Plačková, Karel Doležal, Taoufik Bettaieb, Stefaan P.O. Werbrouck** |
| **15:15 – 15:30** | Plant Growth regulators in somatic embryogenesis of Norway spruce and Scots pine - testing the effect of cytokinin oxidase inhibitors<br>**Saila Varis, Stijn Maris, Sakari Valimaki, Stefaan P.O. Werbrouck, Tuija Aronen** |
| **15:30 – 16:00** | **Coffee Break** |
| **WORKING GROUP 5 - COMMUNICATION<br>Moderators: Valbona Sota; Branislav Cvjetkovic** | |
| **16:00 – 16:15** | Using micropropagated trees in a community-owned revegetation project on neglected land<br>**Carlos Sobrino, Lucía Saborido, David García, Conchi Sánchez, Anxela Aldrey, Puri Covelo, Mª José Cernadas, Bruce Christie, Nieves Vidal** |
| **16:15 – 16:30** | PInK-net: Resuming the network idea of *in vitro* tree labs in Germany<br>**Andrea Rupps, Juliane Raschke, Madlen Walther, Emma Ehmke, Lena Safranek, Julia Eckard, Antje Schmidt, Winston Beck, Ben Bubner, Anne-Mareen Eisold, Franka Thiessen** |
| **16:30 – 16:45** | Exploiting the untapped potential of fruit tree wild DIVersity for sustainable agriculture<br>**Monika Hoffer** |
| **16:45 – 17:00** | Epigenetics as a regulator of tree specialized metabolites *in vitro* production<br>**Stéphane Maury** |
| **17:00 – 17:15** | Present and future perspective on knowledge-sharing and stakeholder engagement strategies on CopyTree<br>**Valbona Sota, Lucie Fischerová, Maurizio Lambardi, Stefaan P.O. Werbrouck** |
| **17:15 –** | **WG1 and WG2 meetings** |

| **SECOND DAY – April 23, 2024** | |
|---|---|
| **WORKING GROUP 3 - AUTOMATION<br>Moderators: Nieves Vidal; Liva Purmale** | |
| **09:00 – 09:45** | **Keynote Speaker: Ander Castander-Olarieta**<br>Somatic embryogenesis in pines: from mass propagation to stress response and adaptation<br>**Ander Castander-Olarieta, Catia Pereira, Mikel Hurtado, Itziar A. Montalbán, Paloma Moncaleán** |

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|---|---|
| **09:45 – 10:00** | Production of high-value molecules using apple cell suspension cultures<br>**Xuan Xu, Salma Halime, Kjell Sergeant, Diego Rios, Salgado, Samuel Jourdan, Jean-Francois Hausman, Jenny Renaut, Gea Guerriero, Sylvain Legay** |
| **10:00 – 10:15** | Temporary immersion system and addition of silver nanoparticles to eliminate pathogens in apricot (*Prunus armeniaca* L.)<br>**Cristian Pérez-Caselles, Marina Martín-Valmaseda, Javier Alfosea, Nuria Alburquerque, Lorenzo Burgos** |
| **10:15 – 11:00** | **Coffee Break** |
| **11:00 – 11:15** | Overcoming challenges for micropropagation of *Prunus domestica* cv. Tropojane in various TIS bioreactor systems<br>**Brunilda Çuko, Ledina Shkëmbi, Valbona Sota, Efigjeni Kongjika** |
| **11:15 – 11:30** | Recent advances in somatic embryogenesis induction in *Aesculus* species<br>**Snezana Zdravkovic-Korac, Dusica Calic, Jelena Milojevic, Maja Belic, Sladjana Jevremovic** |
| **11:30 – 11:45** | Morpho-physiological evaluation of *Solanum betaceum* Cav. *in vitro* cloned plants: a comparison of different micropropagation methods<br>**Mariana Correia, Tércia Lopes, Ana Patrícia Puga, Glória Pinto, Jorge Canhoto, Sandra Correia** |
| **11:45 – 12:00** | Somaclonal variation in *in vitro* culture<br>**Yildiz Aka Kacar** |
| **12:00 – 12:30** | **Poster session** |
| **12.30 – 14.15** | **Lunch Break** |
| **14:15 – 14:30** | Vineyard evolution: advancing with embryogenic callus technology<br>**Pierre Videau, Katerina Labonova, Emma Coulonnier, Camille Mietton, Olivier Zekri** |
| **14:30 – 14:45** | Genetics, genomics, and machine learning applications in the propagation of woody plants: Case studies on Laurel and Peruvian Rosewood<br>**Faheem Shehzad Baloch, Durmuş Alpaslan Kaya, Gülşah Karataş, Nafiz Çeliktaş, Muhammad Asim, Muhmmad Azhar Nadee** |
| **WORKING GROUP 4 - RISK ASSESSMENT**<br>**Moderators: Yesim Yalçin Mendi; Pilar S. Testillano** | |
| **14:45 – 15.30** | **Keynote Speaker: Selim Çetiner**<br>The impact of regulatory oversight on the development and adoption of plant biotechnology |
| **15:30 – 16:00** | **Coffee Break** |
| **16:15 – 16:30** | Somatic embryogenesis in conifers: Present state and application in Germany<br>**Madlen Walther, Juliane Raschke, Jana Seifert, Andrea Rupps** |

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|---|---|
| **16:30 – 16:45** | Micropropagation of *Phalaenopsis* using temporary immersion system<br>**Bora Onur Hallaç, Yeşim Yalçın Mendi, Soner Yağ** |
| **16:45 – 17:00** | Strategic innovations in *in vitro* woody crop production: Balancing technical excellence with market and stakeholder dynamics<br>**Buhara Yucesan, Ahmet Tigrel** |
| **17:00 – 17:15** | Addressing forest seed challenges: Exploring somatic embryogenesis and tissue culture for tree propagation<br>**Branislav Cvjetkovic** |
| **17:15 – 18:30** | **WG3, WG4 and WG5 meetings** |
| **19:30 –** | **SOCIAL DINNER** |

## THIRD DAY – April 24, 2024

### WORKING GROUP 2 - SANITATION and CONSERVATION

### Moderators: Elif Aylin Ozudogru; Claudia Ruta

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|---|---|
| **09:00 – 09:45** | **Keynote Speaker: Bart Panis**<br>Cryotherapy, breeding instrument, commercial stock deposit; Cryopreservation is more than a long-term conservation tool for of plant genetic resources |
| **09:45 – 10:00** | 3D plant imaging in woody tissue culture practice<br>**Corina Catana, Paula Oros** |
| **10:00 – 10:15** | Chestnut gene soldiers against *Phytophthora cinnamomi* infection<br>**Saleta Rico, Jesús Vielba, Beatriz Cuenca, Nieves Vidal, Conchi Sánchez** |
| **10:15 – 11:00** | **Coffee Break** |
| **11:00 – 11:15** | Detection of plant viruses with high-throughput sequencing and *in vitro* elimination of detected viruses<br>**Ine Dewitte, Elien Guldentops, Maaike Heyneman, Yoika Foucart, Kris De Jonghe, Stefaan P.O. Werbrouck** |
| **11:15 – 11:30** | Susceptibility genes: the new frontier of improving plant tolerance to pathogen<br>**Vera Pavese, Andrea Moglia, Lorenzo Antonio Marino, Anna Maria Milani, Elena Corredoira, Mª Teresa Martínez, Daniela Torello Marinoni, Roberto Botta** |
| **11:30 – 11:45** | Enhancement of medicinal bioactive compounds of *Gynura procumbens* by silver nitrate and phytohormones: Perspective in phytotherapy for diabetes and cancer<br>**Viktor V. Husak, Olha A. Bulii, Yurii O. Vakiv, Volodymyr I. Lushchak** |
| **11:45 – 12:00** | Dormant bud cryopreservation to supplement elm genetic resources conservation in Finland<br>**Sakari Välimäki, Leena Yrjänä, Mari Rusanen, Tuija Aronen** |

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|---|---|
| **12:00 – 12:45** | **Poster session** |
| **12:45 – 14:15** | **Lunch Break** |
| **14:15 – 14:30** | CopyAlderTrees — propagation of selected genotypes tolerant to *Phytophthora*<br>**Daniela Cordeiro, Alberto Pizarro, María Teresa Cervera, Carmen Díaz-Sala** |
| **14:30 – 14:45** | Micropropagation tools for the characterization and conservation of valuable agri-food germplasm: case studies from the CULTIVAR Project in Portugal<br>**Tércia Lopes, Ana Pedrosa, Elsa Baltazar, Mariana Correia, Daniela Duarte, Sandra Caeiro, Alberto Cardoso, Hugo Paiva, Jorge Canhoto, Sandra Correia** |
| **14:45-15:00** | Micropropagation and *in vitro* conservation of *Zizyphus spina-christi* L. germplasm by using abscisic acid<br>**Doaa Elazab, Claudia Ruta and Maurizio Lambardi** |
| **15:00 – 15:15** | **Closing talk** |
| **15:15 – 15:30** | **Break** |
| **15:30 – 17:30** | **Management Committee Meeting** |

| | **Poster session: Working Group 1** |
|---|---|
| **P1** | Biotechnological advances for *Melia volkensii*, a climate-resilient tree for reforestation in East Africa<br>**Stefaan Werbrouck** |
| **P2** | Transgene-free genome editing in *Quercus ilex* L.: a way to improve traditional breeding<br>**Vera Pavese, Andrea Moglia, Lorenzo Antonio Marino, Anna Maria Milani, Elena Corredoira, Mª Teresa Martínez, Daniela Torello Marinoni, Roberto Botta** |
| **P3** | How does secretome composition affect the embryogenic capacity in *Pinus nigra* cell lines?<br>**Miroslav Pernis, Terezia Salaj, Jana Bellova, Maksym Danchenko, Peter Barath, Katarina Klubicova** |
| **P4** | *In vitro* propagation of alternative species to the radiata pine in the Basque Country<br>**Alejandra Rojas-Vargas, Ander Castander-Olarieta, Paloma Moncaleán, Itziar A. Montalbán** |
| **P5** | Exploring the role of an rRNA methyltransferases gene family during somatic embryogenesis in dicot species<br>**Ricardo Ferraz, Cláudia Marinho, Patrícia Fernandes, Sandra Correia, Sílvia Coimbra, Jorge Canhoto** |
| **P6** | Isolation and characterization of extracellular vesicles in *Solanum betaceum Cav.* somatic embryogenesis<br>**Miguel Rito, Beatriz César, Jorge Canhoto, Sandra Correia** |
| **P7** | Nanoparticle controlled gradual release of plant growth regulators in tissue culture<br>**Saba Taheri, Bogdan V. Parakhonskiy, Andre G. Skirtach, Stefaan P. O. Werbrouck** |

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| **P9** | Regeneration capacity - key feature for application of modern biotechnological techniques in fruit trees<br>**Susan Schröpfer, Ofere Emeriewen, Andreas Peil, Henryk Flachowsky** |
| **P10** | Transcriptomic analysis of auxin and gibberellin-inhibitor treatments during adventitious rooting in chestnut<br>**Ricardo Castro-Camba, Conchi Sánchez, Saleta Rico, Nieves Vidal, Puri Covelo, María José Cernadas, Anxela Aldrey, Jesús Vielba** |
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| **P14** | Possible applications of new approaches to minimize recalcitrance in woody plant species<br>**Vladislava Galovic, Tatjana Vujović, Saša Orlović** |
| **P15** | Imidazole fungicides in fruit tree tissue culture: impact and potential applications<br>**Tatjana Vujovic, Tatjana Marjanovic, Djrdjina Ruzic** |
| **P16** | *In vitro* propagation of lingonberry *Vaccinium vitis-idaea* L.<br>**Signe Tomsone, Madara Lazdāne, Jeļena Kalniņa** |
| **P17** | Response of mature somatic embryos of hybrid larch to cold and desiccation treatments<br>**Kateřina Eliášová, Parisa Savane, Petre I. Dobrev, Zuzana Vondráková, Václav Motyka, Caroline Teyssier, Marie-Anne Lelu-Walter** |
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| **P2** | Use of high throughput sequencing and cryopreservation in raspberry virus study in Norway<br>**Zhibo Hamborg, Xiaoyan Ma, Dag-Ragnar Blystad** |
| **P3** | Propagating of resistant specimen of *Ulmus glabra*<br>**Liina Jürisoo** |
| **P4** | Effects of copper and silver ions on growth and biochemical characteristics of paulownia plantlets at hydroponic cultivation<br>**Oksana V. Pasat, Viktor V. Husak, Angelika M. Pitukh, Volodymyr I. Lushchak** |
| **P5** | Survey of viral and phytoplasma diseases of grapevine in Georgia<br>**Iveta Megrelishvili, Zurab Khidesheli, Levan Ujmajurideze** |
| **P6** | Cryopreservation of dormant raspberry buds for further *in vitro* cultivation<br>**Olena Bobrova, Alois Bilavcik, Milos Faltus, Jiri Zamecník** |
| **P7** | Endogenous bacterial contamination of date palm during tissue cultures: Deglet Nour varieties<br>**Amal Rabaaoui, Stefaan Werbrouck** |
| **P8** | *In vitro* forest trees: tissue culture as proper tool for breeding and conservation<br>**Anne-Mareen E. Eisold, Franka Thiesen, Tobias Brügmann, Tina Ruhland, Cornelia Bäucker, Volker Schneck, Ben Bubner** |

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| **P9** | Synthetic seeds conversion to plantlets in *Punica granatum* L. cv. Devedishe after storage in different temperature regimes<br>**Yllka Lala, Klara Meta, Brunilda Çuko, Valbona Sota, Carla Benelli, Maurizio Lambardi, Efigjeni Kongjika** |
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| **P1** | Endophytic bacteria in date palm tissue culture: impact on the large scale micropropagation using Rita Bioreactors and remedies<br>**Ameni Nasri, Emna Baklouti, Emna Graja, Ahlem Ben Ahmed, Hazar Akrimi, Riadh Drira, Alain Rival, Noureddine Drira, Lotfi Fki** |
| **P2** | Effect of LEDs illumination on the *in vitro* micropropagation of peach rootstock GF 677 (*Prunus amygdalus* × *Prunus persica*)<br>**Lilyana Nacheva, Nataliya Dimitrova, Diyana Aleksandrova, Snezhana Milusheva, Ivaylo Tsvetkov** |
| **P3** | Unlocking the secrets of stable European Beech *in vitro* propagation<br>**Franka Thiesen, Ben Bubner** |
| **P4** | Stem cutting propagation of *Sideritis* and *Salvia* spp. and survival rating under different rooting hormone concentrations<br>**T. Tselegkaridis, Eirini Sarroy, Sampson Panajiotidis, Eleni Abraham** |
| **P5** | Application of topolin cytokinins in mutation- assisted breeding of *Coffea arabica*: role in somatic embryo germination and regeneration<br>**Radisras Nkurunziza, Joanna Jankowicz-Cieslak, Ivan L. Ingelbrecht, Pooja Mathur, Stefaan Werbrouck** |
| **P6** | Enhancing woody plant micropropagation: insights and strategies from progressive research at Deroose Plants<br>**Sofie Van Gijseghem, Lucas Vanhaelewyn, Filip Vandenbussche** |
| **P7** | Micropropagation of two commercial varieties of apple in bioreactors<br>**Simon Miranda, Mickael Malnoy, Stefano Piazza, Anxela Aldrey, Mª José Cernadas, Conchi Sánchez, Nieves Vidal** |
| **P8** | Enhanced natural stilbene production in *Arachis hypogaea* callus culture through fungal elicitation<br>**Hajer Ben Ghozlen, Sven Mangelinckx, Stefaan P.O. Werbrouck** |
| **P9** | Physiological and biochemical responses of clonally propagated *Prunus* spp. rootstocks during water stress and recovery: molecular insights into GF677 post-stress responses<br>**Mariana Correia, Tatiana Soares, Tércia Lopes, Elsa Baltazar, Maria Celeste Dias, Jorge Canhoto, Mónica Zuzarte, Sandra Correia** |

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| **P10** | Optimizing propagation process of hybrid aspen *in vitro* cultures by the use of LEDs<br>**Toms Kondratovičs, Mārtiņš Zeps** |
| **P11** | Strategy to produce stable clonal lines of *Dittrichia viscosa* for phytoremediation applications<br>**Capaci Piergiorgio, Anglana Chiara, Barozzi Fabrizio, Stigliano Egidio, Gian Pietro Di Sansebastiano** |
| | **Poster session: Working Group 4** |
| **P1** | Micropropagation of *Philodendron Birkin* using temporary immersion system<br>**Soner Yağ, Bora Onur Hallaç** |
| **P2** | How to capture thousands of genotypes – initiation of SE in Norway spruce<br>**Saila Varis, Jaanika Edesi, Mikko Tikkinen, Tuija Aronen** |
| **P3** | Micropropagation of some *Anthurium* varieties for commercial *in vitro* production<br>**Yeşim Yalçın Mendi, Yıldız Aka Kaçar, Belgin Biçen, Melike Cengiz, Çağlar Yıldız** |
| | **Poster session: Working Group 5** |
| **P1** | Joint project OPAL in Germany: development and evaluation of methods for the efficient production of hybrid larch and fir clones<br>**Juliane Raschke, Jana Seifert, Madlen Walther, Emma Ehmke, Andrea Rupps** |
| **P2** | The wonderful forest world inside a glass jar: micropropagation as an educational tool<br>**Puri Covelo, Conchi Sánchez, Mª José Cernadas, Anxela Aldrey, Ricardo Castro, Carmen Fuentes, Nieves Vidal** |
| **P3** | The history and present status of *in vitro* culture research at the institute of nature and forest research (INBO)<br>**Hannes Wilms, Marijke Steenackers, Linda Meiresonne, Bart De Cuyper** |

# WORKING GROUP 1:

# Recalcitrance

# Biotechnological advances for *Melia volkensii*, a climate-resilient tree for reforestation in East Africa

**Stefaan P.O. Werbrouck**

*University Ghent, Belgium, Fac. Bioscience Engineering, Valentin Vaerwyckweg 1, 9000 Ghent, Belgium*

Stefaan.werbrouck@ugent.be

## Abstract

*Melia volkensii* is a valuable Kenyan savanna tree, known for its resilience to climate change, high-quality timber, and resistance to pests like termites and locusts. Overexploitation has caused a decline in its natural populations, but successful reforestation efforts over the past 20 years have reintroduced millions of trees, generating valuable genetic diversity for future breeding programs. *In vitro* cloning offers a promising alternative to traditional seedling-based reforestation, but *M. volkensii in vitro* culture presents challenges such as poor seed germination, stunted shoot growth without rooting, and excessive callus formation. Acclimatization of *in vitro* plantlets to ex vitro conditions has also been a major hurdle. This is a review about the solutions that we developed to improve *M. volkensii in vitro* biotechnology, including the use of meta-topolin derivatives for improved micropropagation, modification of rooting media for better root development, and the application of ABA homologues to regulate acclimatization. Additionally, field trials incorporating beneficial microbes and mycorrhizae are underway. We have also developed techniques for adventitious shoot and somatic embryo induction, which have been applied to polyploidy induction, genetic transformation, and early embryo rescue. These advancements collectively contribute to a more efficient and effective approach to propagating and re-establishing *M. volkensii* in its native habitat.

***Keywords:*** *Melia, reforestation, mahogany, in vitro*

## 1. INTRODUCTION

*Melia volkensii*, a member of the Meliaceae family, is endemic to the semi-arid and arid regions of Kenya, Somalia, and Tanzania. Its natural habitat ranges from dry bushland to wooded grassland at altitudes between 400 and 1600 meters above sea level. This species is notable for its combination of drought resistance and rapid growth, making it a valuable resource for reforestation efforts in arid environments. The primary objective of establishing new *M. volkensii* plantations is to harvest its termite-resistant, mahogany-like timber after 15-20 years of growth. Additionally, its leaves and seeds contain bioactive compounds with medicinal and insecticidal properties, offering potential economic benefits for farmers (Jaoko et al., 2020). Despite its local significance, *M. volkensii* propagation presents challenges due to overexploitation, habitat fragmentation, and the loss of wild elite trees (Runo et al., 2004).

The species' recalcitrant seeds, encased in a woody endocarp, are susceptible to damage during extraction and exhibit poor germination rates. Seedling production has been insufficient to meet the growing demand for reforestation. In response to these challenges, plant tissue culture techniques have been explored over the past decade as a means of propagating *M. volkensii*. These techniques have facilitated the development of protocols for micropropagation, somatic embryogenesis, and adventitious shoot and root induction. Furthermore, they have opened avenues for investigating advanced biotechnological approaches such as polyploidy induction and genetic transformation.

## 2. *IN VITRO* INITIATION

Following the removal of the endocarp, young seeds with intact seed coats are subjected to a surface sterilization procedure involving a 70% ethanol rinse, a 15-minute immersion in a 10% sodium hypochlorite (NaOCl) solution containing 0.005% detergent (Teepol), and three subsequent rinses with sterile distilled water. In addition to removing the seed coat at the micropylar end, longitudinal incisions are often made to scarify the seed coat and enhance germination. Germination typically commences within one week on Murashige and Skoog (1962) (MS) medium supplemented with 20 g/L sucrose and 20 g/l Plant Agar (Duchefa, The Netherlands) at pH 5.4. For all the experiments described herein, explants were consistently cultured under a 16-hour light/8-hour dark photoperiod, with a photosynthetically active radiation (PAR) of 40 µmol photons $m^{-2}$ $s^{-1}$ and a constant temperature of 23°C. Each cotyledon possesses an axillary meristem capable of generating additional shoots if the main shoot axis is compromised. The presence of cotyledons is crucial for robust seedling development. Approximately two weeks post-germination, the seedling can be divided into nodal segments to initiate micropropagation.

## 3. MICROPROPAGATION

While *Melia volkensii* can be propagated on Murashige and Skoog (MS) medium supplemented with 20g/l Plant Agar and benzyladenine (BA), superior shoot multiplication and quality are achieved with the meta-topolin riboside (mTR) (Dushimimana et al. 2022a). The use of filter containers, such as well-closed polypropylene Microbox® containers (Sac O2, Belgium) with integrated filters, effectively prevents excessive leaf curling, indicating improved overall shoot quality. This is likely due to enhanced gas exchange within the container. The largest leaf surface area is consistently obtained when filters are present, regardless of their size.

## 4. ADVENTITIOUS SHOOT REGENERATION

Werbrouck et al. (2017) successfully induced adventitious shoot formation in *M. volkensii* leaf explants using 10 µM INCYDE-Cl, INCYDE-F, thidiazuron (TDZ) or N-

(2-chloro-4-pyridyl)-N′-phenylurea (CPPU). Notably, INCYDE-Cl and INCYDE-F where early developed cytokinin oxidation (CKX) inhibitors, reducing the catabolism of endogenous cytokinins like zeatin (Z) and 2iP through oxidative removal of their side chains (Zatloukal et al., 2008). Interestingly, the INCYDEs demonstrated a unique ability to activate dormant meristems located in the secondary axils of *M. volkensii* compound leaves.

## 5. *IN VITRO* ROOTING

*In vitro* rooting of *M. volkensii* shoots has proven challenging. Supplementing ½ MS medium with 30 g/l sucrose and 7g/l Plant Agar and 0.1-1 mg/L IBA induces abundant callus formation at the stem base without root initiation. Hormone-free ½ MS medium results in only 10% of shoots producing adventitious roots, albeit with minimal callus (Fig. 7). Additionally, leaf yellowing and abscission are common in this medium. Pulse treatments with high indol butyric acid (IBA) concentrations (200 mg/L for 4 hours) followed by transfer to hormone-free medium increased rooting to 56 % (unpublished results). However, the resulting roots often grew rapidly at the expense of shoot development and were non-functional due to their brittle nature and separation from the shoot vascular system by callus tissue. Ultimately, 100% rooting success was achieved using a modified woody plant medium with half-strength salts, supplemented with 7 g/l Plant Agar, 30g/l sucrose and either IBA or indole-3-acetic acid (IAA). The addition of silver thiosulfate (STS) to this medium reduced callus formation during rooting. Rooting response varied significantly among clones, with an average survival rate of 85%.

## 6. ACCLIMATIZATION

*In vitro* propagated plants, including *M. volkensii*, often encounter challenges during acclimatization to ex vitro conditions due to anatomical and physiological adaptations acquired in the controlled *in vitro* environment. These adaptations, including thin cuticles and abnormal stomata, impair their ability to regulate water balance effectively. Abscisic acid (ABA), a phytohormone known for its role in stomatal closure and water balance regulation, could potentially facilitate acclimatization, but its inherent instability limits practical applications. Pyrabactin, a stable synthetic analog of ABA, offers a promising alternative.

Rooted plantlets were carefully rinsed with tap water to remove residual medium and transplanted into 15.5 cm x 15.5 cm x 7.5 cm black polyethylene pots containing an autoclaved mixture of natural forest soil, animal manure, and 4 mm gravel in a 5:2:1 ratio. Immediately after transfer to an acclimatization greenhouse maintained at an average temperature of 24 ± 2°C, the substrate was sprayed with 20 ml of either 0, 10, or 15 µM ABA or pyrabactin solutions. Plants were then covered with clear polyethylene foil to maintain humidity. Supplementation of the substrate with pyrabactin significantly enhanced the survival rate of rooted **M. volkensii** plantlets,

accompanied by improvements in leaf number, plant height, and chlorophyll content (unpublished results). These findings suggest potential applications of pyrabactin as an anti-transpirant or priming agent to enhance drought tolerance in a wide range of plant species during acclimatization.

## 7. FIELD SURVIVAL

The high mortality rate of *M. volkensii* plantlets during acclimatization has been a major obstacle in utilizing tissue culture for mass micropropagation. To address this, two experiments were conducted to evaluate the effects of various biological agents, including Trichotech® WP, Bio-cure B®, Rhizatech™, indigenous arbuscular mycorrhizal fungi (AMF), *Bacillus subtilis*, and *Trichoderma*, on the establishment of *in vitro* plantlets in greenhouse and field conditions. All these biological agents significantly improved plantlet survival, with some treatments achieving 100% survival. Notably, *in vitro*-raised plants outperformed seedlings in the semi-arid field conditions of Kiambere (Kenya), particularly when inoculated with beneficial microbes. The root anchoring and binding capacity of *in vitro* regenerated plantlets after 18 months in semi-arid conditions were comparable to seedlings, demonstrating the effectiveness of the developed protocols for propagation, planting, and breeding (Dushimimana et al., 2022b).

## 8. EARLY EMBRYO RESCUE

Current breeding program**s** for ***M. volkensii*** focus on growth rate, habit, and shape, but are hampered by the tree's long juvenile period and slow seed development. To address this, early embryo regeneration has been explored as a means to bypass slow seed maturation (Kimani et al., 2024). Zygotic embryos isolated 60 days after pollination were cultured on Murashige & Skoog (MS) media BAP at 0.75, 1.0 and 1.5 mg/l; TDZ at 0.25 and 1.0 mg/l; IAA at 2.5, 5.0 and 10 mg/l and a combination of 1.0 mg/l TDZ and 5.0 mg/l IAA. The media were also supplemented with 3% sucrose and 0.2% Gelrite® as a gelling agent. The pH was set to 5.8 ± 0.1 before autoclaving at 121 ºC for 15 min. The cultures were incubated at 25 ± 1 ºC.

After 147 days, survival rates ranged from 2% to 44%, with PGR-free media yielding thick fleshy shoots and TDZ inducing somatic embryo formation and shoot clusters. Shoots also developed from non-embryogenic callus on media containing BA and IAA. These shoots were successfully rooted and seedlings were ultimately regenerated within 207 days, a significant reduction compared to the natural seed development period of nearly 390 days. This study demonstrates that early embryo regeneration can substantially accelerate progeny generation and clonal propagation in *M. volkensii*, facilitating breeding and testing of valuable traits.

## 9. POLYPLOIDY

Polyploidy induction was successfully achieved (Dushimimana et al. 2022). Cotyledon explants from diploid *M. volkensii* (2n=2x=28) were treated with oryzalin, a microtubule inhibitor, with or without prior exposure to TDZ t Treatment with 10 mg/L oryzalin for three hours resulted in a 40% yield of tetraploid plants. Pretreatment with 1.1 µM TDZ for 18 days further enhanced tetraploid production to 52.5%, but also increased the occurrence of mixoploid individuals. Tetraploid *M. volkensii* plants exhibited distinct morphological features compared to their diploid counterparts, both *in vitro* and as young potted plants. *In vitro*, tetraploids displayed a more compact growth habit with thicker stems, wider leaves, and a lower density of longer and wider stomata. Ongoing field trials will assess the performance of these tetraploid plants and their potential for future breeding programs aimed at improving the species' resilience and productivity.

## 10. GENETIC TRANSFORMATION

An improved protocol for *Agrobacterium tumefaciens*-mediated transformation of *Melia volkensii* was established, utilizing a novel binary vector, pVK001. This vector was constructed by combining the robust backbone of pTJK136 with the T-region of pSIM24-eGFP, a vector known for its high efficiency in gene transfer. The pVK001 vector incorporates a redesigned M24::eGFP gene as a visual reporter for successful transformation and the pNOS::nptII gene, conferring kanamycin resistance, as a selectable marker. The pVK001 vector and associated transformation protocol provide a valuable tool for introducing desirable traits into *M. volkensii*. Additionally, this transformation system could be adapted for CRISPR/Cas9-mediated genome editing in *M. volkensii*, enabling targeted modifications of specific genes for further improvement of the species.

## 11. CONCLUSION

The high demand for *Melia volkensii* plants, driven by their valuable termite-resistant timber and rapid growth in challenging semi-arid conditions, underscores the importance of developing efficient and scalable propagation methods. Current research efforts are focused on translating the knowledge gained from *in vitro* studies to establish commercial-scale micropropagation systems for elite *M. volkensii* trees. A critical aspect of this endeavor is the development of a robust rooting and acclimatization protocol to ensure the successful transition of *in vitro*-grown plants to field conditions. Achieving this goal will enable the establishment of vast multiclonal forests, composed of genetically superior individuals, which have the potential to transform the landscape of the savannas and contribute to both economic development and ecological restoration. Furthermore, the successful implementation of *in vitro* propagation techniques, coupled with advancements in genetic transformation and polyploidy

induction, offers promising avenues for further improving the traits of *M. volkensii*. These could include enhancing growth rates, disease resistance, and drought tolerance, ultimately maximizing the species' potential for reforestation and sustainable resource utilization. The continued development and refinement of *M. volkensii* biotechnology hold significant promise for transforming the landscape and livelihoods of communities in East Africa.

## Acknowledgments

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# Transcriptomic analysis of auxin and gibberellin-inhibitor treatments during adventitious rooting in chestnut

**Ricardo Castro-Camba, Conchi Sánchez, Saleta Rico, Nieves Vidal, Puri Covelo, María José Cernadas, Anxela Aldrey, Jesús M. Vielba**

*Plant Production Department, Misión Biológica de Galicia, Spanish National Research Council (MBG-CSIC) Avda de Vigo s/n, Santiago de Compostela (Spain) CP-15705*

*jmvielba@mbg.csic.es*

## Abstract

Adventitious rooting is a complex post-embryogenic process necessary for the mass propagation of sweet chestnut (*Castanea sativa* Mill.), a tree with key ecological and socio-economic roles in Europe. Auxin is the main plant growth regulator involved in adventitious rooting. However, other hormones, such as gibberellins, also influence the adventitious rooting process. Experiments were carried out to determine the effects of auxin and gibberellins on the initiation of adventitious roots. The analysis was performed in a juvenile-like chestnut clone derived from basal shoots. Auxin and the gibberellin biosynthesis inhibitor paclobutrazol (PBZ) were applied to microshoots and the results suggest that PBZ stimulates root formation in chestnut, even in the absence of exogenous auxin. A transcriptomics analysis was developed to elucidate the genetic basis of the responses to auxin and PBZ. Around 600 Differentially Expressed Genes (DEGs) were detected in Indole-3-Butyric Acid (IBA)-treated microshoots, while 900 DEGs with significant differential expression were found in PBZ-treated microshoots. IBA-induced genes were mainly related to the auxin signalling pathway. In PBZ-treated tissues, significant expression was found for genes linked to the hormone signalling. These findings increase our understanding of the genetic basis of adventitious rooting regulation in chestnut.

***Keywords:*** *adventitious roots, auxin, chestnut, gibberellins, paclobutrazol*

## 1. INTRODUCTION

The ability of chestnut (*Castanea sativa* Mill.) shoots to form adventitious roots is directly linked to the generation of auxins gradients and the activation of related signalling pathways. The asymmetrical distribution of auxins in neighboring tissues of cells from which roots are formed triggers the expression of specific genes whose action determine the establishment of root initial cells in the vascular bundles or close tissues (Vielba *et al*., 2020). However, the activity of auxins is also influenced by their interactions with other plant growth regulators. Among them, gibberellins (GAs) are particularly relevant because of their ability to control plant growth. Nonetheless, although they are mainly linked to seed dormancy and plant height regulation, they also

take part in the control of other processes which are fundamental for plant development. For instance, GAs activity is linked with the promotion of xylogenesis, floral and fruit development (Castro-Camba *et al.*, 2022a) as well as different stress responses (Castro-Camba *et al.*, 2022b). Notably, GAs also influence root developmental processes. They seem to repress the formation of adventitious roots (Busov *et al.*, 2006) and cause a reduction in root diameter (Wang *et al.*, 2015). Besides, GAs promote root elongation (Rizza *et al.*, 2017) and lateral root development (Busov *et al.*, 2006). Therefore, a plausible link between auxins and GAs might be present in the control of root growth and the induction of adventitious roots, but this link has not been analyzed in chestnut.

The aim of the present study was to test the putative effect of GAs on adventitious roots induction in sweet chestnut, one of the most important forest species in the Mediterranean basin (Martín *et al.*, 2012). For this purpose, chestnut microshoots were treated with the GA biosynthesis inhibitor paclobutrazol (PBZ) and the results were compared with the previously optimized root induction treatment using IBA (Castro-Camba *et al*., 2024b). Afterwards, a transcriptomics analysis was carried out at different time points to identify genes related to the promotion of adventitious rooting in chestnut. Our results suggest that GA inhibition might trigger adventitious rooting through an auxin-related mechanism. Particularly, the expression of *CsPIN1*, an auxin polar transporter, and *Cs22D*, a gene involved in auxin signalling, was induced during the formation of adventitious roots under both treatments, suggesting a link between the activity of auxins and GAs in this developmental process.

## 2. MATERIAL AND METHODS

### 2.1. Plant material and culture medium

Shoot cultures of P2BS clone (derived from basal shoots) established *in vitro* 30 years ago from an 80-year-old chestnut tree (*Castanea sativa* Mill.) were used in this study (Sánchez and Vieitez, 1991). Microshoots were grown in GD medium (Gresshoff and Doy, 1972) supplemented with 0.075 mg $L^{-1}$ benzyladenine, 30 g $L^{-1}$ of sucrose and 7 g $L^{-1}$ of Bacto Agar as gelling agent. Every 4 weeks, well-developed microshoots were selected and used for a new proliferation cycle on fresh medium or for rooting experiments. Basal rooting medium was composed by GD medium with 1/3 of macronutrients concentration, 30 g $L^{-1}$ of sucrose and 7 g $L^{-1}$ of Bacto Agar. Both proliferation and rooting media were adjusted to pH 5.6 - 5.7 and autoclaved at 121ºC for 20 min.

### 2.2. Rooting experiments

Root induction was performed in basal rooting medium during five days in darkness conditions, and treatments were carried out by supplementing the basal rooting medium with IBA 2.5 µM or PBZ 40 µM. The shoots were then transferred to fresh basal rooting medium for another 25 days under a 16 h/ 8 h photoperiod with a light intensity of 90-

100 µmol $m^{-2}$ $s^{-1}$ provided by cool-white fluorescent lamps. The jars were placed in plant growth chamber at 25ºC and 80-90% relative humidity. The essays were carried out in glass jars, with 6 explants per jar, three jars per replicate and three repeats per treatment (6 explants per replication x 3 replications x 3 repeats = 54 explants per treatment). After the 30 days rooting period, the percentage of rooted shoots, the root number and the length of the longest root were recorded. Besides, the time passed until half of the shoots formed roots was also evaluated (T50).

Data normality was evaluated using the Shapiro-Wilk test, and the assumption of homoscedasticity was assessed using the Levene test from the "car" package (Fox and Weisberg, 2019). When the required assumptions were met (Root length, Root number), the data were analyzed using the ANOVA test, and the Tukey's HSD test was used as a post hoc test. When normality was not met (Rooting rate, T50), the non-parametric Kruskal-Wallis test followed by Dunn's test - from the "FSA" package (Ogle *et al.*, 2023) - with Bonferroni adjustment as a *post hoc* test was applied. All statistical analyses were performed using the R software (R Core Team, 2021).

### 2.3. Transcriptomic experiments

Total RNA from the basal portion (1 cm) of the stems of the explants treated with IBA 2.5 µM (IBA), PBZ 40 µM (PBZ) and untreated wounded shoots (W) was extracted with the "FavorPrep Plant Total RNA Purification Mini Kit (for woody Plant)" (Favorgen Biotech corp., Taiwan) at three time points – 24, 48 and 72 h after the beginning of the treatment. Sequencing libraries were constructed with the PCR-cDNA Barcoding kit (SQK-PCB109, Oxford Nanopore Technologies) and were sequenced in a MinION device using Flow Cells R9.4.1. The software Guppy (v3.3.1) was used for basecalling and demultiplexing. The obtained reads were trimmed with Porechop and the quality was evaluated with Nanoplot (De Coster and Rademakers, 2023). Obtained reads were aligned and mapped using minimap2 (Li, 2018a) against the *Quercus suber* reference genome with the option -ax splice. The transcriptomes were assembled and quantified using StringTie2 (Kovaka *et al*., 2019) and the Differentially Expressed Genes (DEGs) were detected with the DESeq2 R package (Love *et al.*, 2014).

Quantitative Real-Time PCR experiments were performed as previously described (Castro-Camba *et al*., 2024a; 2024b), with the same primer sequences for target (*CsPIN1*, *Cs22D*) and reference genes (*CsACT-2*, *CsELF-1*) as in the aforementioned studies. Briefly, 1 µg of total RNA was reverse-transcribed and then 1 µL of cDNA template (8.33 ng of input RNA) was analyzed with 2x Power SYBR® Green PCR Master Mix (Applied Biosystems) in a final volume of 20 µl. Three biological and three technical replicates were assessed for each sample. Relative expression values were expressed as fold-change using the comparative CT method (ΔΔCT Method) (Schmittgen and Livak, 2008).

## 3. RESULTS

IBA-treated microshoots showed a high-rooting rate, achieving 90.7% of rooted shoots (Table 1). Meanwhile, PBZ-treated shoots showed a 40% rooting rate without the use of exogenous auxin. Microshoots that were only wounded and used as controls for the transcriptomic analyses (see below) did not root. Interestingly, shoots rooted with PBZ showed a significant lower number of roots than auxin-treated shoots, although the length of the roots was similar (Table 1). Besides, the rooting kinetics were not affected by the treatments, as the time passed until half of the shoots formed roots was not significantly different for both treatments (T50, Table 1).

**Table 1.** Rooting parameters of IBA- and PBZ-treated chestnut microshoots.

| Treatment (μM) | Rooting rate (%) | Root number | Root length (cm) | T50 |
|---|---|---|---|---|
| IBA 2.5 | 90.7 ± 2.3 a | 4.1 ± 0.2 a | 3.6 ± 0.1 a | 11 ± 0.4 a |
| PBZ 40 | 40.7 ± 3.7 b | 1.2 ± 0.0 b | 3.0 ± 0.3 a | 12 ± 0.0 a |

IBA: Indole-3-Butyric Acid. PBZ: Paclobutrazol. Table shows mean values plus/minus standard error. Different letters indicate significant differences ($p \leq 0.05$).

The transcriptomic analysis produced 21,033,111 reads for the 27 libraries (W, IBA and PBZ at three timepoints and three biological replicates) with a total yield of 21.4 Gb. The average score quality (Q) was 9.2 with no reads below Q<8, and the mean sequence length was 599 bp. After adapters' removal and quality check, the obtained FastQ files were mapped against the *Q. suber* reference genome with a mean 83.2% of reads successfully mapped.

The differential expression analysis was performed comparing the detected reads in IBA or PBZ samples with the reads identified in the wounded plants, therefore allowing for the identification of rooting-related genes in both treatments (Fig. 1).

In the IBA vs Wounded comparison, 624 DEGs were identified, including several genes from families linked with auxin signalling and homeostasis (Aux/IAA, GH3, PIN). The top 25 genes with a higher level of expression from the transcriptomic analysis are shown in Fig. 1, including *CsGH3* genes, *Aux/IAA* genes (*Cs22D*, *CsIAA6*, *CsIAA29*), or the LOB family gene *CsLBD16*. On the other hand, comparison of PBZ and control samples revealed 926 DEGs, with several genes linked with hormone signalling and homeostasis, including the gibberellin receptor *CsGID1B* or the auxin transporter *CsPIN1*. The transcriptomics expression profile of the top 25 more expressed genes showed the presence of genes linked with auxin (*Cs22D*) and cytokinin (*zeatin o-glucosyl tranferase*) signaling, as it's shown in Fig. 1. In addition, several genes related to cell signalling molecules (inositol-3-phosphate, calcium) showed a high level of expression.

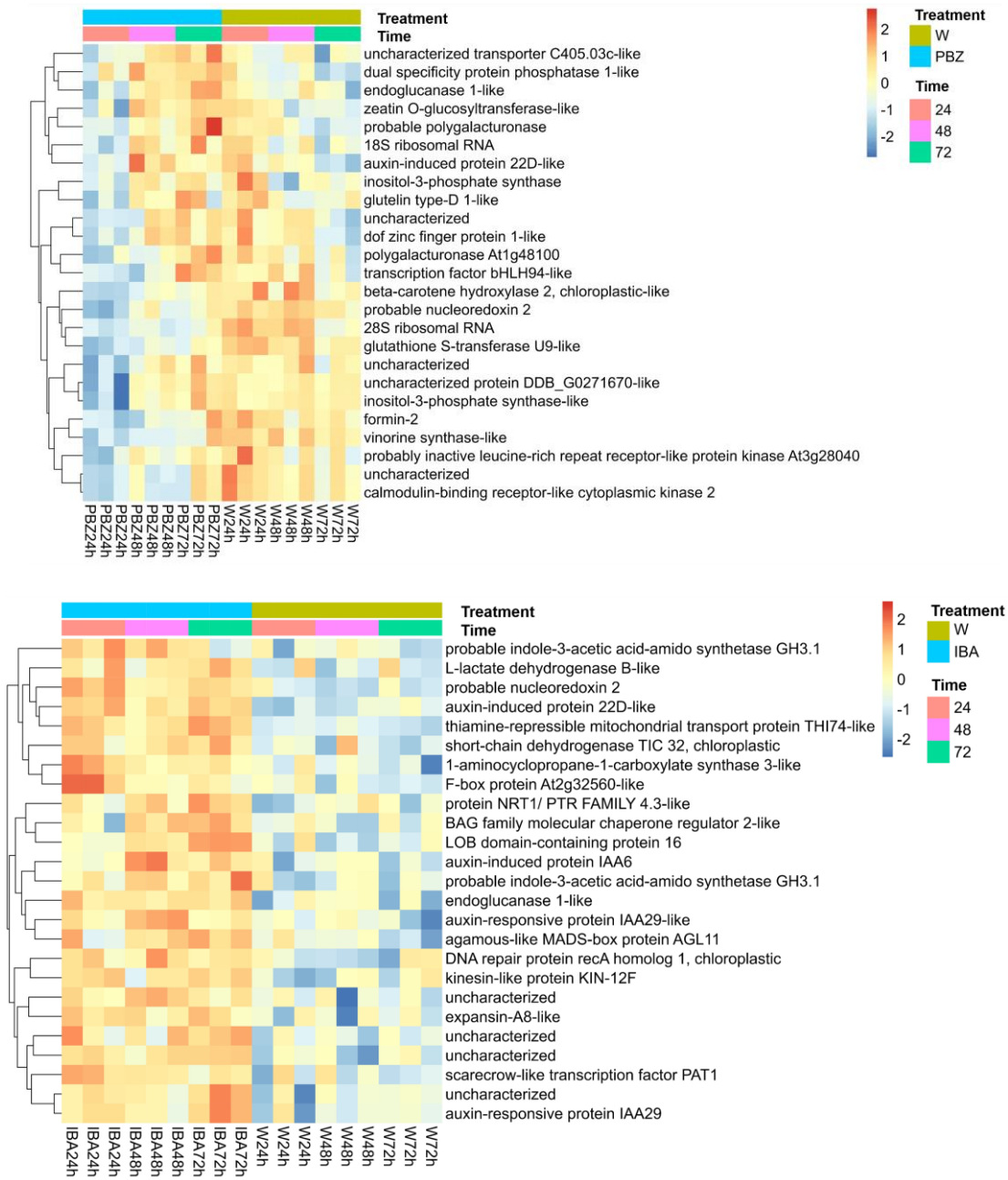


**Figure 1.** Heatmaps showing the normalized expression of the 25 top-expressed genes from the transcriptomic analysis. Top: Top-expressed genes in the PBZ vs W analysis. Bottom: Top-expressed genes in the IBA vs W analysis.

To verify the results from the transcriptomic analyses, specific genes were selected to validate their expression through qRT-PCR experiments. The expression of *Cs22D* was upregulated by both PBZ and IBA (Fig. 2). However, expression in IBA treated shoots increased steadily and reached a maximum at 72 h, while in PBZ-treated shoots the

level of expression did not change significantly over the three days. Interestingly, there seems to be a direct relation between the level of expression of *Cs22D* and the rooting rate in chestnut microshoots. Similarly, for *CsPIN1* the increase of expression induced by IBA was higher than the induction by PBZ treatment (Fig. 2). The highest level of expression for *CsPIN1* in IBA-treated samples was found at 24 h, decreasing afterwards. In wounded samples, expression of both genes was much lower than in PBZ- and IBA-treated shoots.

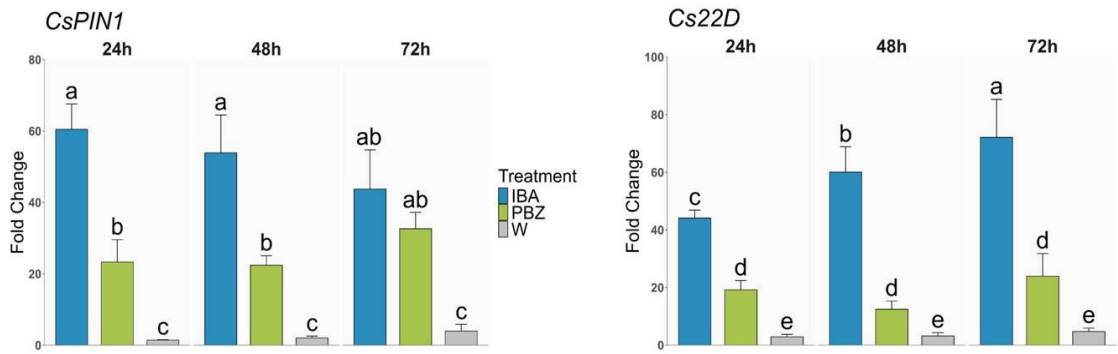


**Figure 2.** qRT-PCR analysis of the expression of *CsPIN1* (Left) and *Cs22D* (Right). Mean expression values for three biological replicates plus/minus standard errors are shown. Different letters indicate statistical differences at $p \leq 0.01$. W: Wounded.

## 4. DISCUSSION

Auxin treatments are necessary to induce adventitious roots in hard-to-root species, including chestnut (Gonin *et al.*, 2019; Vielba et al., 2020). However, in chestnut microshoots, PBZ treatment can induce adventitious roots in the absence of exogenous auxin treatment, although at a lower percentage. Similar results have been obtained in other woody species such as *Rubus brasiliensis* (Bueno *et al.*, 2021). The mechanisms through which PBZ induces adventitious roots remain uncharacterized, but our results suggest that the inhibition of GAs biosynthesis alters the auxin/GAs balance, which in turn partially eliminates the need for an external auxin source. PBZ is believed to modify abscisic acid and cytokinin content, besides its effects on GAs (Desta and Armare, 2021). However, the identity of the detected DEGs suggests that induction of genes related to auxin signalling is the main mechanisms for PBZ-triggered rooting in chestnut.

The transcriptomic approach performed in this study allowed for the identification of genes related to the responses to IBA and PBZ rather than to the wounding procedure, although the latter is also relevant for the adventitious rooting process (Castro-Camba *et al.*, 2024b). Several genes potentially involved in the development of new roots were found in both IBA- and PBZ-detected DEGs, indicating a common inductive

mechanism for the two treatments. Most of the genes identified were related to hormone signalling. Besides auxin-linked genes, *zeatin O-glucosyltransferase-like* was found in both analyses. This gene acts lowering the levels of active cytokinins, which are believed to block adventitious root induction by different means including down-regulation of *PIN* expression (reviewed in Li, 2021). Therefore, a reduction in the pool of active cytokinins might be one of the mechanisms shared by PBZ and IBA to induce adventitious roots.

Among the DEGs, two genes related to auxins stand out: *Cs22D* and *CsPIN1*. *Cs22D* is a member of the Aux/IAA family related to different developmental processes such as flowering (Xu *et al.*, 2016), fructification (Su *et al.*, 2021) and plant responses to stress (Krishnamurthy *et al.*, 2017; Ortiz *et al.*, 2019). *Aux/IAA* genes are essential for auxin signalling, modulating the activity of many transcription factors including members of the Auxin Response Factor family. Recently, the gene *22D* has been related to the acquisition of rooting competence in Huangshan Bitter tea (Fu *et al.*, 2023) as well as on root development in peach (Wu *et al.*, 2021). In apple, the expression of the gene *Cs22D* is upregulated in IBA-treated microshoots (Meng *et al.*, 2019). In our experiments, *Cs22D* was induced in response to PBZ, suggesting that the same signalling pathways, linked to auxin activity, are triggered by both treatments to induce adventitious roots.

On the other hand, *CsPIN1*, a polar auxin transporter, was also responsive to IBA and PBZ, however the expression was higher in auxin-treated shoots. This gene has been previously related to adventitious rooting (Estrella-Maldonado *et al.*, 2016; Velada *et al.*, 2020). The establishment of auxin gradients by means of the activity of auxin transporters is essential in different developmental processes, as it enables the expression of specific genes governing the initiation of formative divisions. This role for *PIN1* genes in adventitious rooting has been shown in other woody plant species such as apple (Guan *et al.*, 2020) or papaya (Estrella-Maldonado *et al.*, 2016). IBA treatment boosted *CsPIN1* expression levels, thus helping in the establishment of the aforementioned gradients. Nonetheless, inhibiting GA synthesis also contributed to generate auxin gradients through *CsPIN1* activity, therefore enabling auxin transport as a key process in the crosstalk between auxins and GAs. These results reinforce the previous hypothesis by which GA inhibits adventitious rooting through *PIN1* modulation (Mauriat *et al.*, 2014; Li *et al.*, 2018b).

## 5. CONCLUSIONS

The inhibition of GAs biosynthesis by the action of paclobutrazol was able to produce adventitious roots in chestnut microshoots in the absence of exogenous auxin treatments. Both treatments, IBA and PBZ, produced higher expression levels of auxin-related genes (*Cs22D* and *CsPIN1*) compared to the expression in the control shoots. These results confirm the putative cross-talk between GAs and auxins in adventitious

rooting regulation, and suggest that the manipulation of GA content might be a valuable tool to improve vegetative propagation protocols in hard-to-root species.

**Author´s contributions.**- Conceptualization: R.C.-C., J.M.V. and C.S. Methodology: R.C.-C., J.M.V. and C.S. Validation: R.C.-C., S.R., P.C. and M.J.C. Formal analysis: R.C.-C., S.R. and N.V. Investigation: R.C.-C., S.R., P.C. and M.J.C. Writing—original draft preparation: R.C.-C. Writing—review and editing: J.M.V. and C.S. Visualization: R.C.-C. and J.M.V. Supervision: J.M.V. and C.S. Funding acquisition: N.V. and C.S.

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# *In vitro* propagation of alternative species to radiata pine in the Basque Country

**Alejandra Rojas-Vargas[1,2], Ander Castander-Olarieta[1], Paloma Moncaleán[1], Itziar Aurora Montalbán[1]**

[1]NEIKER-BRTA. Centro de Arkaute. N 104 - km355, 01192 Arkaute, Álava.

[2]Instituto de Investigación y Servicios Forestales, Universidad Nacional, Heredia 86-3000, Costa Rica

*imontalban@neiker.eus

## Abstract

In the Basque Country, a region in the northeast of Spain, *Pinus radiata* is the most important forest species. In recent years this species has been affected by several diseases caused mainly by fungi: pine pitch canker, caused by *Fusarium circinatum*; red spot needle blight caused by *Dothistroma septosporum* and *D. pini* and brown spot needle blight caused by *Lecanosticta acicola*. In this sense, radiata pine plantations in the Basque country have decreased from 123,921 ha (2016) to 102,488 ha (2022); decrease that coincides with a historical outbreak of needle blight diseases during the years 2018-2019. Therefore, among other actions, it is necessary to look for alternative species with better adaptation to our edaphoclimatic conditions and that can be profitable for the stakeholders and industry. The objective of our work was the development and/or optimization of organogenesis protocols for elite individuals of alternative forest species such as *Sequoia sempervirens*, *Cryptomeria japonica* and *Pinus ponderosa*. For this purpose, apart from conventional growth regulators such as benzyladenine, and fluorescent illumination, other cytokinins (meta-Topolin, thidiazuron or kinetin) and light sources (different LED colours) were assayed.

***Keywords:*** *Cryptomeria japonica, cytokinins, LED lights, micropropagation, organogenesis, Pinus ponderosa, Pinus radiata, Sequoia sempervirens.*

## 1. INTRODUCTION

*P. radiata* has a natural distribution from the Central Coast of California to Mexico. The versatility and quality of the wood, together with the rapid growth of the species, has promoted the expansion of *P. radiata* plantations throughout countries such as Australia, Chile, New Zealand, South Africa and Spain. In Spain, the largest plantations of *P. radiata* are in the Basque Country, north of Spain. (HAZI, 2022). The edaphoclimatic conditions of this region have allowed for the development of an important forestry sector, being *Pinus radiata* the most cultivated species. The first plantation was established in 1897 (Puerta et al., 2004). From then until nowadays, its

cultivation has increased in this region having in 2022 a total wooded area of 25.9% and representing the80-85% of the annual cuts (HAZI, 2022). However, in recent years, radiata pine in the Basque Country has been affected by several diseases caused mainly by fungi: pine canker caused by *Fusarium circinatum*, Dothistroma red spot needle blight caused by *Dothistroma septosporum* and *Dothistroma pini*, and brown spot needle blight caused by *Lecanosticta acicola*. In the Basque country, the presence of *L. acicola* dates to 1942, but for unknown scientific reasons, the disease has expanded quickly in the last years. In this context, the plantations of radiata pine in this region have decreased from 123,921 ha (2016) to 102,488 ha (2022), a decrease that coincides with the historical outbreak of this type of needle blight diseases (HAZI, 2022).

For this reason, both the private sector and administrations have requested the search for species that can be used as alternatives for the establishment of plantations valid for the forestry industry and compatible with the characteristics of our soil and climate. To carry out our experiments, three alternative species were chosen to work with: *Sequoia sempervirens* (D. Don), *Cryptomeria japonica* (Thunb. ex L.f.) D. Don, and *Pinus ponderosa* P. Lawson and C. Lawson.

*S. sempervirens*, commonly known as coastal redwood, naturally occurs in the West Coast of North America; It is an important species due to the economic value of its wood (Sillett et al. 2020). Several countries such as France, Chile, New Zealand and Germany have established redwood plantations with timber production purpose (Breidenbach et al. 2020). In the Basque Country, it occupies an area of 1,586 ha (HAZI, 2022).

*Cryptomeria japonica*, also known as Japanese cedar or Sugi, occurs naturally throughout East Asia (Taniguchi et al. 2020) and Portugal. This species has been introduced in different countries as an alternative species due to its valuable timber and fast-growing (Koguta et al. 2017).

*P. ponderosa*, ponderosa pine or western yellow pine, is native to the United States, Southern Canada and Northern Mexico (Ellis and Bilderback 1991). Ponderosa pine cultivated forests have been established in California with lumber purposes on private lands, while in countries as Chile and Argentina has been introduced as a promising species to optimize and diversify the wood industry (Espinoza et al. 2021).

These species show interesting features, but most of them (height, shape, tolerance to stress, etc.) are expressed during maturity, a point when most of these species have lost the ability to be propagated by traditional techniques (cutting, grafting, etc.). For this reason, in the last years we focused our research on the development of *in vitro* culture protocols for elite individuals of alternative forest species for the Basque Country such as *S. sempervirens* (Rojas Vargas et a. 2021), *C. japonica* (Rojas-Vargas et al. 2023a) and *P. ponderosa* (Rojas-Vargas et al. 2023b). In addition, we studied the effect of various physical-chemical factors on the success of the process. In this paper a summary of last years´research will be presented.

## 2. MATERIAL AND METHODS

For the three species studied a similar approach was followed with some variations depending on the degree of existing knowledge and the material available on our region. First, the most suitable explant was determined. This was done by testing apical and basal parts of different sizes (equal to or smaller than 10 mm, or equal to or bigger than 15 mm) of shoots from adult trees in the case of *S. sempervirens* and *C. Japonica*; for *P. ponderosa* whole zygotic embryos or isolated cotyledons were used as initial explants.

Then, it was necessary to elucidate the best basal medium. In the case of *S. sempervirens*, the basal medium had been already developed and optimized by Arnaud et al. in 1993 (half strength ARN medium for multiplication and 1/3 strength ARN medium for rooting), so we centred our study in the other two species. Three basal media [DCR (Gupta and Durzan 1985), MS (Murashige and Skoog 1962), and QL (Quoirin and Lepoivre1977) all of them supplemented with 8.8 µM BA for four weeks] for *C. japonica* and two basal media [LP (QL modified as in Castander-Olarieta et al. 2023) and HLP (half strength LP)] for *P. ponderosa* were tested. In this latter species different concentrations of BA were analyzed (4.4, 22 and 44 µM) along shoot induction.

After basal media assays, different plant growth regulators were evaluated for bud induction.These were 6-benzyladenine (BA), meta-Topolin (m-T) or kinetin (K) at a concentration of 4.4 µM for *S. sempervirens*, BA, m-T or thidiazuron (TDZ) at 8.8 µM for *C. japonica*, and BA or m-T at 13.1 µM for *P. ponderosa*.

All media were supplemented with 3% (w/v) sucrose, the pH adjusted to 5.8, and 8 to 8.5 $gL^{-1}$ Difco Agar® granulated was added. The explants were cultured on the different induction media for four weeks in the growth chamber at a temperature of 21 ± 1°C, under 16-h photoperiod with 120 µmol $m^{-2}$ $s^{-1}$ of light intensity cool white fluorescent tubes [(FL), TLD 58 W/33; Philips, France].

After bud induction (four weeks in all the experiments), the explants from the three species were cultured on the same basal media but avoiding plant growth regulators, supplemented with 2 $gL^{-1}$ activated charcoal, and the agar (Difco Agar®) concentration raised to 8.5 to 9 $gL^{-1}$.

Once the best basal medium for each species was determined (QL for sugi and HLP for ponderosa pine) rooting experiments were carried out. Rooting in *C. japonica* was carried out by adding 50 µM 1-naphthylacetic acid (NAA) for five weeks and testing different sucrose concentration [1,5 or 3% sucrose w/v)], whereas different auxins or auxins mixtures were assayed for the other two species: 50 µM NAA, 50 µM indole-3-butyric acid (IBA) or a mixture of 40 µM NAA + 10 µM IBA for one week in *S. sempervirens* and 5 µM NAA (for two weeks); 10 µM NAA or a mixture of 5 µM NAA and 5 µM IBA for four weeks in *P. ponderosa*.

For sugi and ponderosa pine the effect of different lights in micropropagation was also studied. For the first one, two different light treatments, FL or red light (peak

wavelength 630 nm; RB4K Grow Light LEDs) at 60 μmol $m^{-2}s^{-1}$ light intensity were assayed at rooting stage. The photoperiod and the temperature of the growth chamber were the same as previously described. For the second one the effects of different lights were tested throughout the entire micropropagation process (from bud induction to root elongation). The light treatments comprised: FL, red light, blue light (peak wavelength 470 nm), and white light (colour temperature 4000 K) at 60 μmol $m^{-2}s^{-1}$ light intensity.

## 3. RESULTS AND DISCUSSION

For the three species bigger explants led to better results: shoots equal or bigger than 15 mm for redwood and sugi (Rojas-Vargas et al. 2021, 2023a) and whole zygotic embryos for *P. ponderosa* (Rojas-Vargas et al. 2023) (Fig. 1). In this sense, our experiments have confirmed that the explant size is an important factor affecting bud induction and proliferation (Renau-Morata et al. 2005). As hypothesized by Desai et al. (2015), bigger explants may have more mineral nutrient reserves and endogenous hormones to support the culture process. When considering micropropagation from seeds, even if isolated cotyledons offer the advantage of having more than one explant per genotype, newly formed buds developed slowly and failed to elongate when using this type of explant. A better performance of whole embryos compared to isolated cotyledons is in accordance to the results reported by Lambardi et al. (1993) in *P. halepensis*.

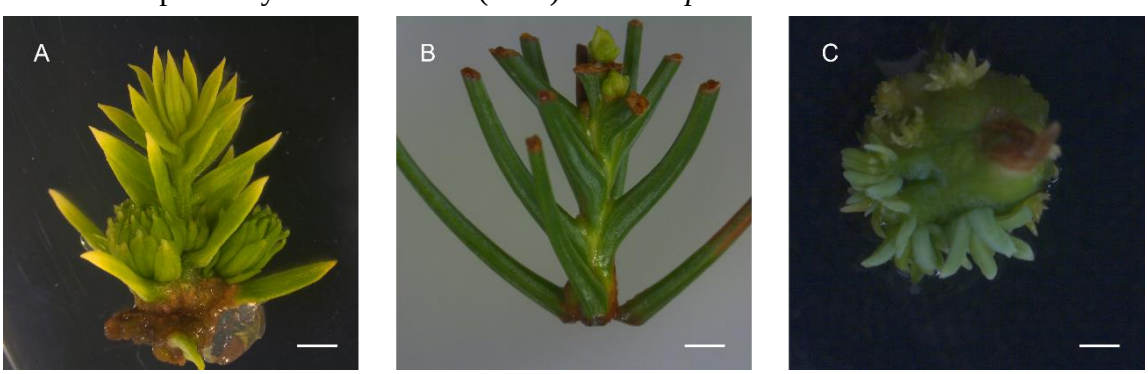


**Figure 1.** Bud induction in (A) *Sequoia sempervirens*, bar scale = 2.5 mm; (B) *Cryptomeria japonica*, bar scale = 1 mm; and (C) *Pinus ponderosa*, bar scale = 1.5 mm

As a general trend, no substantial improvement was observed when other cytokinins than BA were used for bud induction. Among cytokinins, BA is the most commonly used cytokinin in plant tissue culture due to its effectiveness and affordability. Although this hormone has been reported to cause hyperhydricity and other unwanted side effects, this was not the case in our cultures. Contrary to our work, Cortizo et al. (2009) obtained a better organogenic response in *P. pinea* adult trees when they used TDZ. In previous research, when applying m-T to induction medium, we found different results depending on the species; in *P. pinaster* (De Diego et al. 2011) and *P. sylvestris* (De Diego et al. 2010), we obtained higher organogenic response when using m-T instead of

BA. On the contrary, no improvement was found for the induction of axillary shoots in *P. radiata* with this hormone (Montalbán et al. 2011, 2013). However, the application of one cytokinin versus another may have implications in later phases of the process. That was the case for *C. japonica* where root induction percentage was significantly higher in explants coming from a m-T treatment. This effect was also observed by Naaz et al. (2019) in *Syzygium cumini*. This was not the case in our experiments with redwood where shoots induced with BA showed significantly higher *in vitro* rooting rates.

When developing micropropagation protocols one of the major bottlenecks is rooting. In the case of redwood and ponderosa pine, where the effects of NAA or IBA alone, or a mixture or both was compared, no differences were found for *P. ponderosa*. *S. sempervirens* explants showed higher rooting rates when they were cultured with NAA. Contrary to this, in previous organogenesis studies with radiata pine we did not find significant differences for rooting when NAA was used alone or in combination with IBA (Montalbán et al 2011a, 2011b).

Apart from plant growth regulators, the effect of other physico-chemical factors such as sucrose concentration or light sources where studied for sugi explants during *in vitro* rooting. These factors did not have any effect on rooting rates, however statistical differences were found for the number and length of the roots developed. These results demonstrated that adventitious root regeneration can also be influenced by growth regulators among other chemical factors (Zarei et al., 2020), and that the effect of them is species dependent.

The different LED light treatments assayed in ponderosa pine had a significant effect only when considering the number of shoots that elongated enough to be rooted; the existing literature on this topic suggests that the morphogenic effect of light treatments is highly dependent on the species and the *in vitro* phase analyzed. Moreover, Allalaq et al. (2020) reported that different lights had a different effect in *Picea abies* vegetative propagation through modulation of hormone homeostasis. It would be interesting for future research to check the hormonal status of our tissues at different stages and combined LED lights treatments.

Summarizing, although *in vitro* culture protocols must be optimized and adapted to different species, plant tissue culture could be used as a complementary propagation method for elite trees of *S. sempervirens*, *C. japonica* and *P. ponderosa* in future breeding programs to improve the health of the basque forest under the current climate change conditions.

## 4. CONCLUSIONS

We have found that bigger explants were better for shoot induction. Different physical-chemical conditions affected the micropropagation success in the three species evaluated. *S. sempervirens* rooting was affected by the cytokinin used for shoot

induction. *C. japonica* explants induced with m-T and rooted with 1.5% sucrose displayed the best results in terms of root induction, and plantlets exposed to red LEDs showed a significantly higher number of roots. *P. ponderosa* explants growing under blue LEDs produced higher number of shoots big enough to be rooted.

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# Induction of *in vitro* culture of walnuts in Georgia

**Maia Kukhaleishvili, Iveta Megrelishvili, Ekaterine Bulauri**

*Georgian Technical University, Biotechnology Center, Kostava St. 77, 0160, Tbilisi, Georgia*
*maia.kukh@gmail.com*

## Abstract

The cultivation of walnut culture in Georgia is quite actual, facilitated by our black earth soils. Due to the lack of a walnut seed production system, there is a shortage of walnut planting materials in Georgia. Besides, the traditional propagation of elite varieties of walnut is associated with many difficulties, in particular, the need for large areas for cuttings, problems of low rooting, the existence of separate rootstocks, high labor costs, and low production rates. Therefore due to a shortage of high-quality walnut seedlings in Georgia, in the last few years, elite walnut seedlings (rootstock and parent) have been imported from abroad. Hence, today, the most actual direction for growing walnut plantations is considered the usage of biotechnological methods, especially the development and implementation of the *in vitro* cloning technology in Georgia.

***Keywords****: walnut, in vitro, propagation, rooting*

## 1. INTRODUCTION

Walnuts (*Juglans regia* L.) are widely consumed, especially in the form of dried fruit. The bark, fruit peel, green fruit peel, and leaf parts of the walnut plant are widely used in the pharmaceutical and cosmetic industry (Sharma et al., 2022). Despite a very ancient culture of walnut species (*Juglans* spp.), its breeding started in the twentieth century. The important walnut-producing countries in the world are China, the United States of America, Iran, and Turkey. Walnut culture was introduced in Georgia in the 16th century, and due to favorable climatic conditions, it is widespread in most ecological zones of the country. In terms of consumption, it occupies one of the leading places in food products. However, walnut seedlings obtained by local technologies cannot meet the demands of farmers, and high-quality walnut seedlings are in short supply, and accordingly as the existing walnut trees cannot produce adequate yields, the import of walnuts is very high. This is also influenced by the fact that the country does not receive clean planting material, which is a big problem for the country from an economic point of view.

Plant tissue culture, a time-tested approach for establishing and maintaining a broad range of plant species, is a valuable alternative approach for propagation of plants, when

traditional methods are not sufficient. Tissue culture procedures cover the methods used to obtain plants and / or novel plant products aimed at research from cells, tissues, and organs transferred to a sterile nutrient media (Hussain et al., 2012; Kumar & Loh, 2012). . One of the most important steps in plant tissue cultures is the sterilization process (Kyte, 1987). Microorganisms, both exogenous and endogenous, such as several species-specific bacterial, fungal, and viral organisms, can contaminate plant cultures (Varghese & Joy, 2016). These contaminants may show themselves immediately, or they may not show their effects in any way for a long time (Ray & Ali, 2016). According to Leal et al. (2007), endogenous contamination is a significant problem in micropropagation studies using explants from mature walnut trees. Furthermore, they indicated that darkening due to oxidation in the tissues during the surface sterilization step resulted in a significant reduction in shoot production, which could be avoided by using immature shoot explants that are more resistant to disinfection.

This study aimed the induction of walnut *in vitro* culture to facilitate the production of planting materials.

## 2. MATERIALS AND METHODS

The *in vitro* culture of walnuts was induced using the well-known apical meristem method (Caboni & Damiano, 2006), and was as follows:

**Plant material:** Walnut samples were collected in early spring from a 3-4-year-old orchard in the village of Dzevera, Gori region of Georgia. In the initial stage, to prevent bacterial and fungal contamination, 20 cm-long young stems with buds were treated with 3% Ridomil Gold, and 0.3% Captan solution for 30 seconds and were stored in a refrigerator until use. For induction of sprouts, samples recovered from storage in refrigerator were covered with polyethylene bags, and placed in growth chambers with controlled conditions (temperature 25 ± 1°C, humidity 75-80%, 5000 lux, 16/8h light/dark photoperiod) (Fig. 1).

**Explants' sterilization:** The initial material, i.e., shoot tips, for the *in vitro* culture initiation and establishment was obtained from newly growing shoots of the "Chandler" walnut cultivar. Initiated explants were sterilized, at first in 0.8% (v/v) sodium hypochlorite for 15-20 minutes, followed by 70% (v/v) ethanol solution for a short time and washed 4 times in sterile deionized water.

Second sterilization was carried out with 1% mercuric chloride for 5-6 minutes, followed by 70% (v/v) ethanol solution for a short time, and washed 4 times in sterile deionized water. Afterward, shoot tips were soaked in an antioxidant aqueous solution of PVP (polyvinylpyrrolidone) (500 mg/l) and L-cysteine (50 mg/l) for 2 hours before being transferred to culture media in test tubes.

**Nutrient media:** Two different basal media was used for initiation of the *in vitro* culture of walnut:

- DKW nutrient medium (Driver and Kuniyuki, 1956) and
- half-strength MS (Murashige and Skoog, 1962)

Both media were supplemented with growth regulators and additives: 1 mg/l of benzylaminopurine (BAP), 0.5 mg/l of indole-3-butyric acid (IBA), 500 mg/l PVP, 40 g/l glucose, pH of the medium was set to 6.0 before autoclaved.

Different concentrations of phytohormones were used to improve root formation under phytotron-regulated conditions (temperature: 23-24°C, humidity - 75-80%, 5,500 lux, 16/8h light/dark photoperiod). 0.005 mg/l, 0.05 mg/l, and 0.5 mg/l IBA in combination with 0.5 g/l and 1g/l BAP were used to evaluate the *in vitro* propagation of walnuts.

**Figure 1.** Young stems of walnut for initiation of buds in controlled conditions

## 3. RESULTS AND DISCUSSION

The explants presented a high percentage of survival and only 34.4% fungal contamination was observed in the case of 3% Captan compared to 3% Ridomil Gold.

Regarding initial explant treatments, the micropropagation of walnut (*Juglans regia* L.) the regeneration rates ranged from 25.57% to 18.72%. Thus overall, the 3% Captan + 1% mercuric chloride combination provided the highest survival rate (24.27%) among the treatments in DKW medium.

Over the years, we have been able to determine the best antiseptics and different combinations of their concentrations. It was found that the contamination rate when using 0.1% mercuric chloride was relatively lower (65%) than when using different concentrations of sodium chloride (85%). But, the new protocols including antifungal treatments with Captan 0.3% seems to be more effective in the overall survival rate, thus raising the regeneration rates as well.

Because different varieties have different effects on several mediums, the modification of medium composition is always effective and successful for *in vitro* propagation. Various modifications of DKW and MS media have been tested and determined. Mainly, the change of medium is related to the quantitative and qualitative change of plant growth regulators.

The concentrations of 0.05 mg/l IBA and 1g/l BAP combination had a positive effect on the *in vitro* development of walnuts compared to others. As a result, apparently healthy *in vitro* plants of walnut were obtained (well-developed root system, stem, and green leaves) (Fig. 2).

**Figure 2.** *In vitro* plants of walnuts

One of the most important limitations in micropropagation of walnut species is the low rooting rate. Researchers have tried various methods to overcome this problem. For instance, Payghamzadeh and Kazemitabar (2011) determined that the use of ¼ strength DKW and vermiculite (250/200 h/h) promoted root elongation and secondary root development in two-week cultures. In the present study, the rooting walnut plants were placed in polyethylene plastic bags (13.0x10.5x4.0 cm) containing soil (2/3 peat and 1/3 vermiculite, pH-5.8), and kept at room temperature. The plants were watered once a week, and they were covered with polyethylene bags (3-4 hours a day) periodically to maintain humidity. Under such conditions, 30% of the plants continued to develop, which is the best result when compared to the data of researchers from different countries (Vahdati et al., 2022; Kaur et al., 2006) (Fig. 3).

**Figure 3.** Walnut culture in soil with vermiculite and peat

## 4. CONCLUSION

*In vitro* propagation of walnut trees requires careful management of various factors to successfully clone and produce healthy plants. For the first time in Georgia, *in vitro* rooted, strong walnut plants were established in the Biotechnology Center of the Georgian Technical University. The obtained results are the first steps for the production of healthy seedlings of this very valuable culture in Georgia. Despite challenges such as contamination, and media optimization, this technique supports conservation of valuable cultivars and enables the commercial production of uniform, disease-free planting material.

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# Transgene-free genome editing in *Quercus ilex* L.: a way to improve traditional breeding

**Vera Pavese[1], Andrea Moglia[1], Lorenzo Antonio Marino[1], Anna Maria Milani[1], Elena Corredoira[2], Mª Teresa Martínez[2], Daniela Torello Marinoni[1], Roberto Botta[1]**

[1]*Dipartimento di Scienze Agrarie, Forestali e Alimentari—DISAFA, Università degli Studi di Torino, Largo Paolo Braccini 2, Grugliasco, 10095 Torino, Italia*

[2]*Misión Biológica de Galicia, Sede en Santiago, Consejo Superior de Investigaciones Científicas, Avd. Vigo, s/n, 15705 Santiago de Compostela, La Coruña, Spain*

*vera.pavese@unito.it*

## Abstract

The CRISPR/Cas9 technology represents a revolution in the biotechnology area, facilitating the production of cultivars deprived of negative traits. The CRISPR/Cas9 system can be delivered through the Agrobacterium tumefaciens strain; this method may lead to the Cas9 integration into the host genome and remain active, causing off-target events. To circumvent these issues, the CRISPR/Cas9 machinery can be introduced as a ribonucleoprotein (RNP) complex, targeting the specified gene before being rapidly degraded. This approach represents a new era of gene editing, paving the way for the production of plants without transgenes. In woody species, especially *Quercus ilex* L. traditional breeding poses significant challenges due to prolonged juvenile phases, recalcitrance to clonal propagation and high heterozygosity level. Consequently, the adoption of new genomic technology can support and improve breeding. In the present work, we present the first protoplast isolation protocol in holm oak, highly valued in the European Mediterranean zones. Protoplasts were successfully extracted from both *in vitro* leaves and proembriogenic masses. Proembryogenic masses represented the best source for high protoplast yield (11 x 10 6 ± 2 x 10 6 protoplasts/ml) and viability (92% ± 0.5). CRISPR/Cas9 RNPs targeting the phytoene desaturase gene were successfully delivered into protoplasts, demonstrating an editing efficiency of 5.6% ± 0.5. Protoplasts were then cultured in semi-solid media and after a 45-day embryogenic calli development were observed.

***Keywords****: holm oak; protoplasts; CRISPR/Cas9*

## 1. INTRODUCTION

*Quercus ilex* L. (Holm oak) is an abundant species in the Iberian Penisula and other Mediterranean regions, which is the main constituent of the *dehesas*, a particular type of agrosilvopastoral system in Spain, where several crops coexist (Pulido et al., 2001). For Spain, the *Quercus* spp. species are economically valued for their timber and acorns,

used to feed the Iberian pigs, from which several gastronomical products are produced (Canellas et al., 2007). This renowned species has been damaged by oak syndrome decline in the last decades, caused by different abiotic and biotic factors. Among them, *Phytophthora cinnamomi* Rands infection is considered the principal reason for the oak decline. Therefore, the conventional breeding programs in woody plant species, are challenging due to the long biological cycles and the high heterozygosity level. To overcome traditional breeding, new genetic frontiers are explored to produce holm oak plants resistant to *P. cinnamomi*, to safeguard the *dehesas* agroecosystem. The CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) methodology stands as one of the innovative and precise techniques in the plant biotechnology area, which can generate a single-point mutation in a specific area of the genome. Usually, the CRISPR-Cas9 delivery is performed using the A*grobacterium tumefaciens* strain into the plant cells. However, the *A. tumefaciens* delivery method led to the vector integration into the plant genome and remained active, causing off-target effects. Therefore, to avoid this matter, the CRISPR-Cas9 can be delivered as ribonucleoprotein (RNP) which acts directly on the specific gene and then it is rapidly degraded by the cell nucleases and proteases, generating a transgene-free plant according to the European directives.

The main objectives of the study were:

- to set up an isolation protoplast protocol from different tissues, such as proembryogenic masses (PEMs) and young leaves, to obtain a high protoplast yield;
- to test the efficiency of the transfection ability of protoplast with the Green Fluorescence Protein (GFP);
- to test the CRISPR/Cas9 ribonucleoprotein (RNP) targeting the phytoene desaturase (pds) marker gene, whose silencing causes an albino phenotype.

## 2. MATERIAL AND METHODS

### 2.1. Protoplast isolation

For the protoplast isolation, PEMs and young leaves were used as starting material to select the most suitable explant for good protoplast yield and quality. The protoplasts extraction from *in vitro* leaves was performed using six concentrations and combinations of enzyme solutions and two different base mineral solutions, which are the CPW solution (Kuzminsky et al., 2016) and NEW solution (Pavese et al., 2022; Pavese et al., 2024). The CPW solution consisted of 0.2 mM $KH_2PO_4$, 1 mM $KNO_3$, 10.1 mM $CaCl_2$ x $2H_2O$, 1 mM $MgSO_3$ x $7H_2O$, 0.96 mM KI, 0.16 mM $CuSO_4$ x $5H_2O$, 11% D-Mannitol, 0.1% BSA (Bovine serum albumine; pH 5.7), meanwhile the NEW solution was composed by 20 mM morpholinoethane sulfonic acid (MES), 0.5 M mannitol, 20 mM KCl, and 10 mM $CaCl_2$ (pH 5.7). The leaves were treated with

different enzymes, enzyme concentrations and combinations by adding directly to the CPW and NEW solutions: a) 3% Cellulase R-10 (Duchefa Biochemie, Netherlands), plus 1.5% Macerozyme R-10 (Duchefa Biochemie, Netherlands), 1.5% Hemicellulase from *Aspergillus niger* (SigmaAldrich, St. Louis, MO); b) 2% Cellulase R-10, 0.75% Macerozyme R-10 and 0.75% Hemicellulase; c) 1% Cellulase R-10, 0.5% Macerozyme R-10 and 0.5% Hemicellulase; d) 3% Cellulase R-10, 1.5% Macerozyme R-10 and 1.5% Pectolyase Y-33 (Duchefa Biochemie, Netherlands); e) 2% Cellulase R-10, 0.75% Macerozyme R-10 and 0.75% Pectolyase Y-33; f) 1% Cellulase R10, 0.5% Macerozyme R-10 and 0.5% Pectolyase Y-33.

For PEMs, 1.5% Cellulase R-10 and 0.5% Macerozyme R-10 were used as previously evaluated by Pavese et al. (2022). PEMs were dissected into small pieces before adding the enzymatic solution. Protoplasts were then extracted using the protocol standardized for woody plant species by Pavese et al. (2022). The protoplast yield was evaluated through the hemocytometer while the Trypan blue assay was used to perform the viability test. The full protocol is already available in Pavese et al. (2024).

### 2.2. GFP protoplast transfection

The protoplasts extracted were then transfected with the plasmid pAVA393 (Ochatt et al., 2005) with the GFP marker gene. For the GFP transfection, protoplasts were incubated with 10 or 20 mg of GFP plasmid DNA and 100 mL of 40% (w/v) polyethylene glycol (PEG). After 20 min, the protoplasts were rinsed and the GFP transfection was evaluated at different time points (12h, 24h, and 48h) using the fluorescence microscope (Nikon Eclipse Ti2, Japan).

### 2.3. Cas9 ribonucleoprotein protoplast transfection

For the transfection via Cas9 ribonucleoprotein, the gene *phytoene desaturase* (*pds*) was identified as a target. The single guide RNA (sgRNA) targeting the *pds* gene of *Castanea sativa*, designed (5:GAGTCAAGAGATGTGCTAGG:3) by Pavese et al. (2022), was evaluated to target the holm oak *pds*, due to the homology sequence. The transfection via RNP was performed using the protocol by Pavese et al. (2022). Then, the DNA was extracted from the transfected protoplast after 1 day, the PCR reaction was performed and products were purified through DNA/RNA Clean Up E.Z.N.A.® kit (Omega Bio-tek, Norcross, GA, USA) and sequenced using the Sanger method. The chromatograms were then analyzed through the bioinformatic software TIDE (Tracking of Indels by Decomposition) (https://tide.deskgen.com, accessed on 08/03/2023).

### 2.4. Protoplast regeneration

Protoplasts were cultured in three different regeneration media: Q1 which was supplemented with 0,2 mg/L BAP, 0,1 mg/L cefotaxime, 0,1 mg/L carbenicillin, 0,2 mg/L $AgNO_3$, and 2,46 g/L WPM (Woody Plant Medium); Q2 medium, composed of 2,6 g/L MS3B, 0,1 mg/L BAP, and 0,01 mg/L 2,4-D; Q3 medium, supplemented with

2,6 g/L MS3B, 0,1 mg/L BAP, and 0,1 mg/L 2,4-D. To the three media, 90 g/L D-Mannitol, 5 g/L sucrose, and 6 g/L Plant agar were added (Pavese et al., 2024).

## 3. RESULTS AND DISCUSSION

### 3.1. Protoplast isolation

The protoplast isolation represents a tricky and challenging step, especially in woody plant species. Indeed, the donor material is considered one of the crucial factors for the successful isolation of protoplast, influencing the yield and viability (Reed and Bargmann, 2021). Among the different enzyme solution combinations, the mixture with 2% Cellulase R-10, 0.75% Macerozyme R-10 and 0.75% Pectolyase Y-33 lead to a protoplast yield of $1.2 \times 10^6 \pm 0.3 \times 10^6$ protoplasts/mL with 82% ± 1 viability after 12 h from *in vitro* leaves. Using the PEMs, as starting material, the highest protoplasts yield was detected ($11x10^6 \pm 2x10^6$ protoplasts/mL) after 6 h of enzymolysis treatment in the NEW mineral solutions (92% ± 0.5 viability) compared to the number of protoplasts ($8.8x10^6 \pm 5x10^6$ protoplasts/mL) obtained using the CPW mineral solution (Pavese et al., 2024). Although leaves are the most used explants for obtaining protoplasts, they are rich in lignin and phenols, affecting negatively the activity of the cell-wall degrading enzymes (Brandt et al., 2020; Fizree et al., 2021). Nowadays, cell suspensions or embryogenic calli are considered the most efficient plant material for protoplast isolation and regeneration (Bertini et al., 2019), obtaining high-quality protoplasts.

### 3.2. GFP protoplast transfection

PEMs derived protoplasts, were transfected with 10 and 20 µg of pAVA393:GFP applying PEG-mediated editing because the ratio between protoplast and plasmid DNA influences the transfection efficiency (Burris et al., 2016). The results showed that the highest transfection percentage (62%) was recorded with 10 µg of plasmid and the highest expression of GFP was detected at 24 h from the transfection process (Figure 1). These results are comparable to that obtained in European chestnut (Pavese et al., 2022). Meanwhile, the holm oak protoplasts transfected with 20 µg of pAVA393:GFP showed lower GFP expression in all the tested time points (20%) (Pavese et al., 2024).

### 3.3. Cas9 ribonucleoprotein protoplast transfection

After the confirmation of the sequence homology between the *C. sativa* and *Q. ilex phytoene desaturase* gene, using the Sanger sequencing and the MEGAX alignment, the transfection via CRISPR/Cas9 RNPs of PEMs-derived protoplasts was achieved using the crRNA designed on the chestnut *pds*. These two species are closely related and belong to the Fagales order, demonstrating the possibility of transferring the technology to related species. The protoplast DNA was isolated and the *pds* target region was sequenced using the Sanger sequencing method. The TIDE software was used to analyze the Sanger chromatogram and the editing efficiency was 5.6%±0.5%. The

transfection was performed in three biological replicates (P1, P2, and P3), and the P1 and P2 samples showed an insertion (-1) and a deletion (-3), meanwhile the sample P3 showed a single deletion (-1) (Pavese et al., 2024) (Table 1). This editing efficiency result reached in holm oak was lower than in other species like potato (9-25%; Andersson et al., 2018), *Arabidopsis thaliana* (16%), and rice (19%; Woo et al., 2015). On the contrary, comparable editing frequencies were reported in apple (0.5-6.9%) and grape (0.1%; Malnoy et al., 2016).

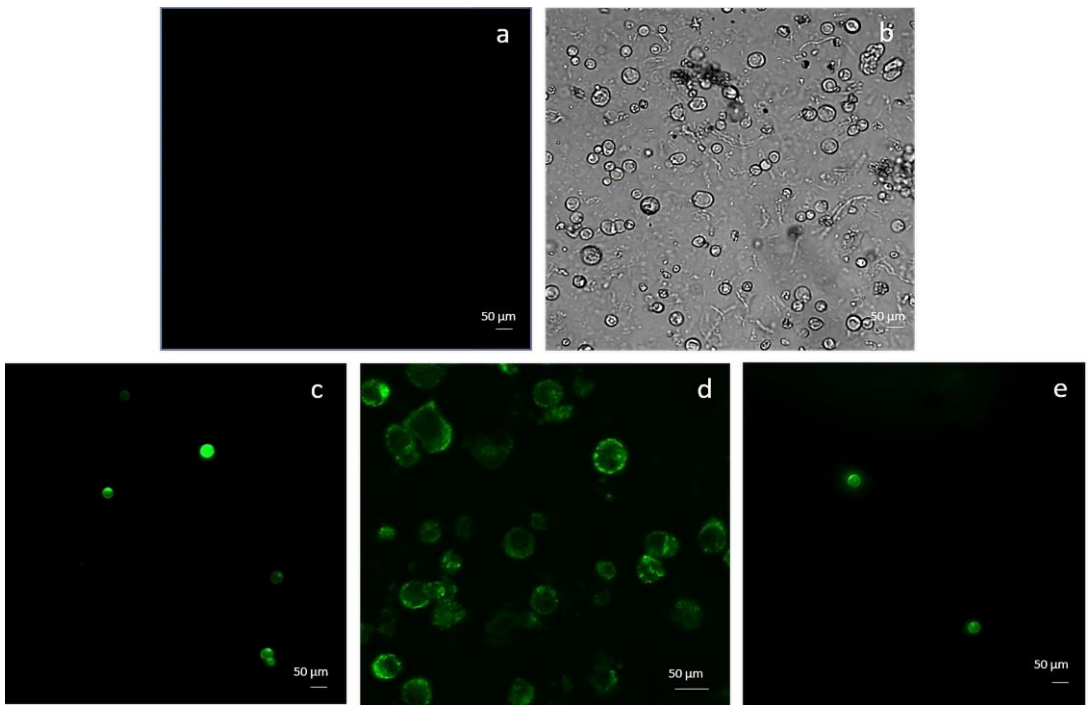


**Figure 1.** Transfection with pAVA393:GFP plasmid. a-b) Negative control. c) Transfected protoplast with GFP after 12 h, d) 24 h, and e) 48 h (From Pavese et al., 2024).

**Table 1.** Results of the CRISPR/Cas9 RNPs editing in protoplast samples: editing efficiency, goodness-of-fit measure ($R^2$), and mutation types are evaluated (From Pavese et al., 2024).

| Samples | Efficiency (%) | $R^2$ | Mutation |
|---|---|---|---|
| P1 | 6.2 | 0.99 | +1; -3 |
| P2 | 5.5 | 0.98 | +1; -3 |
| P3 | 5.2 | 0.98 | -1 |
| mean ± SD | 5.61 ± 0.5 | | |

### 3.4. Protoplast regeneration

The protoplasts were cultured in three different semi-solid medium (Q1, Q2, and Q3). After the culture in semi-solid media, microcalli appeared visible after 6 weeks only in the Q3 medium, which is suitable for regeneration (Pavese et al., 2024). The regeneration step is the major bottleneck for woody species (Corredoira et al., 2019), in particular starting from protoplasts (Attre et al., 1989; Papadakis and Roubelakis-Angelakis, 2002) due to their high recalcitrance to the *in vitro* culture procedures (Martinez et al., 2017). The Q3 medium, composed of MS3B medium with 1/2 concentration $NH_4NO_3$ and $KNO_3$, is supplemented with 0.1 mg/L BAP and 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and this phytohormone ratio has been demonstrated to be efficient for the microcalli formation; indeed the 2,4-D, a growth regulator with simil-auxin action, led to the cell wall formation and the initial protoplast division and growth, as observed in other plant species (Shi et al., 2016; Tu et al., 2023) (Figure 2). However, due to the recalcitrance issue of the woody plant species, the formation of somatic embryos and plantlet regeneration is challenging and requires protocol optimization to overcome the recalcitrance and achieve a successful propagation method.

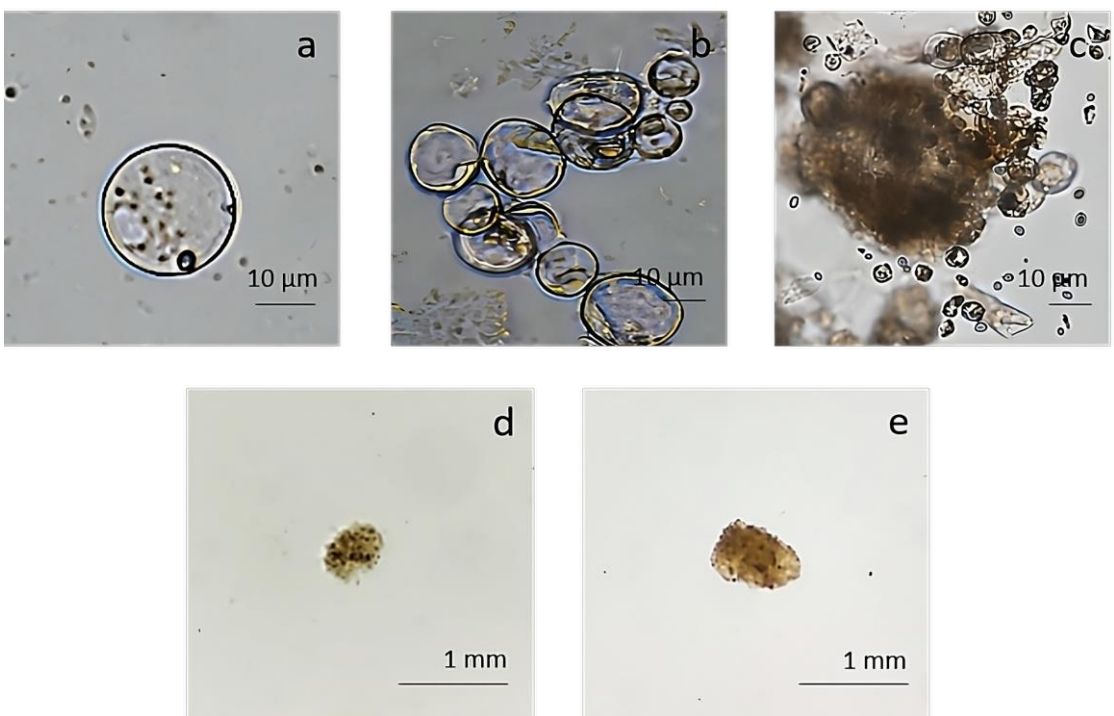


**Figure 2.** Regeneration from protoplast cultured in Q3 medium. (a) Protoplast; (b) protoplast division (after 5 days); (c) microcolonies (after 15 days); (d, e) calli development (after 45 days) (From Pavese et al., 2024)

## 4. CONCLUSIONS

In this study, we developed the first method for isolating protoplasts from *in vitro* leaves and PEMs from holm oak. PEMs yielded the highest quality and quantity of protoplasts. Our protocol also enabled successful DNA transfection of these protoplasts. Notably, we applied the CRISPR/Cas9 system using RNPs in holm oak for the first time, achieving transgene-free protoplasts and showing great potential for improving holm oak breeding. Future research will focus on refining the protoplast regeneration protocol.

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# Imidazole fungicides in fruit tree tissue culture: Impact and potential applications

**Tatjana Vujović, Tatjana Marjanović, Đurđina Ružić**

*Fruit Research Institute, Kralja Petra I No. 9, 32000, Čačak, Serbia*

tvujovic@institut-cacak.org

## Abstract

The application of imidazole fungicides in *in vitro* culture has revealed that certain fungicidal substances can exhibit various uncommon side effects across different tissue-cultured plant species. The effect of the imidazole fungicide Prochloraz on the micropropagation of fruit tree species (pear rootstock Pyrodwarf and cherry rootstock Gisela 5) that had previously demonstrated poor multiplication and/or rooting capacity *in vitro* will be discussed in this paper. Prochloraz at 1, 5, and 10 μM concentrations significantly increased the shoot-inducing effects of N-benzyladenine (BA) in both genotypes, but no cytokinin-like effect was observed in cytokinin-free media. Furthermore, shoots of both rootstocks were the longest and had the largest fresh and dry weights on media containing this fungicide in combination with BA and $GA_3$. Prochloraz, either alone or in combination with indole-3-butyric acid, also affects root induction under *in vitro* conditioms. Prochloraz alone stimulated the growth of lengthy roots; however, when combined with IBA, short, thick, white, radially spread roots emerged. These results strongly indicate that Prochloras could be recommended for micropropagation of these rootstocks.

***Keywords:*** *fruit tree rootstocks, multiplication, rooting, Prochloraz*

## INTRODUCTION

Fungicides are mostly employed to prevent fungal contamination in *in vitro* culture, as saprophytic species of fungi may interfere with the growth and development of plant cells and tissues. In that case, they act like "vitropathogens", whose metabolites, toxines, or enzymes can change the pH value and the osmotic potential of the medium, poison and macerate the *in vitro* tissues (Kowalik and Gródek, 2002). However, some azole fungicides, including those belonging to the imidazole and triazole classes, can induce morphogenetic and organogenetic responses in *in vitro* plants (Werbrouck et. al., 2001). This dual action can lead to various developmental changes in plants cultured *in vitro*, and a number of side effects of the imidazole fungicides were observed in diverse tissue culture plant species: inhibition of bushiness in Gerbera (Toppoonyanont et al., 1999), histogenic instability in *Ficus benjamina* chimeras, or restoration of normal embryonic development in *Citrus* (Werbrouck et al., 2001). It has also been found that imidazole

fungicides such as Imazalil, Prochloraz, and Triflumizole, as well as the triazole retardant Paclobutrazol, enhance the effect of exogenously added cytokinins on the multiplication of representatives of the Araceae family - *Spathiphyllum floribundum* Schott and *Anthurium andreanum* Schott (Werbrouck and Debergh, 1995; Werbrouck and Debergh, 1996; Werbrouck et al., 1996). A similar effect of Imazalil has been observed in other ornamental plant species, such as *Curcuma alismatifolia* Gagnep. (Toppoonyanont et al., 2005). In contrast, Sarropoulou and Maloupa (2019) found that Imazalil cannot be used in the tissue culture system of the *Sideritis raeseri* Boiss & Heldr. subsp. *raeseri* medicinal plant as promoter of shoot proliferation; however, it could be recommended for enhancing root elongation. In their research benzimidazole fungicide Carbendazim proved useful in shoot proliferation of this species.

Although the interaction between imidazole fungicides and 6-benzyladenine (BA) has been most commonly studied, research conducted on some representatives of the Araceae family has shown that imazalil, Prochloraz, and Triflumizole also enhance the effect of other cytokinins (zeatin, meta-topolin) as well as substances with cytokinin-like activity (thidiazuron) (Werbrouck and Debergh, 1996).

Studies on the mechanism of action of imidazole fungicides in *in vitro* culture of *Spathiphyllum floribundum* have shown that they do not alter the metabolism of exogenous cytokinins such as BAP (Werbrouck et al., 1999). Instead, their effect on representatives of the Araceae family is partly based on the inhibition of gibberellic acid ($GA_3$) biosynthesis (Werbrouck et al., 1996).

In this paper, we discuss the results of the influence of the imidazole fungicide Prochloraz on the multiplication and rooting of two fruit tree rootstocks – *Pyrus communis* L. 'Pyrodwarf' and *Prunus cerasus* × *P. canescens* 'Gisela 6'.

## 1. EFFECT OF PROCHLORAZ ON *IN VITRO* MULTIPLICATION OF FRUIT TREES

Despite the varying plant growth regulator (PGR) compositions of the growth media, the 'Pyrodwarf' pear rootstock displayed a very low multiplication capacity (Ružić et al., 2004), while cherry roostock Gisela 6 hardly rooted under *in vitro* conditions on media containing 5µM IBA or NAA, which were mostly employed for *Prunus* roooting (non-publish data). In an effort to improve and develop *in vitro* propagation and considering the activity of imidazole fungicides, we focused on investigating the influence of Prochloraz on the multiplication and rooting of these popular low-vigorous rootstocks. Nineteen combinations of 1, 5, or 10 µM Prochloraz applied alone or combined with different PGRs: 4.4 µM BA, 5 µM indole-3-butyric acid (IBA) and 0.3 µM gibberellic acid ($GA_3$) were tested in experiments (Table 1).

In both 'Pyrodwarf' (Ružić et al., 2008) and 'Gisela 6' rootstocks (Ružić et al., 2009; Vujović et al., 2009), Prochloraz was only effective in the induction of shoot formation

in the presence of cytokinin BA and not in se (Figures 1a and 2a). Adding Prochloraz (especially at 5 µM and 10 µM) to the medium that contains 4.4 µM BA significantly improves shoot induction in both genotypes as compared with results obtained on the medium containing BA alone. These results are in line with those obtained by Werbrouck et al. (1996) and Werbrouck and Debergh (1995; 1996). In their experiment, neither imidazole fungicides such as Imazalil, Prochloraz, and Triflumizole nor Paclobutrazol showed cytokinin effects on cytokinin-free medium. In addition, these fungicides did not significantly alter the metabolism of exogenous cytokinins, such as BA in *S. floribundum* and comparing the endogenous cytokinin pool with or without Imazalil did not lead to conclusions (Werbrouck et al., 1999). All these findings exclude the possibility that the cytokinin-like effect of imidazole fungicides is involved.

**Table 1.** Experimental design

| Type and concentration of PGRs and PRO | Combination | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** |
| BA 4.4 µM | | | | | | | | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | | |
| $GA_3$ 0.3 µM | | ■ | | ■ | | ■ | | ■ | | ■ | | ■ | | ■ | | ■ | | | |
| IBA 5 µM | | | | | | | | | | | | | | | | | ■ | ■ | ■ |
| PRO 1 µM | | | ■ | ■ | | | | | | | ■ | ■ | | | | | ■ | | |
| PRO 5 µM | | | | | ■ | ■ | | | | | | | ■ | ■ | | | | ■ | |
| PRO 10 µM | | | | | | | ■ | ■ | | | | | | | ■ | ■ | | | ■ |

PGRs – plant growth regulators; PRO – prochloraz; BA - 6-benzyladenine; $GA_3$ – gibberellic acid; IBA - indole-3-butyric acid

The structural similarity of imidazole fungicides with triazole types of plant growth retardants suggested their interference in the gibberellin pathway (Werbrouck et al., 1999). It is widely recognized that gibberellins and cytokinins have antagonistic effects on various developmental processes, such as shoot and root elongation, cell differentiation, shoot regeneration in culture, and meristem activity (Weiss and Ori, 2007). According to Werbrouck et al. (1996), endogenous gibberellins inhibit the full expression of exogenous cytokinins like BA, which have the potential to induce shoot growth. In experiments by Ružić et al. (2008; 2009) and Vujović et al. (2009), 4.4 µM BA applied alone was not able to induce proliferation in both analyzed fruit tree genotypes. Adding Prochloraz to the medium with BA probably removes the block by inhibiting gibberellin biosynthesis, which increases shoot proliferation. Additionally, a significant increase in the shoot multiplication index in both genotypes was observed when the Prochloraz concentration was increased from 1 to 10 µM while maintaining a constant BA content of 4.4 µM (Figures 1a and 2a). On the other side, the absence of shoot multiplication when Prochloraz is added to a BA-free medium could indicate that the level of endogenous cytokinins is not adequate to induce shoots, even when the blocking effect of endogenous gibberellins is diminished.

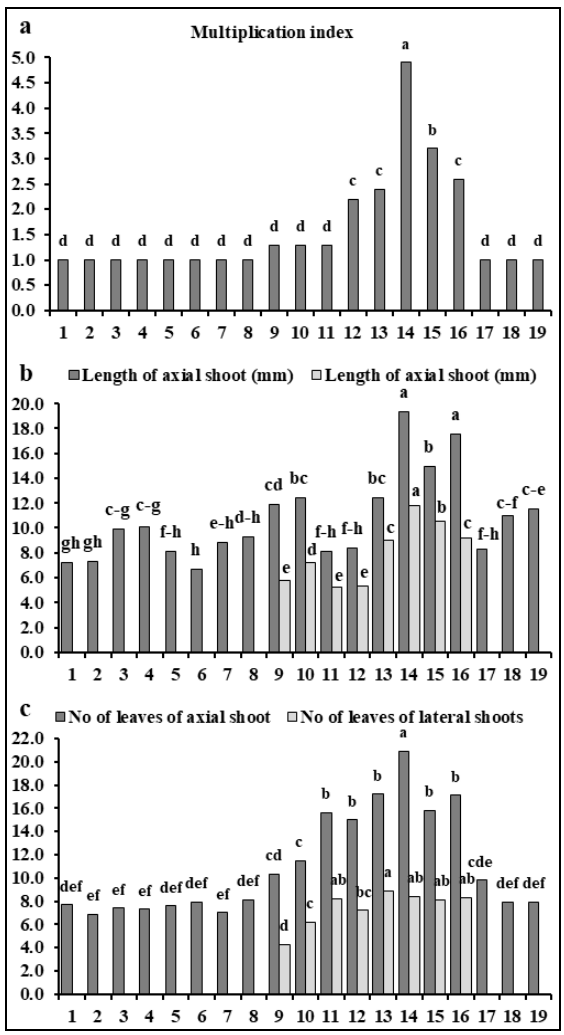



**Figure 1.** Effect of Prochloraz applied either alone or in combination with 4.4 µM BA, and/or 0.3 µM $GA_3$, and/or 5 µM IBA on multiplication capacity *in vitro* of 'Gisela 6' rootstock (Vujović et al., 2009; Ružić et al., 2009)

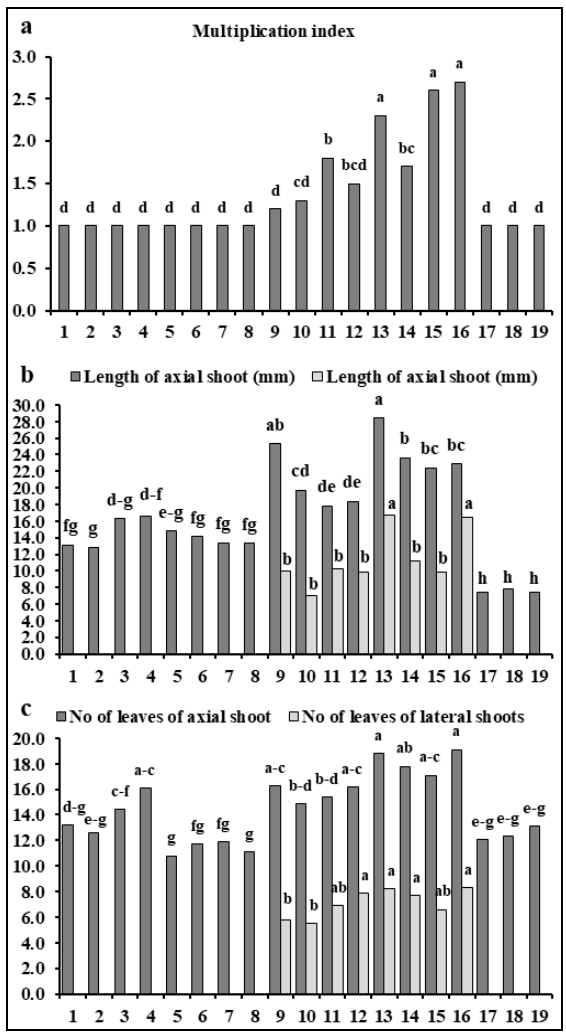



**Figure 2.** Effect of Prochloraz applied either alone or in combination with 4.4 µM BA, and/or 0.3 µM $GA_3$, and/or 5 µM IBA on multiplication capacity *in vitro* of 'Pyrodwarf' rootstock (Ružić et al., 2008; Ružić et al., 2009)

For each parameter means followed by the same letter are not significantly different at the 5% level of significance using Duncan's Multiple Range Test; Type and concentration of PGRs and Prochloraz for each experimental treatment are presented in the Table 1.

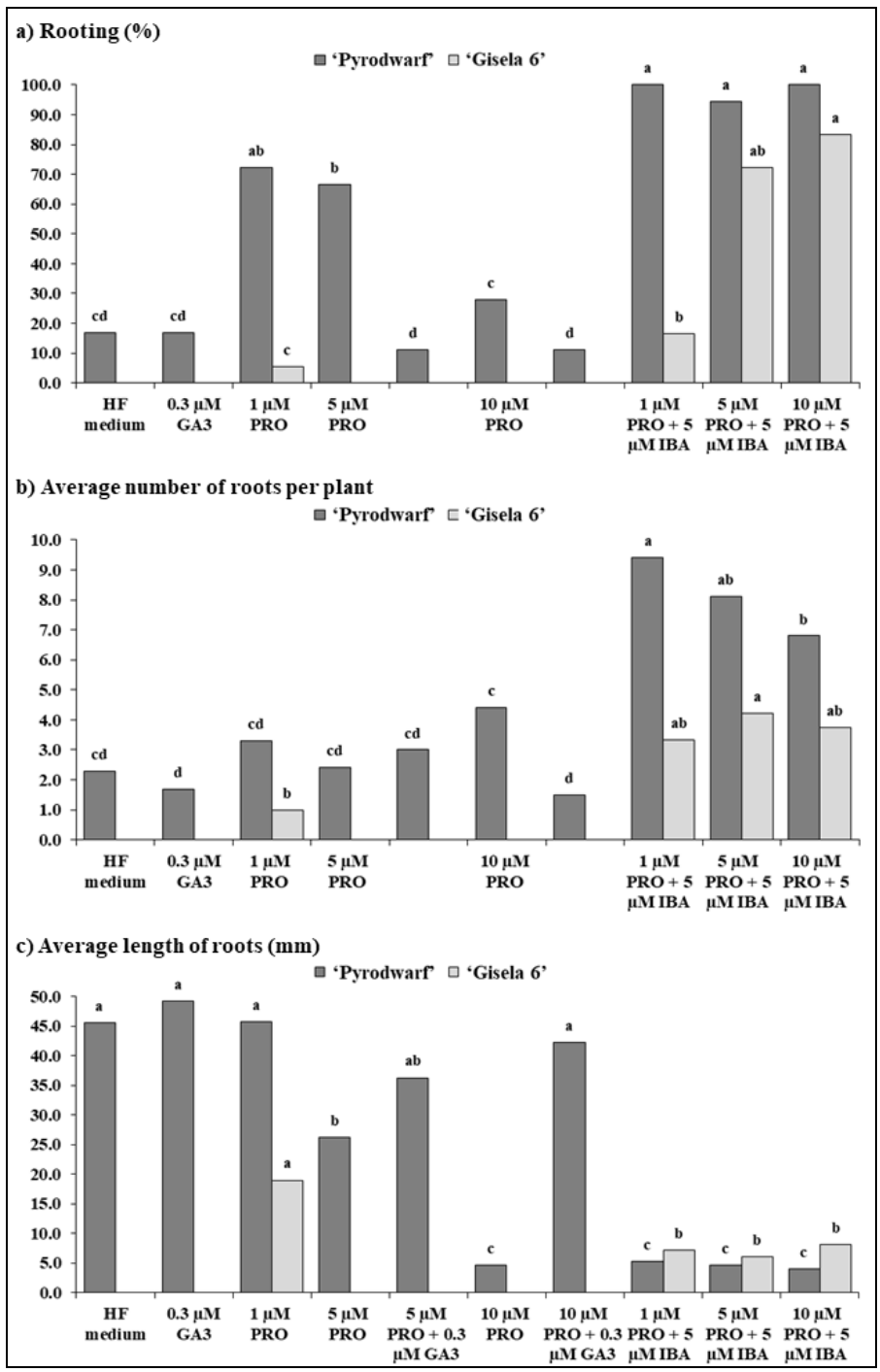
Evidence that adding $GA_3$ counteracts the effect of imidazole, as demonstrated with Imazalil (Werbrouck et al., 1999), supports the hypothesis that $GA_3$ might be involved in this phenomenon. However, in experiments with 'Pyrodwarf' and 'Gisela 6', the effect of adding 0.3 µM $GA_3$ in a medium containing BA and Prochloraz was dependent on both the concentration of Prochloraz and the genotype (Ružić et al., 2008, 2009;

Vujović et al., 2009). A significant decrease in multiplication index was observed in the presence of 10 µM Prochloraz in 'Gisela 6' (Figure 1a) and 5 µM Prochloraz in 'Pyrodwarf' (Figure 2a), but still, the values of this parameter remained higher than those noted on medium with BA applied alone or in combination with $GA_3$. Adding $GA_3$ in media containing BA combined with Prochloraz at two other concentrations (1 µM and 10 µM) did not significantly affect capacity for multiplication in 'Pyrodwarf'. Contrary to this, in 'Gisela 6', when 0.3 µM $GA_3$ was added to media containing BA and Prochloraz (1 µM or 5 µM), a significant increase in multiplication index was noted. Consequently, combining BA and $GA_3$ with 5 µM Prochloraz in 'Gisela 6' (Figure 1a–c) or 10 µM Prochloraz in 'Pyrodwarf' (Figure 2a–c) resulted in the best multiplication in terms of all parameters analyzed (multiplication index, shoot length, and number of axial and lateral shoots).

## 2. EFFECT OF PROCHLORAZ ON *IN VITRO* ROOTING OF FRUIT TREES

In addition to its impact on multiplication, Prochloraz also demonstrated a significant effect on the rhizogenesis *in vitro* of fruit tree rootstocks. The summary results of studies by Ružić et al. (2008; 2009) and Vujović et al. (2009) are presented in Figure 3. For the pear rootstock 'Pyrodwarf', rhizogenesis was observed in 10 out of 19 tested PGR combinations, while for the rootstock 'Gisela 6', rooting was achieved with four PGR treatments.

Prochloraz applied alone induced rooting in 'Pyrodwarf' regardless of the concentration used, although the rooting percentage decreased from 72.2% to 27.8% with increasing concentrations of Prochloraz. Tefera and Wannakrairoj (2004) also noted a decrease in root number and root length in korarima with increasing Imazalil concentration (0–4 mg/l) in the absence of cytokinins. In the study of Sarropoulou and Maloupa (2019), 2.5–10 mg/l of Imazalil, even though it promoted root elongation of *S. raeseri* shoot tip explants, led to a simultaneous decrease in root number and rooting percentage. Adding $GA_3$ to the medium containing Prochloraz either completely inhibited rhizogenesis (1 µM Prochloraz) or led to a statistically significant reduction in the rooting percentage of 'Pyrodwarf' (Figure 3a). $GA_3$ addition did not affect other rooting parameters when applied in medium with 5 µM Prochloraz, but in combination with 10 µM Prochloraz, a significant decrease in the number of roots and an increase in root length were observed (Figures 3b–c). The highest root formation was observed on media containing Prochloraz in combination with IBA, and the concentration of Prochloraz did not significantly affect most of the rooting parameters. Short stems and large, wide leaves of deep green color were common characteristics of all plants rooted in these media. Applied either individually or combined with IBA, Prochloraz influences the formation of a large callus mass as well as a very high rooting rate of the Pyrodwarf rootstock, particularly when combined with IBA. Contrary to these results, in gerbera 'Rosabella' (a bushy cultivar), Imazalil reduced callus formation (Topoonyanont and Debergh, 2001).




For each genotype means followed by the same letter are not significantly different at the 5% level of significance using Duncan's Multiple Range Test

**Figure 3.** Effect of Prochloraz applied either alone or in combination with 0.3 µM $GA_3$ or 5 µM IBA on rooting capacity *in vitro* of 'Pyrodwarf' and 'Gisela 6' rootstocks (Ružić et al., 2008; Vujović et al., 2009; Ružić et al., 2009)

In 'Gisela 6', Prochloraz applied alone at a concentration of 1 µM induced rooting, but at a low percentage (Figure 3a). However, all three tested concentrations of Prochloraz in combination with IBA influenced rhizogenesis, with the highest rooting percentage (83.33%) obtained on the medium with 10 µM Prochloraz (Figure 3a). Adding Prochloraz to the medium with IBA resulted in an increase in the rooting percentage proportional to the increase in its concentration, while other rhizogenesis parameters did not vary significantly (Figures 3b–c). The rooted plants had wide, green leaves and short stems. The callus was small, firm, compact, and dark brown. The obtained results for the rooting percentage of this genotype on medium containing 5 mM IBA in combination with 10 mM Prochloraz are significantly higher than those obtained by Rathore et al. (2024) on medium containing only IBA in a one-step rooting procedure and are comparable to the results achieved using the two-step method. However, the number of roots and particularly the root length on media with Prochloraz were significantly lower compared to the results achieved by the aforementioned authors.

In both rootstocks, rooting on media containing Prochloraz combined with IBA was accompanied by specific characteristics of the formed roots – short and thick radially spread roots. Werbrouck et al. (1999) also observed that imidazole fungicides influence the formation of thicker roots, whose elongation is significantly inhibited, which is in agreement with the results obtained for 'Gisela 6'. However, according to Ružić et al. (2004), rooting of Pyrodwarf shoots on medium with IBA resulted in 92% of plants developing short and thick roots, indicating that Prochloraz was not a contributing factor.

## 3. CONCLUSION

Enhanced propagation through the manipulation of *in vitro* conditions is achievable by the application of imidazole fungicides. The effect of the imidazole fungicide Prochloraz on *in vitro* shoot multiplication of 'Pyrodwarf' and 'Gisela 6' rootstocks is evident only in the presence of exogenous cytokinins such as BA. Procholraz, applied either individually or in combination with IBA, also significantly affects root induction *in vitro*. However, different plant genotypes exhibit varying responses to fungicides, necessitating genotype-specific considerations when designing tissue culture protocols.

While the use of fungicides in tissue culture offers substantial benefits, it also presents challenges. Optimizing the concentration and exposure time of fungicides is crucial to avoiding phytotoxic effects. Furthermore, the possibility of fungicide residues influencing following plant growth or being transferred to regenerated plants should be thoroughly investigated. Advances in this area could lead to the creation of novel fungicide formulations or application strategies that maximize benefits while minimizing adverse effects. A deeper understanding of these interactions could revolutionize plant tissue culture practices, making them more efficient and reliable for a wide range of plant species.

In summary, fungicides play a vital role in tissue culture beyond their antifungal properties. Their ability to influence plant growth and development presents exciting opportunities for improving propagation techniques. However, careful management and further research are essential to realize their full potential safely.

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# *WORKING GROUP 2:*

# Diagnosis, sanitation, and conservation

# Tissue culture as proper tool for forest tree breeding – A case study with wood of value

**Anne-Mareen E. Eisold, Cornelia Bäucker, Volker Schneck**

*Thünen Institute of Forest Genetics, Eberswalder Chaussee 3a, 15377 Waldsieversdorf, Germany*

a.eisold@thuenen.de

## Abstract

Tissue culture techniques play a crucial role as tools for breeding and conservation purposes. Especially for trees, which grow slowly and have long generation cycles, biotechnological methods can be particularly helpful in the preservation and propagation of desired or valuable genotypes. On the basis of a long-term breeding story, we present an overview about the recent relevance of tissue culture for the conservation of trees with specific wood anatomic properties. In detail, we focus on the workflow of the *in vitro* establishment of maple (*Acer pseudoplatanus*) clones with wavy grain figure. Often, a small amount of vegetative explant material is sufficient to generate stable *in vitro* cultures and to produce clonal tree plantlets. These plantlets serve as source for future trees of value in an auction-based timber market where logs with wavy grain consistently achieve top prices. Therefore, *in vitro* cultures of trees with desired valuable wood properties may have positive economic implications for forest owners and forestry in future.

***Keywords:*** *tissue culture, wood of value, forest tree breeding, clonal plantlet production, wavy grain figure.*

## 1. INTRODUCTION

Forest tree breeding plays a strategical role in transformation processes of forestry. Trees are long living organisms with long reproduction cycles. Suffering from increasing biotic and abiotic stresses the acceleration of both natural and assisted reforestation is needed. Therefore, breeding of adapted forest trees and conservation of endangered genetic resources become more a research focus in forestry. Here, biotechnological approaches are still being important to overcome bottlenecks of conventional breeding methods.

At the Thünen Institute of Forest Genetics (Germany), the biotechnological method of tissue culture has a long history and different research questions have been studied in the recent years. These topics were related to (i) conservation and vegetative propagation of threatened forest tree species, (ii) the optimization and up-scaling of *in*

*vitro* propagation in order to provide trees for field trials / commercial issues and (iii) the cultivation and propagation of clones with certain wood characteristics.

Currently, most of the ongoing research projects are addressed to tree species that show serious damage due to drought and/or the spread of pathogens, such as beech (*Fagus sylvatica*) or ash (*Fraxinus excelsior*). Furthermore, there is other research into optimizing the tissue culture method for sycamore maple (*Acer pseudoplatanus*) with wavy grain figure and for curly birch (*Betula pendula* var. *carelica*) (Bäucker and Liesebach 2018, Quambusch *et al.* 2021, Ewald *et al.* 2000). The collection of clones with special wood properties was expanded and supplemented by other tree species, for which all single steps of tissue culture procedure had to be adjusted.

At the institute, the majority of the current research into biotechnological methods on tree species is embedded in research projects funded by the Federal Government through the Fachagentur für Nachwachsende Rohstoffe e.V. (FNR): BUCHETIG (funding code 2219WK60A4), FRAXFORFUTURE (funding code 2219WK21D4), PYROPHOB (funding code 2219WK50E4), TREEEDIT (funding code 2219NR359) and WERTHOLZ (WOOD OF VALUE, funding code 2221NR009C). As the projects usually only run for a few years, the continuation of established *in vitro* cultures can be a challenge.

## 2. *IN VITRO* CULTURE OF WOOD OF VALUE

Timber of sycamore maple (*acer pseudoplatanus*) and other tree species with special grain characteristics, such as wavy grain, is sold at top prices at central European timber auctions. Therefore, methods for conservation and propagation of trees with such value-enhancing wood characteristics can make a substantial contribution to increase the value chain in forestry. The long-term preservation of the genetic clonal material of such trees is achieved by grafting and establishment in *in vitro* culture. Within the framework of current research work, the clone collection is being expanded and the *in vitro* clones are genetically characterized by ssr markers. The long-term aim is the efficient production of approved wavy grained clones for forestry.

### 2.1. Wavy grain wood

Wavy grain is a rare figure type of wood resulting from undulating fiber growth – also known as ”fiddleback” figure. Undulations of the wood fibers in the tree rings result in a systematically modified orientation of cambial cells, which finally leads to the characteristic S-shape of the fibers along the tangential axis (Quambusch *et al.* 2021, Lewandrowski *et al.* 2024). Although this grain differs from the normal straight grain, it is highly appreciated for its decorative effect in high-quality furniture manufacturing and in music instrument making due to its specific physical-acoustic characteristics (Kúdela and Kunštár 2011, Sopushynskyy and Teischinger 2013) (Fig. 2E). The top price levels of wavy grained maple timber at auctions in Germany and Switzerland show the high demand of extraordinary wood and their economic value (Fig. 1A).

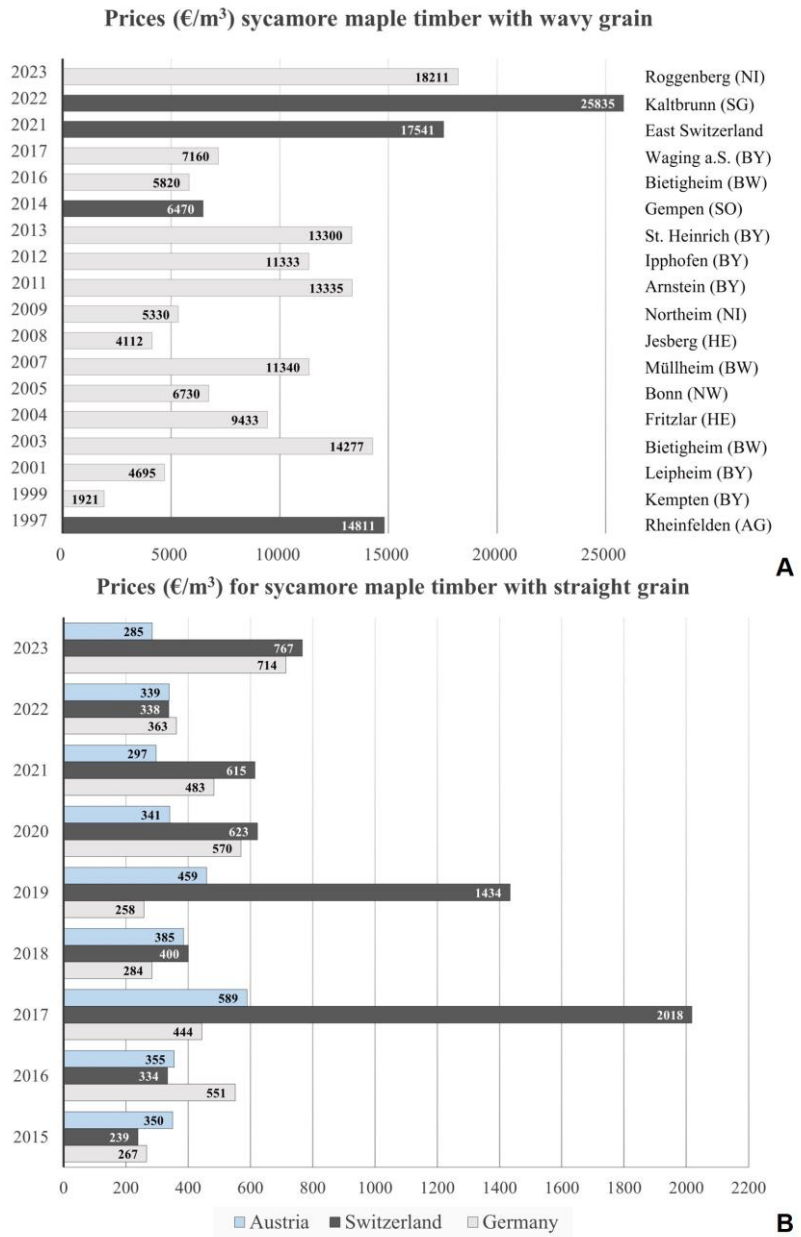


**Figure 1.** Overview about prices (€/m$^3$) for timber of sycamore maple with wavy grained (A) and straight grained wood (B) achieved on auctions in Germany (light grey bars), Switzerland (dark grey bars) and Austria (blue bars). Places of auctions and respectively origins of logs are indicated with corresponding federal state (data collection of the Thünen Institute). AG: Argau; BW: Baden-Württemberg; BY: Bavaria; HE: Hesse; NI: Lower Saxony; NW: North Rhine-Westphalia; SG: St. Gallen; SO: Solothurn. Prices for straight grain timber are achivable on hompages of local auctions in Vogelbeck/Germany (www.landesforsten.de/bewirtschaften/holz/holzverkauf/submissionen/), in Büren a.A/Switzerland (www.fb-bucheggberg.ch/submission), in Heiligenkreuz/Austria (waldverband-noe.at/wp-content/uploads/2023/).

In extreme cases, the timber of fiddleback maple achieves more than the 50fold of the price of straight maple wood, which ranges frequently from 200 to 700 Euro. Some exceptions can be found for straight grained timber of excellent quality. Here, prices up to 2000 Euro per solid cubic meter have been paid at local auctions (Fig. 1B).

As no external morphological hint allows the detection of trees with "fiddleback" figure, the identification of wavy grained timber occurs after felling of the trees by visual inspection and persists sometimes as wavy structure at the stump (Fig. 2D). For this reason, the exact proportion of individuals with wavy grain within a maple population remains unknown, but percentages between 3 and 7% have been reported (Kúdela and Kunštár 2011, Sopushynskyy and Teischinger 2013). Wood anatomic analyses revealed a strong age dependent appearance as well as strong hints for a genetic inheritance of the wavy grained structure (Quambusch *et al.* 2021). The intensity of wavy grain figure appears heterogenous along the stem axis and ranges between low and high levels.

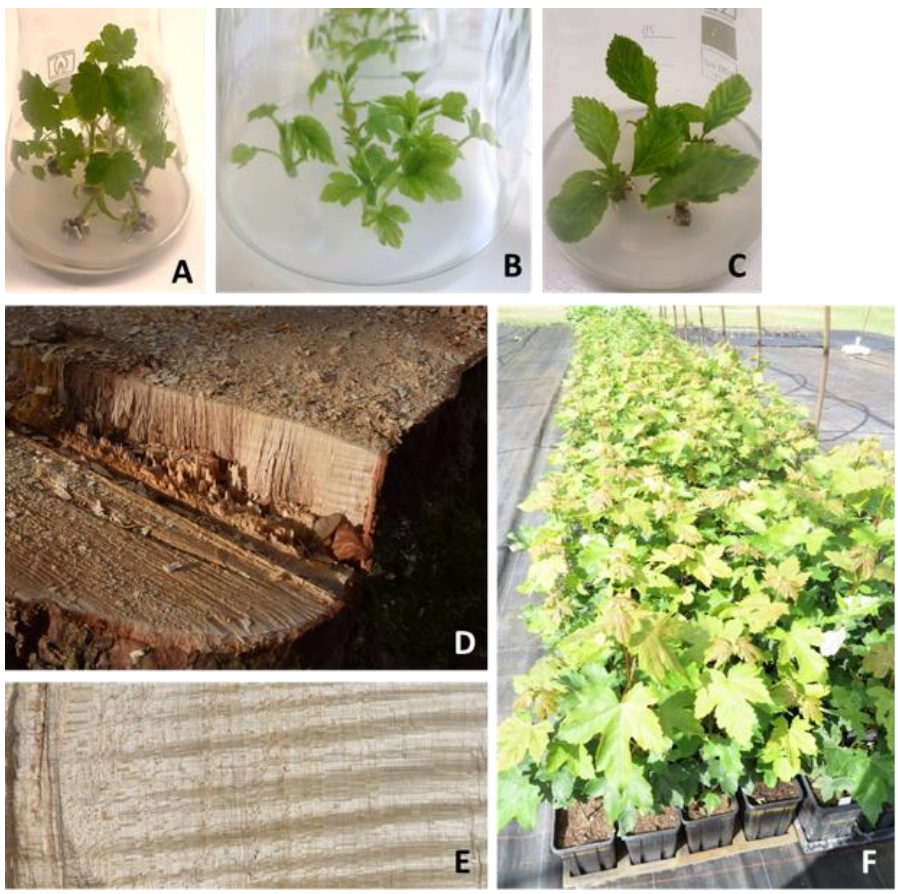


**Figure 2.** *In vitro* clones with wavy grain of *Acer campestre* (A), *Acer pseudoplatanus* (B), *Alnus glutinosa* (C), wavy grained wood visible on the stump of a felled sycamore maple tree (D), a processed piece of wavy grained timber (E), plants from wavy grained maple clones produced for clone tests (F).

## 2.2. Tissue culture as conservation and production method

The *in vitro* conservation of material from wavy grained trees bases on the classical tissue culture scheme, with steps of sterilization and transfer of the explant to culture medium. Once the clones are established and adapted to the sterile microclimate conditions in the tissue culture laboratory, they are supposed to be propagable in order to finally produce high amounts of clonal plantlets (Fig. 2F). Therefore, the plantlets have to be rooted, followed by acclimatization under *ex vitro* conditions (Fig. 3). In total, the tissue culture workflow beginning from the preparation of buds until the acclimatization under natural conditions takes a minimum time period of approximately one year.

A very important step for the successful establishment of plant material in the tissue culture is the surface sterilization of the material. As material we use winter buds from tree branches of *A. campestre*, *A. pseudoplatanus* and *A. glutinosa*. The branches have been cleaned under rinsing water, cut into 2-3 cm pieces and buds have been dissected. After incubation of the buds for 15 min in 0.05% $AgNO_3$ and 2 drops Tween® 20, they have been flushed in *Aqua dest.*, dried and outer bud scales have been removed. The explants of *Acer* spec. have been transferred on establishing media, which consists of double concentrated woody plant medium (WPM, Duchefa) (Lloyd and McCown 1980) supplemented with 0.03 mg/L Thidiazuron, 20 g/L saccharose and 8 g/L Phytoagar. Recently, additives have been tested to decrease the contamination ratio. The medium for the establishment of *A. glutinosa* consists of full concentrated WPM supplemented with 7.65 ml/L Zeatin, 32.8 g/L glucose and 8g/L Phytoagar.

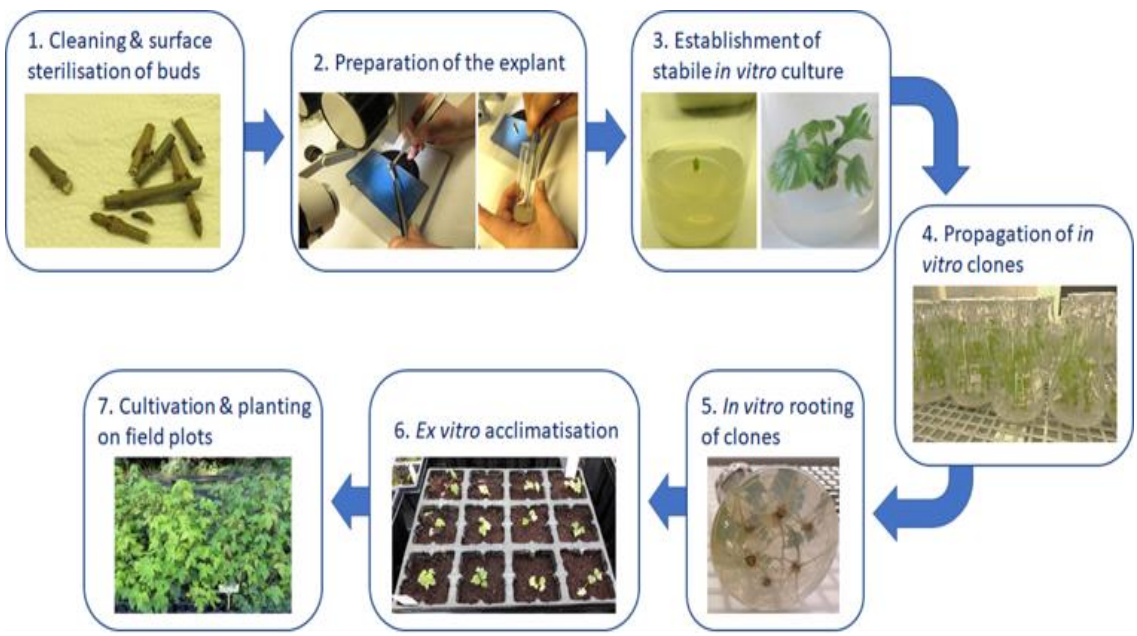


**Figure 3.** Workflow of *in vitro* establishment, propagation and production of clonal plantlets.

During the workflow of tissue culture, procedure details as well as culture media have to be optimized depending on the tree species and, sometimes, genotype specific needs. This means a permanent calibration of hormonal treatment, light conditions and the frequency of transfer cycles. A discontinuity of adjustments, which may occur in the context of non-permanent projects, can cause serious physiological disorders of the clonal plants, visible as browning, hyperhydricity or dieback (Debergh *et al.*, 1992; Polivanova and Bedarev, 2022; Rojas-Martínez *et al.*, 2010). Within the 2-year gap between the funded research projects on wavy grain maple, we recognized significant losses of clonal material (Fig. 4). To our experience, the propagation performance of the plants differs a lot between the clones of the same tree species. So, a continuous transfer to fresh medium is obligatory for the maintenance of the overall clone collection and to ensure stabile nutrition conditions and exogenous hormone levels (Polivanova and Bedarev, 2022). If the time period of the transfer to fresh medium is extended to the double time of the optimum, many clones stagnate in their growth. Finally, only clones with an adequate resilience amplitude are able to show height growth of the shoots and, therefore, are propagable. This underlines the need for a permanent and skilled handling of the clones.

The clone collection of the ongoing project comprises almost 54 steady clones of donor trees with wavy grain. These clones come from the following species: *A. pseudoplatanus* (51 clones), *A. campestre* (1 clone), *Alnus glutinosa* (1 clone) and *Salix caprea* (1 clone), (Fig. 2A-2C). As an unwanted epigenetic effect in tissue culture somaclonal variations has been described, caused by a constant stress during *in vitro* propagation (Leva *et al.*, 2012, Bairu *et al.*, 2011). Therefore, the storage of suitable vegetative material in cryopreservation is supposed to be mandatory for a stabile long-time conservation of the clones, especially for clones of recalcitrant species After thawing, the plant cells are able to recover and to proliferate, which facilitates the restart of *in vitro* culture of the clones. Thus, the genetic identity of clonal material is supposed to be conserved over a long time (Amankwaah *et al.*, 2022).

Before the commercialization, the clones are supposed to be evaluated for certain physiological and wood anatomical characteristics in the field, such as growth performance, physiological fitness, straightness of trunks and the occurrence of "fiddleback" figure (Bäucker *et al.*, 2020). The clone test under field conditions is the prerequisite for the approval of clones in the category "Tested" according to the regulations on the production and marketing of forest reproductive material in Germany. Due to the long growing period that is required for wood maturation, the approval will be possible after 15 years of tree growth earliest. If the clones meet the requirements, the clone stock in the tissue culture will form the basis for commercial propagation and finally clone tree production.

Thus, the *in vitro* conservation of the clones with the valuable potential to form wavy grained wood is essential for future research purposes as well as for the later commercial use of the selected clones.

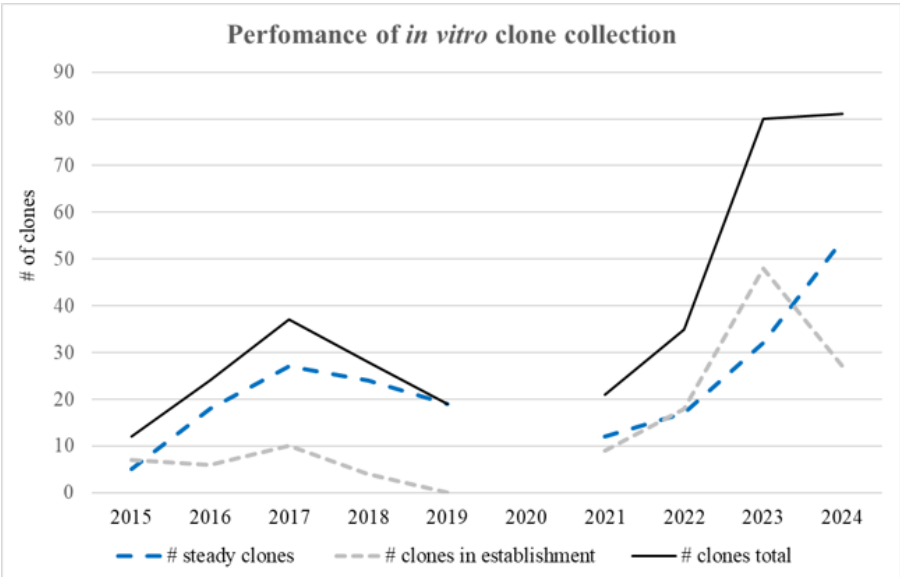


**Figure 4.** Performance of the *in vitro* clone collection over a period of two projects (2015-2019 and 2021-2024) representing the number (#) of steady clones (blue dashed line), of clones in the stage of establishment (grey dashed line) and total number of clones (black line).

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# Cryopreservation of dormant raspberry buds for further *in vitro* cultivation

**Olena Bobrova[1,2], Alois Bilavcik[1], Milos Faltus[1], Jiri Zamecník[1]**

[1]*Plant Physiology and Cryobiology, Crop Research Institute, Drnovska 507, 16106 Prague 6, Czech Republic*

[2]*Institute for Problems of Cryobiology and Cryomedicine NAS of Ukraine*

helen.bobrova.77@gmail.com

## Abstract

The aim of this study was to develop a protocol for the low-temperature storage (cryoconservation) of dormant raspberry (*Rubus idaeus* L.) buds and their subsequent introduction into *in vitro* culture. One-year-old dormant canes of the Sanibelle and Willamette raspberry varieties were used in the experiments. Nodal segments with a single bud in the middle were previously frost-dehydrated until they reached a water content of 20 to 30% of fresh weight. At different stages of dehydration, the water state in the buds was monitored by measuring water activity, water content, and the percentage of crystallized water. The study revealed a high resistance of dormant raspberry buds to dehydration. The dehydrated raspberry buds were then frozen using a two-step cryoprotocol. After thawing, the cryopreserved raspberry buds were rehydrated. The buds were washed and sterilized in several stages and prepared for *in vitro* cultivation. Then, the buds with a portion of the twig were placed on a sterile semi-solid nutrient medium. A sufficient level of *in vitro* plant regeneration after cryopreservation of dormant buds was achieved. Therefore, cryopreservation of dormant buds of woody crops can be successfully utilized to preserve plant genetic resources. These cryopreserved buds can later be used at a convenient time for *in vitro* plant introduction for further multiplication and regeneration.

***Keywords:*** *cryopreservation, Rubus idaeus L., crystallized water, water activity, water content.*

## 1. INTRODUCTION

Cryopreservation involves storing samples of biological plant material at extremely low temperatures, typically in liquid nitrogen, which halts all cell division and metabolic activities. This method is considered ideal for the long-term, reliable, and cost-effective preservation of the germplasm of various plant species without alteration (Benelli, 2021). The ability of woody plant materials to endure cryopreservation is influenced by their physical and biological properties. Shoot tips are the most commonly used tissues for this purpose. Additionally, plant buds, which contain the shoot meristem and mature tissues, can also be utilized as explants. Shoot tips and buds are favoured for preserving

the genetic resources of vegetatively propagated plants due to their greater genetic stability compared to other explants, such as cells and embryogenic tissues. Dormant buds exhibit higher frost resistance due to physiological adaptations (Arias et al., 2017). However, this frost resistance is often insufficient for cryopreservation without prior pretreatment in most woody plants. Many cryopreservation protocols involve reducing the water content of branch nodes or dormant buds before freezing to prevent cryodamage caused by water crystallization (Choudhary et al., 2023). For instance, a protocol for the cryopreservation of dormant apple and mulberry buds includes drying stem explants at -4°C followed by a two-step freezing process (Choudhary et al., 2023; Vogiatzi et al., 2011). Dormant buds of some woody plants can be cryopreserved without prior dehydration. The state of water in dormant plant buds plays a crucial role in their preservation.

The purpose of this work was to study the state of water in dormant raspberry buds during dehydration and to develop a protocol for their cryopreservation for further *in vitro* cultivation.

## 2. MATERIALS AND METHODS

**Materials:** One-year-old dormant canes of the Sanibelle and Willamette raspberry varieties (*Rubus idaeus L.*) were collected during the winter seasons of 2021-2024 from the orchard of the Crop Research Institute in Prague, Czech Republic. Nodal segments (35 mm) with a single bud in the middle were freeze-dehydrated at -4°C in open air until they reached a water content of 20 to 30% of their fresh weight.

**Methods:** The water status in raspberry nodal segments was assessed through measurements of water activity, water content, and the percentage of crystallized water. Water activity in the raspberry node segments was determined using a Water Activity Meter HP23-AW-A (Rotronic, Switzerland). The water content in the samples was determined gravimetrically by measuring the mass change between fresh and dried materials. Low-temperature phase transitions in the raspberry buds were analyzed using a Q2000 differential scanning calorimeter (TA Instruments, USA). Thermal effects were recorded during cooling and heating at a rate of 10°C/min over a temperature range from -90°C to 25°C. Thermal analysis of the buds were repeated three times. Phase transition temperatures and percentages of crystallized water in the plant tissues were determined using TA Instruments Universal Analysis 2000 software.

Freezing in 50 ml tubes covered with aluminum foil was carried out using a two-step cryoprotocol: **1.** Cooling from -4°C to -30°C (cooling rate of 1°C/h) in a computer-controlled freezer, followed by equilibration for 24 hours; **2.** Immersing the tubes into liquid nitrogen.

Thawing of the nodal segments was carried out by slow thawing at +4°C. The nodal segments were then rehydrated in moist white peat for 14 days at +4°C. After

rehydration, the nodal segments were washed under running tap water for 20 min and then sterilized as described by Välimäki et al. (2022). Next, the buds, with a 10 mm segment of the twig, were placed on a semisolid MS medium modified according to Dziedzic and Jagla (2013).

## 3. RESULTS

A study of water activity and water content in dormant raspberry nodal segments during freeze-dehydration showed that the dynamics of change are non linear and the rate of dehydration decreases over time (Fig. 1). For both studied varieties Sanibelle (Fig. 1A) and Willamette (Fig. 1B), after a certain time of dehydration, water activity and water content practically do not change. Due to the fact that dormant raspberry buds have a high resistance to dehydration, they can be cryopreserved over a wide period of time after reaching this level of dehydration.

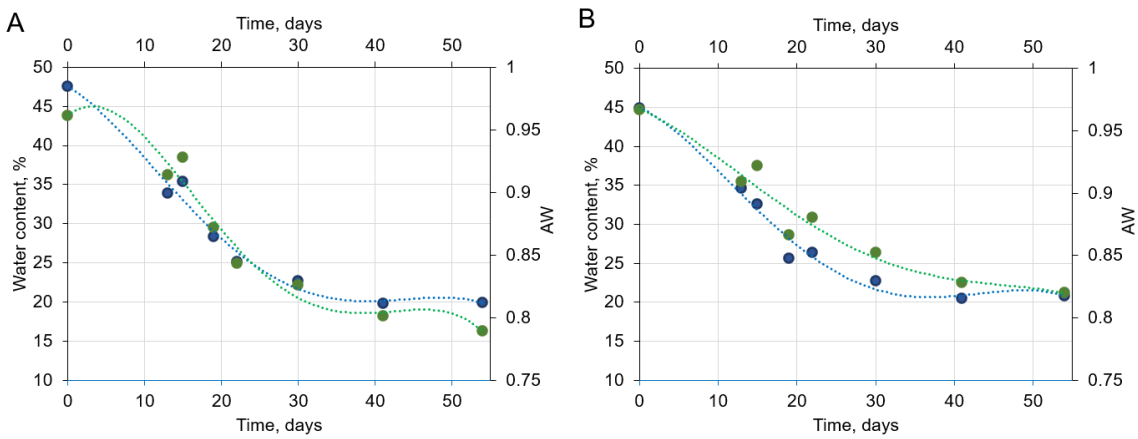


**Figure 1.** Changes in water content (blue markers) and water activity (green markers) of nodal segments for Sanibelle (A) and Wilamette (B) varieties during dehydration

Calorimetric studies revealed that as dehydration progresses, the melting point naturally decreases due to the higher concentration of solutes in dormant raspberry buds (Fig. 2).

Moreover, the crystallization temperature exhibits a more pronounced decrease with increasing dehydration time, indicating a heightened tendency for water to supercool as the moisture content of the buds decreases. At high cooling rates, this supercooling-prone water can vitrify, but insufficient dehydration could result in a metastable glassy state, leading to crystallization during the heating stage. The percentage of crystallized water also decreases with increasing dehydration time and beyond a certain level of dehydration the percentage of crystallized water is less than 1%. This level of dehydration correlates with a reduced dynamics of decrease in humidity and water activity. Raspberry buds dehydrated to this moisture level exhibit high viability and effectively withstand cooling using a two-stage cryoprotocol.

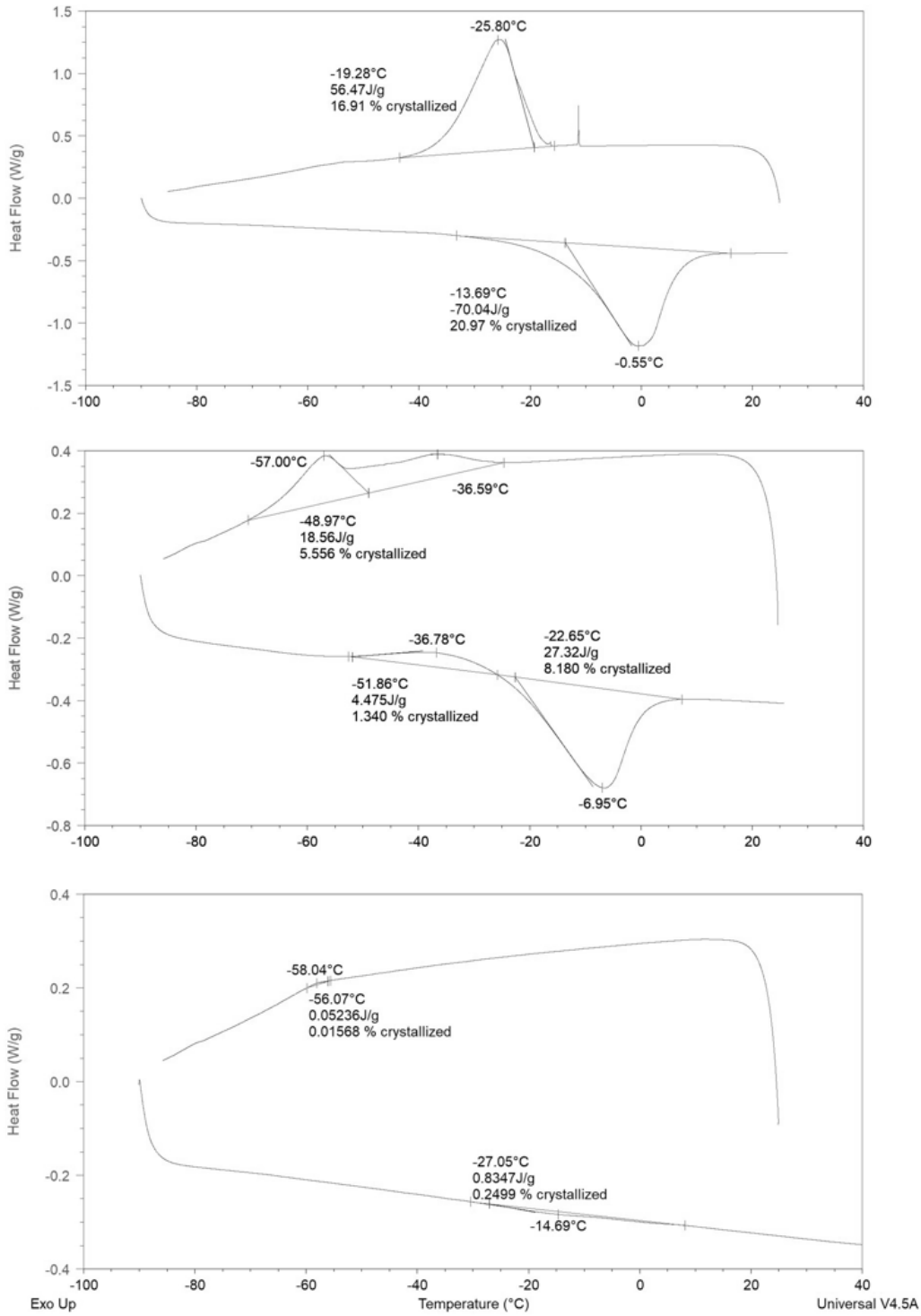


**Figure 2.** Changes in temperatures of phase transitions and percentage of crystallized water in raspberry buds (Sanibelle variety) during dehydration (control at the top and the more dehydrated the buds, the lower in the graph)

Since raspberry buds cannot be grafted, the plants were regenerated *in vitro* (Fig. 3). Non-dehydrated raspberry buds did not recover at all after cryopreservation, but as the level of dehydration increased, an improvement in bud viability was observed. A sufficient level of viability to regenerate plants *in vitro* was achieved after cryopreservation of strongly dehydrated dormant buds.

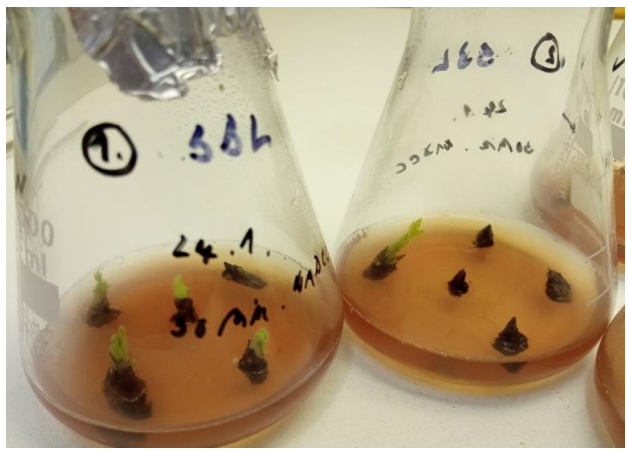

**Figure 3.** Regenerating of cryopreserved dormant raspberry buds of Sanibelle *in vitro* (13 days)

## 4. DISCUSSION

For plant buds, the primary mechanism of protection against low temperatures is extraorgan freezing (Ishikawa *et al.*, 2015; Pramsohler and Neuner, 2013). Bud scales, which possess high ice-forming activity, undergo crystallization with minimal supercooling, while flowers and meristematic tissues remain supercooled, acting as water sources for extraorgan crystallization. This phenomenon likely explains the appearance of two peaks of crystallization during the cooling of dormant raspberry buds (Fig. 2). However, the cooling rates used in the study using the DSC method (10°C/min) are too rapid for gradual freezing of water into the extraorgan space. The presence of two peaks of crystallization during cooling is a characteristic feature of buds in various plants (Kader and Proebsting, 1992). This inclination towards extraorgan freezing is exploited in cryopreservation techniques for dormant buds, which involve freeze-drying and two-step cooling to dehydrate the meristematic parts. This method is applicable to many fruit trees (Bilavcik *et al.*, 2021; Choudhary *et al.*, 2023; Rantala *et al.*, 2021; Vogiatzi *et al.*, 2011). Further research is needed to optimize the cryopreservation protocol for dormant raspberry buds by exploring variations in annealing times during the first step of cooling and warming rates.

## 5. CONCLUSIONS

Two-stage freezing of dormant raspberry buds after preliminary dehydration allows sufficient preservation for plant regeneration *in vitro* post-cryopreservation. Thus,

cryopreservation of dormant buds of tree crops can be successfully utilized to preserve plant genetic resources. These cryopreserved buds can later be used at a convenient time for the introduction of plants *in vitro* for further propagation and regeneration.

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# Synthetic seeds conversion to plantlets in *Punica granatum* L. cv. Devedishe after short-term storage at 4°C

**Yllka Lala[1], Klara Meta[1], Brunilda Çuko[1], Valbona Sota[1,2], Carla Benelli[3], Maurizio Lambardi[3], Efigjeni Kongjika[2]**

[1]*Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Boulevard "Zog I" Tirana 1001, Albania*

[2]*Biotechnology & Genetics Scientific Research Unit, Section of Natural and Technical Sciences, Academy of Sciences of Albania, "Murat Toptani" Promenade, Tirana 1000, Albania*

[3]*IBE-CNR, Istituto per la BioEconomia, Consiglio Nazionale delle Ricerche, via Madonna del Piano 10, 50019 Sesto Fiorentino (Firenze), Italia*

*yllka.lala@qttbfushekruje.gov.al*

## Abstract

*Punica granatum* L. (pomegranate) is an important fruit tree cultivated in Albania that has many uses in the food industry and for medical purposes. Due to climate change, agricultural systems face problems, and some cultivars of interest are at risk of extinction soon. For this reason, optimizing biotechnological methods for *ex situ* conservation of important pomegranate varieties is considered an emergency. This study aimed to preserve encapsulated explants of the Devedishe pomegranate variety for short-term periods at 4°C. As initial explants zygotic embryos were used and after their proliferation under aseptic conditions, the shoots were encapsulated using a 3% sodium alginate solution and a 1.1 mM calcium chloride ($CaCl_2$) solution. One group of the obtained synthetic seeds was incubated for 3 months at 4°C, and another group, considered as the Control one, at 25°C. After storing for 3 months, the synthetic seeds were transferred at 25°C, and conversion into plantlet rates were monitored and compared between groups. A high rate of conversion into plantlets (96.34%) was observed two weeks after encapsulation for the synthetic seeds incubated at 25°C; thus, it cannot considered a suitable technique for germplasm storage, but it can be beneficial for germplasm exchange. Meanwhile, the rate of conversion into plantlets of synthetic seeds incubated at 4°C was at 70.33% after 3 months of storage in these conditions. The micropropagation coefficient during further subcultures was not negatively affected. However, more extended conservation periods should be tested to further optimize slow-growth conservation strategies for pomegranates. Optimizing *ex situ* conservation protocols will make the sustainable utilization of pomegranate genetic resources cultivated in Albania possible for further use in preservation and genetic improvement programs.

***Keywords:*** *encapsulated explants, pomegranate, minimal growth techniques, ex situ conservation*

## 1. INTRODUCTION

With various climatic conditions and positioned in the Mediterranean basin's center, Albania is considered one of the most concentrated countries regarding tree species, forms, and populations. The pomegranate is one of the most important species from which many local populations and varieties originate. In addition to wild pomegranate populations in regions up to 700 m above sea level, several varieties are cultivated in Albania. The *Punica* genus belongs to deciduous shrubs or small trees. The best-known species of this genus is the pomegranate (*Punica granatum* L.). Currently, pomegranate is the focus of research, including cancer prevention or curative potential (Bhatia et al., 2013) and their antioxidant activity and anti-inflammatory properties (Sharma et al., 2017; Larrosa et al., 2010). Albania has its own autochthonous varieties of pomegranate, also known as "red gold", which are cultivated by many families of the Coastal Lowlands, starting from Malësia e Madhe to Saranda, (Koka, 2012; Xhuveli, 2012). Although suitable climatic conditions are found in most areas of Albania, it, as in other countries, has always been considered a second culture. Interest in its cultivation has increased in recent years. With the government's program to increase walnut and pomegranate crops, it is expected that the number of pomegranate plants in the next four years will increase 10-fold and production will increase 15-fold.

Conservation of plant genetic resources under *in vitro* conditions is one of today's priority issues. These genetic resources are endangered for various reasons. Despite the great wealth in these local varieties and forms, today, the market is mainly oriented towards foreign varieties, causing a gradual loss of autochthonous genetic resources of special importance, such as pomegranate, blueberry, and plum. Socio-economic changes and environmental exploitation are the basis of this process of erosion of autochthonous genetic biodiversity. This irreversible loss is of great concern to the national economy and, therefore, it is necessary to take measures and implement strategic methodologies for the collection, characterization, conservation and sustainable utilization of the valuable autochthonous genetic resources of the main fruit tree species. Alternative strategies are needed to conserve plant genetic resources. For this reason, it is necessary to develop short-term and long-term strategies for the conservation of plant genetic resources using *in vitro* techniques and to create genetic banks in the long term. In this context, *in vitro* propagation efficiently produces homogeneous collections of populations of interest (Kongjika et al., 2002; Debnarh et al., 2006; Neuman et al., 2009). The technology of synthetic seed production is considered an effective method for the propagation and conservation of plant species, which is of high economic importance due to their multiple uses and unique properties (Ara et al., 2000; Ravi and Anand, 2012). The real challenge that is highly affected by the genotype is plantlet recovery after maintaining the synthetic seeds in different physical or chemical conditions for short- or mid-term conservation purposes. This study aimed to establish a

short-term conservation protocol for encapsulated shoots of *Punica granatum* L. cv. Devedishe and plantlet recovery in post-conservation.

## 2. MATERIAL AND METHODS

### 2.1. Stabilization of aseptic cultures and regeneration of *in vitro* plantlets

***Plant material:*** Zygotic embryos of *Punica granatum* L. Devedishe variety that were used as primary explants were collected from populations in Milot, in northern Albania. Undamaged and uniform zygotic embryos were selected, peeled from the pulp, and allowed to dry for about two hours. The seeds were left in running water for 12-24 hours to swell the embryos and facilitate their isolation.

***Sterilization protocol:*** A meticulous sterilization process was followed to ensure the purity of the explants. Mercury bichloride (0.1%) was used for 15 min, followed by three rinses with double-distilled water to remove any $HgCl_2$ residues from the plant tissues.

***Explant proliferation:*** After isolation, the zygotic embryos were inoculated in WPM (Lloyd & McCown, 1981) basal medium supplemented with 1 mg/l of 6-Benzylaminopurine (BAP) and 0.1 mg/l of 1-Naphthaleneacetic acid (NAA). The concentration of sucrose was 3% and of agar 0.7%. The pH value was established at 5.6.

***In vitro chamber conditions:*** The explants were grown in a controlled environment to mimic their natural habitat. The growth chamber maintained a temperature of 25° ± 2°C, with a 16 h light/24 h regime using cool, white fluorescent light, providing optimal conditions for their development.

### 2.2. Production of synthetic seeds and their conversion to plantlets

***Production of synthetic seeds by the encapsulation technique:*** The apical shoots from the *in vitro* obtained plantlets were isolated under aseptic conditions and immersed in a 3% (w/v) sodium alginate solution. Both explants and sodium alginate solution were then dropped in the 100 mM $CaCl_2$ x $2H_2O$ solution, and were left in such conditions for 30 min.

***Incubation conditions:*** The obtained synthetic seeds were inoculated in above-mentioned WPM medium and were incubated at +4°C for three months and then transferred to the growth chamber at +25°C for conversion into plantlets. Synthetic seeds incubated directly at +25°C after their production were used as a control group.

***Conversion of synthetic seeds into plants:*** Synthetic seeds were periodically observed for their survival percentage, possible contamination occurrence, and regeneration into whole plants. This thorough observation ensured the accuracy of the results.

## 3. RESULTS AND DISCUSSIONS

### 3.1. *In vitro* organogenesis and proliferation of the zygotic embryos

Pomegranate zygotic embryos are easily stabilized in culture. Germination of the embryos was observed 3 days after their inoculation under aseptic conditions (Fig. 1a).

There were no signs of necrosis and the contamination percentage was at 4%. This is an essential step of the experimental plan because it ensures the acquisition of aseptic cultures of pomegranate and the provision of high number of plantlets that will be needed for the encapsulation of the shoots and the testing of their survival under minimal growth conditions at 4°C.

WPM basal solution was effective in this case. Different authors have reported using different basal solutions to promote the proliferation of pomegranate explants.

Similarly to our research, Kanwar et al. (2010) reported a high regeneration rate when zygotic embryos were used as primary explants. The efficiency of WPM basal medium for the proliferation of *Punica granatum* explants was also reported by Samir et al. (2009). However, Patil et al. (2011) found the use of MS basal medium to be more effective for the proliferation of pomegranate nodal explants. They also highlighted the importance of a suitable BAP : NAA ratio 15 – 10 : 1 between these PGR categories, which is also in accordance with the protocol followed in our research.

### 3.2. Synthetic seeds conversion after short-term conservation at 4°C

The apical shoots were isolated from *in vitro* stabilized plantlets and encapsulated under aseptic conditions. For this purpose, isolated shoots were dropped on a medium-viscosity sodium alginate solution containing 100 mM $CaCl_2$. After 30 min in these conditions, resistant and easily handled beads were obtained (Fig. 1 b).

After producing synthetic seeds, these explants were transferred to the recovery medium (Fig. 1 c) and incubated at 4°C to evaluate a short-term conservation possibility.

After preserving for 3 months at 4°C, the synthetic seeds were transferred to 25°C, and the rate of contamination, necrosis, and plantlet recovery was recorded. The obtained data were compared with synthetic seeds incubated at 25°C after production, and the ability of their conversions in whole plantlets (Fig. 1 d, e, f) was evaluated).

The obtained data show that in both preserved and control groups, the rate of contamination is less than 2%, indicating good practical skills working under aseptic conditions. However, differences were observed regarding the rate of necrosis which is much higher in the preserved synthetic seeds, affecting the rate of their conversion into plantlets. The synthetic seeds preserved for 3 months at 4°C showed a lower plantlet recovery rate, but this is a good result considering the storage period.

Fig. 3 indicates that 70.33% of the synthetic seeds regenerated successfully after the storage period. The fact that 29.67% of the synthetic seeds did not survive indicates challenges associated with the storage conditions, such as possible damage due to low temperatures or shorter conservation periods. Meanwhile, the plantlets recovery rate was high in the non-preserved seeds (96.34). The conversion into plantlets in the Control group was observed two weeks after encapsulation of the synthetic seeds incubated at 25°C. Thus, it cannot be considered a suitable technique for germplasm storage. However, it can be beneficial for germplasm exchange.

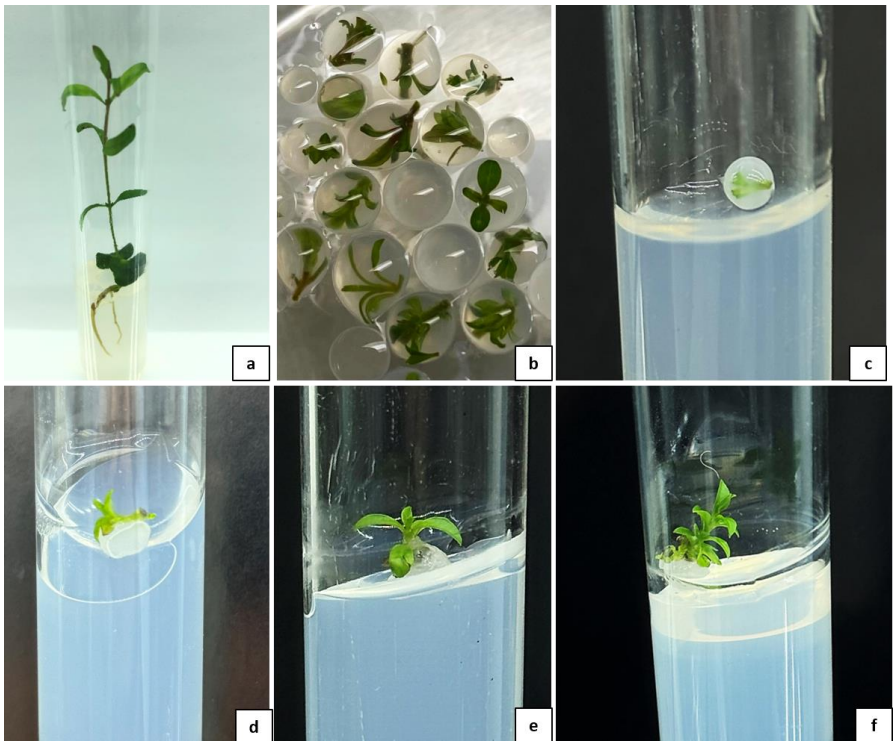


**Figure 1.** a) Germination of *Punica granatum* L. cv Devedishe zygotic embryos incubated at 25°C b) Encapsulation of pomegranate shoots c) Inoculation in the recovery medium d, e, f) Different stages of conversion of synthetic seeds of *Punica granatum* L cv. Devedishe into whole plants under *in vitro* conditions

The findings are particularly relevant to micropropagation, where synthetic seeds are used to mass-propagate plant species. Improving regeneration rates can significantly enhance the efficiency of these techniques. For conservation purposes, improving synthetic seeds' storage and regeneration protocols can help preserve genetic diversity and support the reintroduction of plant species.

Artificial seed technology is considered helpful for germplasm storage and exchange. Many reports indicate the efficiency of such applications for economically important plant species (Piccioni and Standardi, 1995; Sandoval-Yugar et al., 2009; Micheli et al., 2019). However, too many factors must be optimized to obtain high-quality synthetic seeds, like the degree of viscosity of sodium alginate used, $CaCl_2$ concentration, and curing time (Kikowska and Thiem, 2011). A high percentage of encapsulated shoot tips of pomegranate assessed in the present study showed regrowth and satisfactory development time. Incubation for 3 months at 4°C slightly negatively affected the recovering percentages. However, these results show a high possibility for short- or medium-term preservation of pomegranate using encapsulation technology, and these findings can serve as a good start for future implementation of long-term preservation protocols via cryopreservation using encapsulation-dehydration methods.

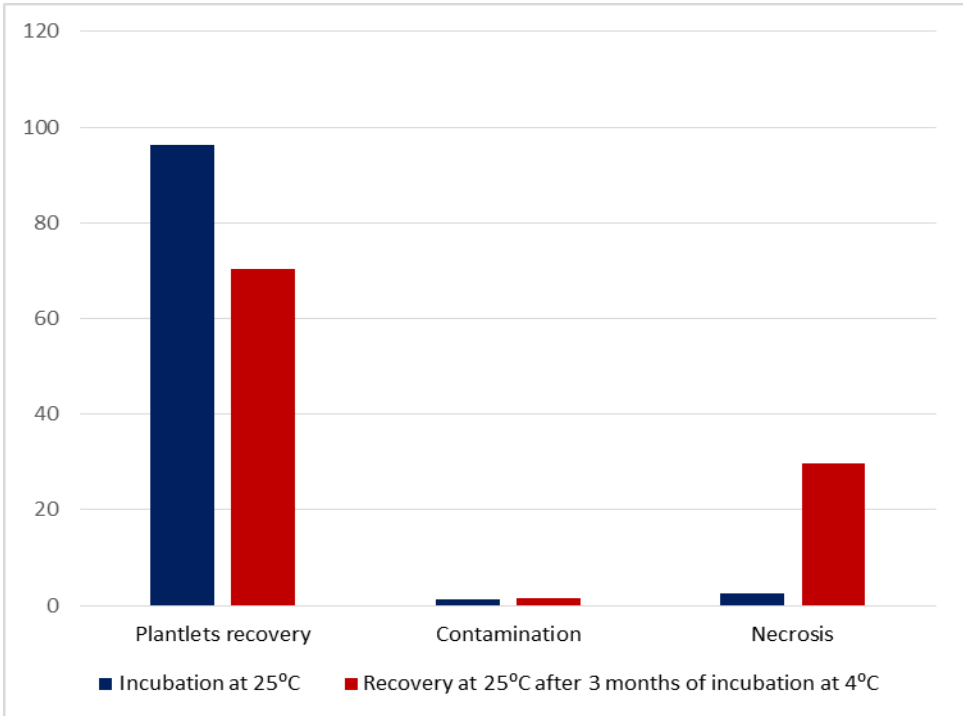


**Figure 3.** Contamination, necrosis and plantlets recovery rate of pomegranate synthetic seeds of *Punica granatum* L. cv Devedishe

## 4. CONCLUSIONS

The findings are particularly relevant to micropropagation, where synthetic seeds are used to mass-propagate plant species. Improving regeneration rates can significantly enhance the efficiency of these techniques. For conservation purposes, improving synthetic seeds' storage and regeneration protocols can help preserve genetic diversity and support the reintroduction of plant species.

Artificial seed technology is considered helpful for germplasm storage and exchange. Many reports indicate the efficiency of such applications for economically important plant species. The wide use of pomegranate for different purposes makes it essential to conserve these plants. The practical application of synthetic seed production methodologies presents multiple advantages in maintaining the regenerative potential of explants after a certain conservation period. Based on this study's results, it can be concluded that plantlet recovery from synthetic seeds is very high during incubation at 25°C, which shows the multiple potential uses of synthetic seeds technology. On the other hand, it may serve for adequate short-term preservation periods using minimal growth techniques via encapsulation and incubation at 4°C. These results proved that such technology might be effective for preservation programs, especially for establishing long-term conservation strategies and genetic collections of *Punica granatum* L. cv Devedishe germplasm.

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2) "Safeguard of Albanian autochthonous fruit germplasm by synthetic seed technology and advanced conservation at ultra-low temperature (Cryopreservation, -196°C)".

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# *In vitro* cultures used in biology of forest tree species – New research opportunities

**Teresa Hazubska-Przybył[1], Paweł Chmielarz[1], Mikołaj K. Wawrzyniak[1], Joao Paulo Rodrigues Martins[1], Joanna-Kijowska-Oberc[1], Hanna Fuchs[1], Ewelina Ratajczak[1], Agnieszka Szuba[1], Leszek Karliński[1], Konrad Forysiak[2], Jarosław Sęktas[2]**

[1]*Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland*

[2]*Prof. Stefan Białobok Forest Arboretum, Syców Forest District, Leśna 6, 56-504 Stradomia Dolna, Poland*

*hazubska@man.poznan.pl*

## Abstract

The micropropagation technique is used extensively in studies on forest tree biology at the Institute of Dendrology PAS. The scope of research conducted includes, among others, the development of propagation and cryopreservation protocols for economically and ecologically important trees (*Picea abies* (L.) H. Karst, *P. omorika* (Pančić) Purk., *Pinus sylvestris* L., *Quercus robur* L., etc.). We have developed a method of somatic embryogenesis (SE) for Norway spruce of Polish origins and for the endangered, endemic Serbian spruce species. We also have developed an innovative method of cryopreservation of embryogenic tissues (ETs) for coniferous species of the *Abies*, *Picea* and *Pinus* genera, based on stepwise dehydration of the material before placement in liquid nitrogen (LN). Recently, we have focused our research on the effects of auxins on the somatic development of *P. abies* and *P. omorika* plants, showing that they can affect embryogenic cultures in different ways. Moreover, we have shown for the first time that it is possible to propagate centuries-old oak specimens *in vitro* and to cryopreserve plums capable of developing into complete plants. We also undertaken the studies on the influence of ectomycorrhization of *Populus* x *canescsens* seedlings with the fungus *Paxillus involutus* on the growth intensity of colonized seedlings on high-throughput metabolomic or proteometabolomic analysis. Our aim is to obtain plants with improved growth and tolerance to stress related to heavy metals, with a view to using vegetative seedlings for afforestation and phytoremediation of polluted areas.

***Keywords:*** *Picea omorika, Quercus robur, cryopreservation, ectomycorrhization*

## 1. INTRODUCTION

The *in vitro* technique has a wide range of applications in forest tree biology research. For example, the tissue culture method based on organogenesis or somatic embryogenesis (SE) has been used to develop protocols for micropropagation and

tissues or organ cryopreservation of *Picea abies* (L.) H. Karst, *P. omorika* (Pančić) Purk., *Quercus robur* L. and *Q. petrea* (Matt.) Liebl. The studies on the influence of ectomycorrhisation level of *Populus* x *canescsens* seedlings with the fungus *Paxillus involutus* and lead (Pb) treatment of these plants *in vitro* have been carried out. We tested whether micropropagation techniques could be potentially used as a supportive method in Polish forest management, and to enable the preservation of biodiversity of both old and endangered tree species. Moreover, we are also interested in whether this biotechnological tool would allow positive mycorrhization effects and production of more resistant *in vitro* derived plants to industrial pollution, i.e. Pb.

Due to changing of the environmental conditions, forest management in Europe (including Poland) is increasingly facing serious problems, which in the near future may significantly affect the efficiency of wood production. At the same time, the demand for this raw material grows continuously, so new possibilities for increasing its production, also based on biotechnological tools, are being worked on. The production of economically important forest tree seedlings, e.g. through SE, would provide high-quality plant material in unlimited quantities for the establishment of plantation crops. Valuable material, obtained *in vitro*, could be stored in gene banks in the form of ETs (*Picea* spp., etc.) or plumul (*Q. robur*, *Q. petrea*) and used in due course for production purposes. An additional source of wood would be environmentally resistant material, planted in post-industrial areas.

The SE is the basic method of micropropagation of coniferous species. The model species for research into this process is the Norway spruce, for which propagation methodology has been developed primarily in Scandinavian countries. The established SE methodology has been tested and modified for the requirements of our native Norway spruce and Serbian spruce, as an endangered but also ornamental and pollution-resistant species. To induce embryogenic cultures of Norway spruce, we used mature zygotic embryos from trees of various provenances mainly. In the case of Serbian spruce, this type of explant was used from trees growing in the Arboretum in Kórnik. We evaluated the effect of various compounds in the culture media (sucrose, abscisic acid, ascorbic acid, Phytagel, and various auxins) on the particular stages of somatic embryo development to improve the existing protocols (Hazubska-Przybył et al., 2016, Hazubska-Przybył et al., 2020, Hazubska-Przybył et al., 2024a). Finally, the modification of these micropropagation methods allowed us to obtain somatic seedlings of both spruce species, capable of growing in non-laboratory conditions. We are currently working on producing more plant material that can be tested in field conditions. To store the obtained cell lines long-term, we have developed a cryopreservation method based on the stepwise dehydration method. It is an alternative method to the other ones routinely used in coniferous plant material cryostorage. In this approach ETs is dehydrated in the presence of sucrose and then drying for a short time in sterile air before freezing in LN. The procedure does not require the use of the cryoprotectant dimethyl sulfoxide (DMSO), which may cause damage to the genetic

material in cryopreserved tissues. We achieved a high level of method efficiency especially for *P. omorika* (Hazubska-Przybył et al. 2010), but recently also for *Abies alba* x *A. numidica* and *Pinus nigra* (Hazubska-Przybył et al. 2024b), after thawing tissues from LN and their proliferation.

Micropropagation technique, based on microshoot induction and proliferation, to propagate several Polish, centuries-old trees of *Q. robur* was applied, as an alternative to the conventional cloning methods (Chmielarz et al., 2023, Rodrigues Martin et al., 2024). In the case of *Q. robur* these techniques are inefficient, and only seeds can be used as a propagule source. Pedunculate oak is a species that occurs in most of Europe and is economically important due to its high-quality timber. Some individuals can live for more than 1000 years and thus are of particular interest, as they managed to survive for so long under environmental conditions that varied over the centuries. However, despite their genetic and historical importance, some ancient individuals over 400 years old have been killed in Poland in recent years as a result of vandalism (e.g., Napoleon oak, Chrobry oak, Mieszko I oak). For this reason, methodologies that allow the cloning and conservation of those forest individuals must be applied. However, it is essential to highlight that the genotype can influence morphogenetic responses. It is also commonly known, that explants from older trees tend to be very recalcitrant or have low regenerative capacity. To test this hypothesis, an experiment was recently conducted using plant material from adult individuals (70 and 600 years old). We also tested whether BAP concentration would improve shoot induction from these explants.

Lately, we developed for the first time a protocol for the cryopreservation of *Q. robur* and *Q. petrea* germplasm as plumules in cooperation with Kostrzyca Forest Gene Bank (Chmielarz 2011, Wasileńczyk et al., 2024). Oaks seeds, which are sensitive to desiccation and freezing temperatures, cannot be stored long-term in gene banks under conventional conditions. In practical forest production, acorns of *Quercus* are stored for up to 18 months at –3°C in well-ventilated containers. Extended storage is unfeasible due to the seeds' active metabolism and rapid decline in viability. Therefore, like other recalcitrant-seeded species, their germplasm can be preserved in LN at –196°C. To date, successful cryopreservation has been reported for 15 oak species, a small fraction of the *Quercus* genus's total diversity. Various plant materials, such as pollen, embryos (both somatic and zygotic), shoot tips, plumules, and dormant buds, have been cryopreserved from *Quercus* spp. (Ballesteros et al., 2020). However, post-cryopreservation regrowth often remains low, resulting in inefficient cryopreservation methods. In our studies, plumules isolated from acorns of both oaks were subjected to cryoprotection, followed by desiccation and LN exposure. After thawing from LN, the growth and development ability of cryogenically stored plumules was assessed under *in vitro* culture conditions.

The *in vitro* culture technique is also a useful tool for studying mycorrhizal symbiosis. We used this technique in analyses of the impact of ectomycorrhization of *P.* x *canescens* seedlings with the fungus *P. involutus* on the growth intensity and Pb tolerance of colonized plants. According to Krpata et al. (2008), mycorrhizae are important in both nutrient-

deficient regions and anthropogenically disturbed environments. As *Populus* spp. has the potential ability to grow in harsh conditions, together with mycorrhizal associations and it may be useful for phytoremediation purposes, especially in regions polluted with Pb. The symbiotic relationship between plants and mycorrhizal fungi results in improvement of host organism tolerance to heavy metals (Szuba, 2015). Therefore, we studied the influence of two strains of *P. involutus* on the effect of $Pb^{2+}$ on poplar tree growth under a controlled, *in vitro* cultures, without the risk of contaminations by other fungal strains. To obtain a more comprehensive view of the condition of inoculated plants, we performed high-throughput metabolomic or/and proteometabolomic analysis (Szuba et al., 2020).

Using this biotechnology tool, we have developed several innovative solutions to the above research problems in the hope that they will find application in science and practice.

## 2. MATERIALS AND METHODS

### 2.1. Materials

ETs of *P. abies* and *P. omorika* were induced from mature zygotic embryos, excised from seeds collected from donor trees growing in "Zwierzyniec" Experimental Forest near Kórnik and in the Kórnik Arboretum, respectively. The innovative cryopreservation method was developed for two-year-old *P. omorika* and one-year-old *P. abies* ETs (Hazubska-Przybył et al., 2010, 2013, 2016, 2020). To induce the tissue cultures of *Q. robur*, the epicormic shoots were used as explant sources. They developed from dormant buds located under the bark of lignified branches. Branches from ca. 20–800-year-old oaks from 67 selected trees were cultivated *ex vitro* in culture pots at 25°C (Chmielarz et al., 2023).

For *Quercus* spp. cryopreservation experiment, *Q. robur* and *Q. petrea* plumules (the shoot apical meristem of an embryo, 1 mm in size) were excised from acorns of various Polish origins. The plumules were taken within 6 weeks or 4 months after the acorns' storage, respectively (Chmielarz et al., 2023, Wasileńczyk et al., 2024).

*P.* x *canescens* (*P. tremula* L. x *P. alba* L., clone 1) tissue cultures were induced from apical and lateral buds, as explants, harvested from the collection of poplars of ID PAN in Kórnik (Bojarczuk et al., 2015). Fungal strains were isolated from fruiting bodies collected under poplar trees. Two strains (with the lowest and the highest tolerance) were selected for the experiments after treatment with 75 mM Pb $(NO_3)_2$ (Szuba et al., 2017).

### 2.2. Methods

**Somatic embryogenesis of *Picea spp.***

Mature zygotic embryos were incubated on half-strength Litvay medium (Litvay et al., 1985), supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3,5,6-trichloropicolinic acid (picloram) or 1-naphthaleneacetic acid (NAA) at 9 µM and benzyl adenine (BA) at 8.8 or 4.5 µM. For the proliferation the same media was used

but with a lowered concentration of BA to 4.5 or 2.2 µM, respectively (Hazubska-Przybył et al., 2016, Hazubska-Przybył et al., 2020). Somatic embryos maturation was carried out on the medium without any plant growth regulators (PGRs), but with the addition of 1% activated charcoal (AC) and 34 g/L sucrose for 7 days. Next, the cultures were transferred on the media supplemented with various concentrations of ABA (10-80 µM), sucrose (17-68 µM) and Phytagel (4-8 g/L) for 5-7 weeks (Hazubska-Przybył et al., 2016). Somatic embryos at the cotyledonary stage were incubated for two weeks in darkness and for the next two weeks in light. Properly developed, germinated embryos were transferred to a mixture of perlite and peat (1:3) to acclimatize them to the *ex vitro* conditions (Hazubska-Przybył et al., 2020).

***Cryopreservation of* Picea *spp. by stepwise dehydration method***

ET of *P. omorika* and *P. abies* were precultured on the medium with increasing concentration of sucrose (0.25-1.00 M) for 7 days (Hazubska-Przybył et al., 2010, 2013). In the case of *P. abies* 10 µM of ABA was added to the preculture media additionally (Hazubska-Przybył et al., 2013). Next, the tissues were air-dried over silica gel for 2 hr. and frozen in LN. After thawing the ET survival and proliferation, and maturation of somatic embryos capacity were assessed. We also analyzed the genetic fidelity of unfrozen and frozen ET, as well as somatic embryos derived from cryostored *P. abies* tissue, based on five microsatellite *loci* (Hazubska-Przybył et al. 2013).

**Micropropagation of centuries-old trees of *Quercus robur***

For the *in vitro* establishment of centuries-old *Q. robur* trees, a WPM (Lloyd and McCown, 1980) medium supplemented with 3.5 µM BAP was initially adopted. This same cultivation medium has been used in plant material's multiplication/maintenance phase. Donor tree age, genotype and *in vitro* regeneration efficiency were assessed in this study (Chmielarz et al., 2023). Recently, the effect of two resources of auxins (indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) in the absence or presence of ascorbic acid (AC) at the morphological level was studied to improve the rooting of *Q. robur in vitro*-derived plants (Rodrigues Martins et al. 2024).

**Cryopreservation of *Quercus* spp. plumules**

Plumules of *Q. robur* were stored in 0.5 M sucrose solution and next subjected to cryoprotection, desiccation and cooling before freezing in LN (Chmielarz et al., 2011). This method was modified by Wasileńczyk et al., (2024) and applied for *Q. petraea* plumules cryostorage. In the experiments, plumules were placed in the wells of a cryo-plate and embedded in calcium alginate gel to form alginate beads (one plumule per bead). For cryoprotection, the encapsulated plumules were then attached to the cryo-plate with calcium alginate gel. The samples were immersed in a solution of 2.0 M glycerol and 1.0 M sucrose, dehydrated in a laminar airflow cabinet for 2 hr. and plunged directly into LN.

**Ectomycorrhization of *Populus x canescens* seedlings *in vitro***

*P.* × *canescens* microcuttings were transferred to jars containing a 3-mm layer of Modified Melin-Norkrans medium (Kottke et al. 1987) on top of Murashige and Skoog medium. For inoculations, mycelial fragments (5-mm diameter) were placed close to the microcuttings. Inoculated or non-inoculated *P.* x *canescens* microcuttings with 0.5 cm roots were treated with 0.75 mM $Pb(NO_3)_2$. After 6 weeks of culture, the root colonization rate of both fungal strains and their impact on poplar growth parameters and Pb accumulation were determined (Szuba et al., 2017). Micro-cuttings characterized by impaired growth in response to colonization by a fungal strain were analyzed using an integrated proteomics–metabolomics approach (Szuba et al., 2020).

## 3. RESULTS

Our studies on *P. abies* and *P. omorika* SE protocol development revealed that mature zygotic embryos used as explants and induced ETs are sensitive to various auxins applied during the induction and proliferation steps. For example, the highest SE induction frequency was observed after 2,4-D application in *P. omorika* and picloram in *P. abies* explants. NAA treatment significantly promoted ET proliferation in *P. abies*. While in *P. omorika* the presence of NAA or picloram in the proliferation medium decreased the further production and maturation of somatic embryos (Hazubskla-Przybył et al., 2020).

The quality of the germinated *P. abies* embryos and their development into plantlets was dependent on the auxin type. We demonstrated that different auxin types can generate various physiological responses in plant materials during particular steps of SE in both *Picea* spp. (Hazubska-Przybył et al., 2020). Our experiments also showed that ABA and osmoticum had a significant effect on the production and maturation of somatic embryos of both spruce species. Decreasing of ABA (10 µM) or sucrose (17 g/L) concentration in the medium resulted in the precocious germination of embryos. On the other hand, adding sucrose to the maturation medium at the highest tested concentration (68 g/L) improved the growth of the radicles of the embryos during germination (Hazubska-Przybył et al., 2016). We also observed that the intensity of the growth of the hypocotyls and radicles of *P. omorika* embryos depended on the Phytagel concentration in the maturation medium.

The stepwise dehydration method enabled efficient cryopreservation of both spruce species. In the case of *P. abies* ET survival was 54.4% maximally, after treatment with sucrose and ABA (Table 1). The addition of ABA during the preculture of ET with sucrose improved their ability to form mature somatic embryos (Hazubska-Przybył et al., 2013). For *P. omorika* ET we obtained almost 100% survival after 3 weeks of *in vitro* cultivation after sucrose treatment alone during a 7-days preculture period (Table 1; Hazubska-Przybył et al., 2010). Genetic analysis of *P. abies* plant material after cryopreservation by stepwise dehydration method did not revealed any changes in tested

microsatellite *loci* (Hazubska-Przybył et al., 2013). We also obtained promising results using similar protocol for *Abies alba* x *A. numidica* and *Pinus nigra* ETs, with the exception of *P. nigra* air-drying time, which was prolonged to 2.5 hr. (Table 1; Hazubska-Przybył et al., 2024). In these experiments we obtained high ET survival rate for *Abies alba* x *A. numidica* (84.4%) and *P. nigra* (86.7%).

**Table 1.** Efficiency of cryopreservation of selected coniferous ET species based on stepwise dehydration method

| **Species** | **Cryopreservation method** | **Method modification** | **ET survival rate (%)** | **References** |
|---|---|---|---|---|
| ***P. omorika*** | sucrose pretreatment – 7 days (0.25M-1 day; 0.5M-1 day; 0.75M-2 days; 1.00M-3 days; air desiccation over silica gel – 2 hr. at 25ºC; LN freezing; rapid thawing in water bath at 42ºC | – | 99.0 | Hazubska-Przybył et al. 2010 |
| ***P. abies*** | | sucrose and ABA pretreatment (10µM) | 54.4 | Hazubska-Przybył et al. 2013 |
| ***A. alba* x *A. numidica*** | | – | 84.4 | Hazubska-Przybył et al. 2024 |
| ***P. nigra*** | | 2.5 hr. air desiccation | 86.7 | Hazubska-Przybył et al. 2024 |

According to Chmielarz et al. (2023) the *in vitro* culture can be a useful biochemical tool to the centuries-old oak trees (even up 800-year-old) propagation. They reported the continuous and increase in the number of *in vitro* cultured shoots both in younger and some old oaks. However, the efficiency of *in vitro* shoot multiplication was strictly dependent on the genotype of the donor plant. Finally only half of the tested old trees were suitable as a source of explants to establish tissue cultures of *Q. robur*. The *in vitro* rooting phase still appears to be partially limiting for some *Q. robur* genotypes.

Root formation can occur without PGRs in the cultivation medium. However, adding low concentrations of auxin (IAA) and AC can assist in the rhizogenesis process. For the 800-year-old genotype, it was possible to verify anatomical changes in the roots depending on whether or not the auxins IBA and IAA were combined with AC. Roots grown in the absence of AC and in the presence of auxins showed larger aerenchymas forming from the regions closest to the root apex.

The same was not verified when AC was present. In fact, this compound was a crucial modulating factor during the rooting of *Q. robur*. In addition to changes in root anatomy, plants rooted with AC showed a more efficient photochemical efficiency of photosystem II, which resulted in plants with greater accumulation of fresh mass (Rodrigues Martins et al., 2024).

Plumules of *Q. robur* isolated from acorns of four provenances showed a 51-76% survival rate and 2-20% regrowth after applied cryopreservation procedure under *in vitro* conditions (Fig.1; Chmielarz et al., 2011).

While *Q. petraea* plumules cryopreserved using modified aluminium cryo-plate method had a bit higher maximal survival rate (up to 83%). Properly growing seedlings with both shoots and roots were derived from cryopreserved plumules of both *Quercus* spp. The experiment for *Q. petraea* was successfully repeated on seeds from various provenances, yielding consistently good results. However, seed quality and storage time post-harvest are crucial factors for plumule regrowth after cryopreservation (Wasileńczyk et al., 2024).

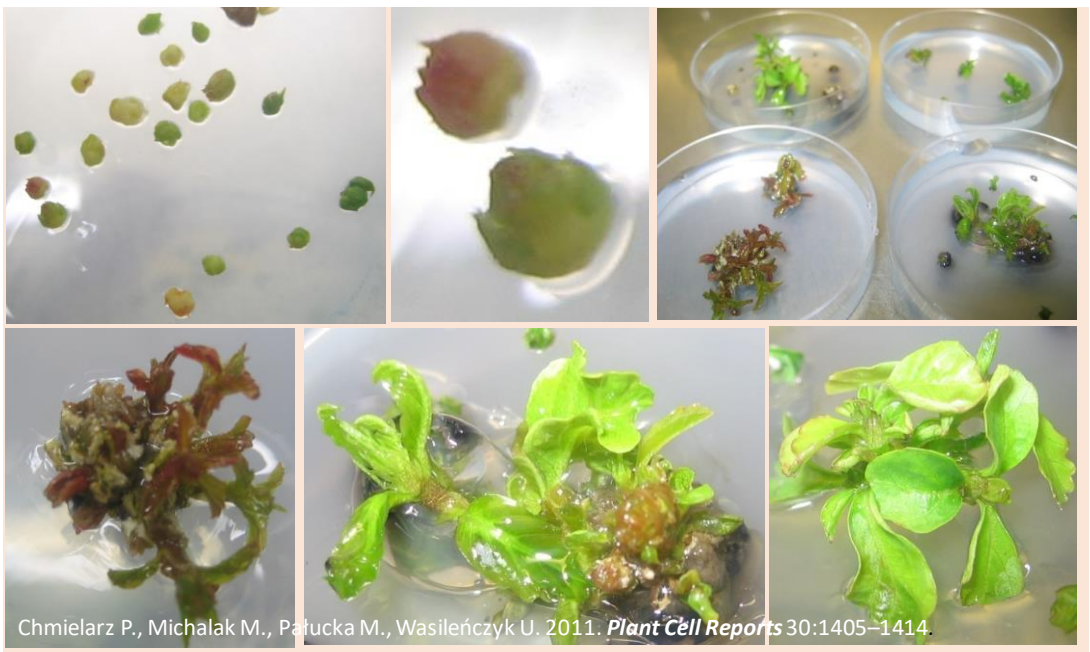


**Figure 1.** Plant regeneration of *Q. robur* plumules after cryopreservation

The experiments on ectomycorrhization of *P.* x *canescens* demonstrated that roots were colonized at different levels: non-mycorrhized, changed (fungal cells were present on the root surface only) and fully mycorrhized (Fig.2; Szuba et al., 2017). According to Szuba et al. (2017) plants inoculated with the tolerant fungal strain were characterized by better growth and Pb accumulation in the roots and stems as compared to the control ones. For example, 217 % increase in dry weight was observed in inoculated plants. However, ectomycorrhizae (ECMs) are not always beneficial for the plant host.

Despite the negative impact of colonization of some *Paxillus involutus* strains on the growth, nutrition and C/N balance of poplars, the mechanisms characteristic of negative pathogen-parasite interactions are not activated, and the fungus is considered a symbiotic partner (details in Szuba et al. 2020).

**Figure 2.** Ectomycorrhization of *P.* x *canescens* seedlings with the fungus *P. involutus* (according to Szuba et al. 2017)

## 4. DISCUSSION

Technology based on micropropagation is widely applicable to the study of forest tree biology, with the prospect of new opportunities to explore this field of science. The exemplary research directions presented in this article have allowed us to solve several research problems in unconventional ways (Hazubska-Przybył et al., 2010, 2013, 2020, 2024a and b, Chmielarz et al., 2011, 2020, Rodrigues Martins et al., 2024, Szuba et al., 2017, 2020, Wasileńczyk et al., 2024). We demonstrated the possibility of obtaining somatic seedlings of an endangered Serbian spruce and also physiological differences in the response to nutrient solution components between this species and a model *P. abies*, especially at the level of response to various auxins added to the media in the first SE stages (Hazubska-Przybył et al., 2020). We have developed an efficient method for cryopreservation of embryogenic tissues, based on the gradual dehydration of material for both spruce species. This method also proved efficient in cryostorage of the tested cell lines *Abies alba* x *A. numidica* and *P. nigra*. The efficiency of the method ranged from 54.4% for *P. abies* to almost 100% for *P. omorika*, in terms of survival of embryogenic tissues (Hazubska-Przybył et al., 2010, 2013). The method still needs to test more TE genotypes, but our results indicate that it can be as efficient as traditionally used cryopreservation methods (Salaj et al., 2007). We have shown that, thanks to the potential inherent in micropropagation techniques, it is possible to propagate even centuries-old trees with unique physiological characteristics as in the studies of Chmielarz et al. (2023) and Rodrigues Martins et al. (2024). Obtaining clonal seedlings of Poland's oldest pedunculate oaks capable of further growth under natural conditions is a spectacular achievement by our scientists. As well as the results obtained in a study to develop a highly efficient cryopreservation procedure for plumules isolated from

acorns of *Q. robur* and *Q. petrea* (Chmielarz et al., 2011, Wasileńczyk et al., 2024). Previously, some authors exposed *Q. robur* embryogenic axes to LN, but with limited success during *in vitro* culture (Berjak et al., 2000, after Chmielarz et al., 2011). Our investigations revealed new possibilities for conserving the biodiversity of pedunculate and sessile oaks based on the cryopreservation of very sensitive plumule tissue. These simple and effective protocols established by our teams will be suitable for use in gene banks. The *in vitro* technique also enabled us to better understand the basics of the process of ectomycorrhization of *P.* x *canescens* with *P. involutus* fungal strains and the various reactions of mycorrhized plants to Pb pollution. We found a positive impact of inoculating poplar trees with Pb tolerant ectomycorrhizal fungal strain on seedling growth and Pb phytostabilization (Szuba et al., 2017). According to Ijaz et al. (2016) this is one of the major types of phytoremediation in plants. Analyses based on the integrated proteomic-metabolomics studies showed that the negative effect of some *P. involutus* strains on the plant condition, nutrition and C/N balance in *P.* x *canascens* did not contribute to the activation of the defence mechanisms, and the fungus was treated as a symbiotic partner (Szuba et al., 2020). The results obtained under controlled *in vitro* conditions provide the basis for further research under uncontrolled environmental conditions. An important question remains to identify whether these non-genetic modifications resulting in improved phytoremediation will find application in the development of effective soil remediation technologies (Szuba et al., 2017).

## 5. CONCLUSIONS

*In vitro* culture technology provides a platform in our unit to bring together research from different scientific fields. Referring to this biotechnological tool, we have developed solutions, often of an innovative nature, to a variety of research problems concerning. We trust that these will have valuable applications in both scientific and practical terms.

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# Effects of potassium dichromate on growth and biochemical characteristics of paulownia plantlets

**Viktor V. Husak[1], Roksolana M. Shcherba[1], Volodymyr I. Lushchak[1,2]**

[1]*Department of Biochemistry and Biotechnology, Vasyl Stefanyk Precarpathian National University, 57 Shevchenko Str, Ivano-Frankivsk, 76018, Ukraine;*

[2]*Research and Development University, 13a Shota Rustaveli Str, Ivano-Frankivsk, 76018, Ukraine.*

*volodymyr.lushchak@pnu.edu.ua*

## Abstract

Paulownia, a deciduous tree with high growth potential, presents an opportunity for the remediation of polluted soils and is a perspective tree for bioenergetics. In Ukraine, where vast areas suffer from pollution of both technogenic and war-related origins, establishing sustainable solutions for land reclamation is crucial. This study focuses on the potential of utilizing paulownia for phytoremediation purposes, particularly in regions like the Ivano-Frankivsk area, known for high potassium dichromate ($K_2Cr_2O_7$) contamination. In the present study, we investigated the morpho-physiological and biochemical responses of microclonally produced plantlets of paulownia Shan Tong (a hybrid resulting from the crossing of two species of paulownia, i.e., *P. tomentosa*, and *P. fortunei*) under exposure to potassium dichromate in various concentrations (50, 100, 200, 300 mg $L^{-1}$) in the growth media. Exposure to 200 mg $L^{-1}$ of $K_2Cr_2O_7$ significantly reduced plantlet growth, as indicated by increased shoot length, stem mass, and leaf mass. It also decreased the content of chlorophylls and carotenoids. At these concentrations, silver nitrate did not affect the content of anthocyanins. Under exposure to 300 mg $L^{-1}$ potassium dichromate, necrosis of the shoots occurred, and the experimental group of plants did not develop. This research underscores the potential of paulownia in phytoremediation, particularly in regions facing chromium contamination. Understanding the biochemical and growth responses of paulownia to K2Cr2O7 can help to develop strategies for optimizing its effectiveness in the remediation of polluted soils, thus promoting environmental sustainability and ecosystem restoration.

***Keywords:*** *Paulownia, potassium dichromate, biochemical characteristics, growth*

## 1. INTRODUCTION

In natural conditions, chromium (Cr) is a component of rocky soils and volcanic dust and is one of the most common environmental pollutants. The chromium concentration in soil varies and ranges from 1 to 1000 mg/kg. Chromium is widely used in industry. High levels of chromium can be found in water bodies and soils near metallurgical

plants, paint and textile factories, leather processing enterprises, and nuclear power plants. According to the International Agency for Research on Cancer, chromium is classified as a strong carcinogen (IARC 1987; Shahid et al., 2017).

Chromium exists in stable trivalent ($Cr^{3+}$) and hexavalent ($Cr^{6+}$) forms, with other valence states being unstable and transient in biological systems. The hexavalent form, $Cr^{6+}$, is recognized as the most toxic, typically occurring as chromate ($CrO_4^{2-}$) or dichromate ($Cr_2O_7^{2-}$) oxyanions. In contrast, $Cr^{3+}$ is less toxic, less mobile, and predominantly associated with organic matter in soils and aquatic environments (Becquer et al., 2003; Husak and Bayliak 2023). The widespread use of chromium in various industrial activities has led to significant contamination of soil and groundwater, raising considerable concern among plant and animal scientists in recent years.

Despite its toxicity, chromium has not been as extensively studied by plant scientists as other trace metals like cadmium, lead, mercury, and aluminum. One major challenge has been the complex electronic chemistry of chromium, which complicates the understanding of its toxic effects on plants. The physiological impact of chromium contamination on plants depends on the specific form of the metal, which influences its mobility, uptake, and overall toxicity within the plant system. Chromium toxicity manifests in plants through various symptoms, including reduced crop yields, inhibited leaf and root growth, disruption of enzymatic functions, and potential mutagenic effects (Shanker et al., 2005; Shanker et al., 2009; Shahid et al., 2017; Husak and Bayliak 2023).

Paulownia Shan Tong, a rapidly growing plant variety, is capable of accumulating significant amounts of various metal ions. This hybrid is notable for its exceptional disease resistance and strong adaptability to a range of environmental stresses, including heat, drought, and cold, with the ability to withstand temperatures as low as -30°C. It is recognized as one of the most frost-resistant varieties among all paulownia species. These unique traits make it an ideal candidate for cultivation in Europe, especially in regions with significant temperature fluctuations and harsh winter conditions. Due to its rapid growth rate and capability to thrive on contaminated soils, paulownia Shan Tong can be effectively used for the remediation of soils polluted with chromium compounds. With these considerations in mind, we specifically selected this paulownia variety for our study. Consequently, this research aims to explore the effects of potassium dichromate ($K_2Cr_2O_7$) on the growth and pigment concentrations in paulownia plantlets.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Phenylmethylsulfonyl fluoride (PMSF) was obtained from Sigma-Aldrich Corporation (USA). All other reagents were received from local suppliers (Ukraine) and they were of analytical grade.

## 2.2. Methods

### 2.2.1. Plant Material and Cultural Conditions

The impact of varying concentrations of potassium dichromate on the growth and biochemical parameters of paulownia Shan Tong was investigated. Paulownia plantlets, previously established *in vitro* as microclonal cultures in our laboratory, were transferred to glass jars containing 25 mL of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The medium was enriched with 30 g $L^{-1}$ sucrose, 100 mg $L^{-1}$ myo-inositol, 6.0 g $L^{-1}$ agar, 0.5 mg $L^{-1}$ 6-benzylaminopurine (BA), and 0.02 mg $L^{-1}$ indole-3-acetic acid (IAA). To assess the effects of $K_2Cr_2O_7$ on paulownia shoot explants, the following concentrations of $K_2Cr_2O_7$ were incorporated into the medium: 0 (control), 50, 100, 200, or 300 mg $L^{-1}$. The pH of the media was adjusted to 5.8 using 1 N NaOH before adding agar, and the media were autoclaved for 30 minutes at 121ºC. The shoot explants were cultured for 40 days in a growth room maintained at 25±2ºC, with a photoperiod of 16 hours of light and 8 hours of darkness, at a light intensity of 50 μmol $m^{-2}$ $s^{-1}$. The experiments were conducted with four independent replications, each consisting of three samples (n=12).

### 2.2.2. Plant morphometric analysis

After 40 days of cultivation, ten *in vitro*-cultivated paulownia plants from each experimental group were randomly selected to evaluate growth characteristics. The plants were washed with sterile distilled water, and morphometric measurements were conducted. The length of the aerial part of the plant was measured from the base of the stalk to the last expanded leaf using a ruler. The fresh mass of the shoots was determined using a precision balance (Husak et al., 2020). For biochemical parameter analysis, all leaves from each plant were collected and frozen in liquid nitrogen.

### 2.2.3. Pigment quantification

To extract pigments, fresh leaves were homogenized with ice-cold 96% ethanol (1:10, w:v) in the presence of 10 mg $mL^{-1}$ $CaCO_3$ to prevent pheophytinization. The homogenates were then centrifuged at 8000×g for 10 minutes at 4°C. The supernatants were collected, and the pellets were re-extracted three times with 1 mL of ice-cold 96% ethanol. The combined extracts were used for spectrophotometric measurement of pigment concentrations. Specific absorption coefficients for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids were employed as per Lichtenthaler (1987). A molecular mass of 570 was used to calculate carotenoid concentrations. The anthocyanin content was determined after acidifying the extract with concentrated HCl to a final concentration of 1%. The anthocyanin concentration was measured spectrophotometrically at 530 nm, using an absorption coefficient of 30 $mM^{-1}$ $cm^{-1}$ (Gitelson et al., 2001; Stambulska and Luschak, 2015; Husak et al., 2020).

### 2.2.4. Statistical analysis

Data are expressed as means ± S.E.M. Outliers were identified and excluded using Shovene's and Dixon's Q tests. The Shapiro-Wilk test was employed to evaluate the normality of the data distribution. Statistical analyses were carried out using the Mynova software (version 1.3), with ANOVA followed by Dunnett's test. Statistical significance was set at $P<0.05$.

## 3. RESULTS

### 3.1. Effect of $K_2Cr_2O_7$ on morphometric characteristics of plants

Potassium dichromate affected morphometric parameters, such as shoot length, stem, and leaf mass (Fig. 1). In particular, we observed a statistically significant reduction in shoot length at 100 and 200 mg $L^{-1}$ of $K_2Cr_2O_7$ compared to the control group. At $K_2Cr_2O_7$ concentrations of 100 and 200 mg $L^{-1}$ in the cultivation medium, the paulownia shoots were 27%, and 57% shorter, respectively, compared to the control plants (Fig. 1a). Additionally, at a concentration of 300 mg $L^{-1}$ potassium dichromate, our studies noted shoot necrosis, with the experimental group of plants failing to develop entirely.

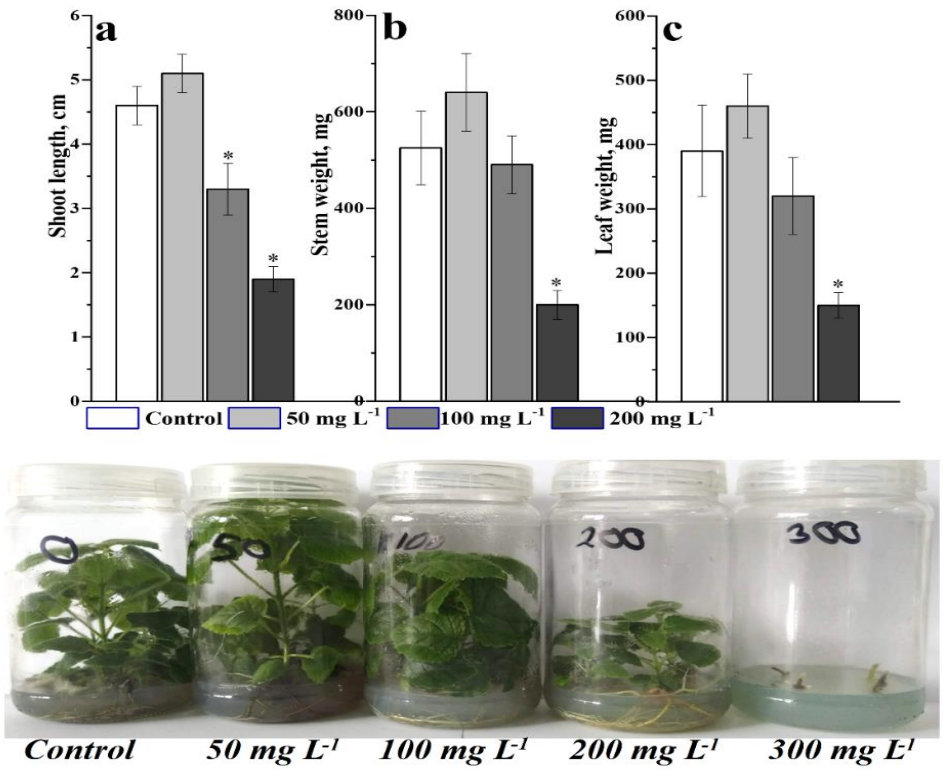


**Figure 1.** The effects of $K_2Cr_2O_7$ (**50, 100, 200, 300 mg $L^{-1}$**) on shoot length (a), stem mass (b), and leaf mass (c) of *in vitro* paulownia plantlets cultivated in MS medium during 40 days. Data are presented as means ± S.E.M, n = 10. *Significantly different from the control (without $K_2Cr_2O_7$) group of plants ($P < 0.05$) according to ANOVA followed by Dunnett's test.

Stem mass in plants cultivated with 200 mg $L^{-1}$ $K_2Cr_2O_7$ concentration was 2.6-times lower than in the control group (Fig. 1b). Similarly, leaf mass was 2.6-fold lower in plants grown on $K_2Cr_2O_7$-supplemented media (Fig. 1c).

### 3.2. Effect of $K_2Cr_2O_7$ on the concentrations of pigments

In our experiments, we measured the levels of Chl *a* and found that exposure to $K_2Cr_2O_7$ at all concentrations had negative effects on pigment content in leaves. At $K_2Cr_2O_7$ concentrations of 50, 100, and 200 mg $L^{-1}$ in the cultivation medium, the Chl *a* levels of paulownia leaves were 44%, 41%, and 32% lower, respectively, compared to the control plants (Fig. 2a). No significant changes were observed in Chl *b* levels in paulownia plantlets exposed to potassium dichromate at 50 and 100 mg $L^{-1}$ (Fig. 2b). However, explants treated with 200 mg $L^{-1}$ of $K_2Cr_2O_7$ demonstrated a decline in chlorophyll *b* concentrations compared to the control (1.12±0.05 µmol $gwm^{-1}$).

Carotenoid content in leaves of paulownia plantlets treated by 50, 100, and 200 mg $L^{-1}$ $K_2Cr_2O_7$ was by 38%, 30%, and 34% lower than that in the control (0.76±0.03 µmol $gwm^{-1}$), respectively (Fig. 2c). No substantial alterations in anthocyanin content were observed within plantlets exposed to $K_2Cr_2O_7$ (Fig. 2d).

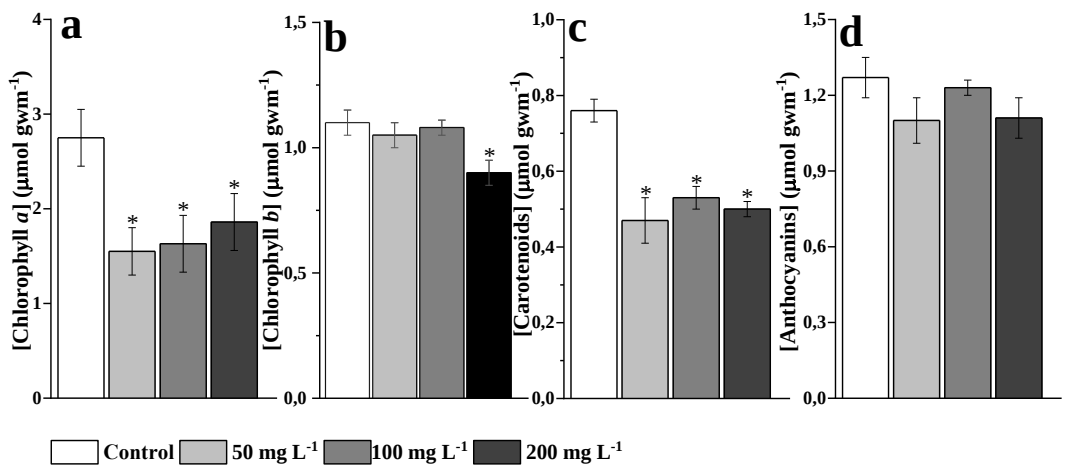


**Figure 2.** Concentrations of chlorophyll *a* (a), chlorophyll *b* (b), carotenoids (c), and anthocyanins (d) in paulownia plantlets, exposed to different concentrations of $K_2Cr_2O_7$. Data are presented as means ± S.E.M, n = 8. *Significantly different from the control (without $K_2Cr_2O_7$) group of plants ($P < 0.05$) according to ANOVA followed by Dunnett's test.

## 4. DISCUSSION

Chromium induces generalized toxic damage in plants, affecting their morphology and overall viability. There is a clear gradient in the size of shoots depending on the concentration of potassium dichromate in the cultivation medium (Fig. 1). Furthermore, at a concentration of 300 mg $L^{-1}$ potassium dichromate, we found necrosis of the shoots,

and the experimental group of plants did not develop at all. Similar results regarding the toxicity of chromium on the growth and biomass accumulation of shoots have been demonstrated in studies with *Triticum aestivum* L. (Ghani et al., 2015), *Pisum sativum* L. (Panday et al., 2009), *Zea mays* L. (Bouhadi et al., 2021), *Lactuca sativa* (Shanker et al., 2005), and *Sinapsis alba* (Hanus and Tomas, 1993).

The toxicity of chromium at these concentrations may indicate the intensification of free radical processes in paulownia shoots and induction of oxidative stress. The direct biochemical mechanisms of chromium toxicity lie in its pro-oxidant properties as a strong oxidizer, damaging enzymes, DNA, and membranes (Panda, 2004). Additionally, chromium accumulates in the roots and submerged parts of the plants in the nutrient gel, impairing the uptake and transport of micro- and macroelements, further deteriorating the condition of the plants (Shanker et al., 2009).

The state of the plant's photoassimilation system – content and ratio of chlorophylls *a/b*, carotenoids, and anthocyanins – is an important diagnostic criterion for the plant's tolerance and adaptation to stress factors. It is known that one of the mechanisms of chronic toxicity involves changes in the photoassimilation structures of plants, which were observed in our studies (Fig. 2a-c). A linear, dose-dependent decrease in chlorophyll content under the influence of dichromate was also observed in *T. aestivum* L. (Subrahmanyam, 2008; Ghani et al., 2015), *P. sativum* L. (Panday et al., 2009), and *Z. mays* L. (Bouhadi et al., 2021). The reduction in chlorophyll content due to $Cr^{6+}$ suggests that chromium toxicity may diminish the size of the peripheral portion of the antenna complex, as noted by Shanker et al. (2005). The identified cause of this effect is a disruption in the functioning of δ-aminolevulinic acid dehydratase, which is involved in the biosynthesis of chlorophylls (Panda, 2004). Furthermore, chromium affects the ultrastructure of chloroplasts, and inhibits the electron transport chain of the photoassimilation system by replacing magnesium ions in chlorophyll with chromium and other metal ions in the active centers of Calvin cycle enzymes (Clijsters and Van Assche 1985). Ali et al. (2006) reported that $Cr^{6+}$ inhibited electron transport activity in *Lemna gibba*, identifying the inhibitory site at the oxygen-evolving complex and $Q_A$ reduction site. The inhibition of PS II electron transport and the formation of reactive oxygen species (ROS) by $Cr^{6+}$ is strongly correlated with a decrease in the D1 protein and oxygen-evolving complex proteins. There is also evidence suggesting that ROS may directly degrade D1 peptide bonds (Lubinková and Komenda 2004). In contrast, Bishnoi et al. (1993) found that $Cr^{6+}$ had a more pronounced effect on PS I activity than on PS II activity in isolated pea chloroplasts, while Dhir et al. (2008) observed increased PS I activity in *Salvinia natans* plants grown in Cr-rich wastewater, which could represent an acclimation response to handle the additional electron flow during stress (Makino et al., 2002). The reduction in Chl *b* (Fig. 2b) due to Cr exposure might result from the destabilization and degradation of proteins in the peripheral part. Additionally, the inactivation of enzymes involved in the chlorophyll biosynthetic pathway could lead

to a general decrease in Chl *b* content in many plants under chromium stress (Vajpayee et al. 2000).

The carotenoid content in paulownia leaves decreased under the influence of all concentrations of potassium dichromate (Fig. 2c). Similar results were obtained for the *Taxithelium nepalense* (Panda, 2004), *Ocimum tenuiflorum* (Rai et al., 2004), *Vigna radiata* (Gautam et al., 2021), *T. aestivum* (Ali et al., 2015), *Brassica rapa* (Fu et al., 2023), and *Oryza sativa* L. (Mishra et al., 2024). The experimental data under the influence of potassium dichromate may indicate intense oxidative stress in the chloroplasts. Despite this, exposure to any of the tested concentrations of potassium dichromate did not result in an increase in anthocyanin content compared to the control group of plants (Fig. 2d), which may indicate a low content of active chromium in the cytoplasm and vacuole due to sequestration (Boonyapookana and Upatham, 2002).

## 5. CONCLUSIONS

The results indicate that potassium dichromate at concentrations ranging from 100 to 200 mg $L^{-1}$ reduced shoot length, stem mass, and leaf mass, thereby impeding the growth and development of paulownia plantlets *in vitro*. Additionally, at a concentration of 300 mg $L^{-1}$ potassium dichromate, our studies detected necrosis in the shoots, resulting in a complete lack of development of the plants. The biochemical responses of the treated plantlets, particularly the levels of chlorophyll *a* and carotenoids, suggest that the phytotoxic effects of $K_2Cr_2O_7$ at concentrations between 50 and 200 mg $L^{-1}$ are likely attributable to the induction of oxidative stress.

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# Gene soldiers against *Phytophthora cinnamomi* infection

**Saleta Rico[1], Jesús Vielba[1], Beatriz Cuenca[2], Nieves Vidal[1], Conchi Sánchez[1]**

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

*[2]Maceda Nursery, Tragsa-SEPI Group, Carretera de Maceda a Baldrei km 2, 32700 Maceda, Spain.*

saleta@mbg.csic.es

## Abstract

European chestnut (*Castanea sativa* Mill.) is significantly affected by ink disease, caused by *Phytophthora cinnamomi*. Resistance to ink disease is a crucial factor in the survival and productivity of chestnuts, and the analysis of molecular responses to infection will provide relevant information for developing tools for its control. This study focuses on understanding the genetic responses of two chestnut genotypes (CS12 and PO11) with different levels of resistance to *P. cinnamomi*, and to identify key genes and signaling pathways involved in defense against this pathogen. CS12 is a pure *C. sativa* clone and is highly sensitive to infection, while PO11, a hybrid *C. sativa* x *C. crenata*, is resistant. Using Next Generation Sequencing (NGS), transcriptomes of inoculated plantlets were analyzed in comparison with the transcriptomes of non-inoculated plantlets. We identified differentially expressed genes in response to infection including genes related to signal transduction, transcription factors and pathogenesis-related genes. Our findings provide insights into the molecular mechanisms underlying plant immune responses and stress adaptation, potentially contributing to the development of effective strategies for ink disease management in chestnut.

***Keywords:*** *Castanea sativa, Phytophthora cinnamomi, ink disease, transcriptomics, DEGs.*

## 1. INTRODUCTION

European chestnut (*Castanea sativa* Mill.) is a long-lived multipurpose tree cultivated worldwide for its nuts and timber (Vielba et al., 2020). Among various threats, chestnut populations are affected by “ink disease", caused by the oomycete *Phytophthora cinnamomi*, which has significantly contributed to the drastic decline of chestnut distribution in Europe. This disease is particularly devastating in water-saturated soils, leading to high mortality rates and significant economic losses in nurseries. Hybrids between European and Asian chestnut species have long been used due to the resistance of Chinese and Japanese species to this pathogen (Fernandes et al., 2022).

Within the oomycetes, *P. cinnamomi* is considered the most dangerous pathogenic species for forest trees, attacking the root system of woody species. Host responses to

infection vary from rapid mortality to several levels of tolerance, suggesting potential genetic control over tolerance (González et al., 2011; Santos et al., 2016). The plant immune system employs different strategies to combat pathogens, primarily through the broad-spectrum pathogen-triggered immunity and the more specific effector-triggered immunity (Pavese et al., 2021). These immune responses involve recognizing pathogen molecules and triggering signaling cascades that allow key genetic modulation to fight the infection. Identification of genes involved in these early stages of the plant response is crucial to understand the molecular mechanisms triggered by plants to defend themselves against biotic stress.

So far, the management of *P. cinnamomi* infection has focused on identifying resistance genes and conducting surveys to detect tolerant genotypes (Fernandes et al., 2022). More comprehensive transcriptomic analyses of genotypes with varying degrees of tolerance in response to oomycete have provided valuable insights into the defense mechanisms against ink disease (Serrazina et al., 2015; Santos et al., 2017; Pavese et al., 2021). Both susceptible (*C. sativa*) and resistant (*C. crenata* Siebold & Zucc.) chestnut species respond to pathogen inoculation by activating genes related to stress response, transcription factors (TFs), and signaling (Serrazina et al., 2015). These findings contribute to understand the molecular mechanisms underlying chestnut's response to *P. cinnamomi* infection and provide a basis for further research and breeding efforts aimed at enhancing resistance in chestnut populations (Santos et al., 2016; Santos et al., 2017; Zhebentyayeva et al., 2019).

The current study aims to analyze the transcriptome differences between two *in vitro* chestnut clones, one resistant and one susceptible, after *P. cinnamomi* infection by using RNASeq. Transcriptome profiles of both genotypes were compared before and 48h after *P. cinnamomi* inoculation. Differentially expressed genes (DEGs) associated with plant defense were upregulated in both genotypes after inoculation. We found that certain DEGs, including receptor-like kinases, TFs (WRKY, ERF), resistance related genes or pathogenesis-related genes of the resistant clone were more significantly up-regulated than those of the susceptible genotype. The analyses provided insights into the genetic mechanisms triggered by this pathogen, potentially contributing to the development of novel strategies to control ink disease.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Two chestnut clones, CS12 and P011, provided by TRAGSA nursery (Grupo TRAGSATEC, Maceda, Spain) were used in this study. They have been previously characterized for their resistance to *P. cinnamomi* (González *et al.*, 2011). PO11 is a highly resistant hybrid genotype (*C. sativa* × *C. crenata*), while CS12 is a non-resistant *C. sativa* genotype. Leaves from rooted plantlets inoculated *in vitro* with *P. cinnamomi* were harvested and used in the transcriptomic analyses.

## 2.2. Methods

### *In vitro P. cinnamomi* inoculation

The inoculation was performed by TRAGSA into a flow chamber under sterile conditions as described by Rial et al. (2008). Briefly, agar fragments (5 mm in diameter) containing sporulated *P. cinnamomi* mycelium with sporangia that would subsequently release zoospores were introduced into each *in vitro* culture tube with a rooted seedling on a paper bridge.

### Total RNA extraction

Total RNA was extracted from leaves of the PO11 and CS12 using the "FavorPrep Plant Total ARN purification Mini Kit (for woody Plant)" (Favorgen Biotech corp., Taiwan) following the manufacturer´s instructions. Samples were collected 48 h post-inoculation and samples from non inoculated (NI) plants were used as control. RNA was treated with DNaseI (Promega, Madison, WI) and phenol:cloroform extraction and etanol precipitation was performed. Quality and quantity were evaluated using a Nanodrop 1000 (Thermo Fisher Scientific™, USA) by detecting absorption at 260 and 280 nm and a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity was assessed using OD260/OD280 and OD260/OD230 ratios, and only samples with ratios between 1.9-2.1 and 2.0-2.2, respectively, were used. RNA integrity was verified by electrophoresis on a 1.2% agarose gel. RNA samples were stored at -80°C for RNA-seq analysis.

### Library Preparation and Sequencing

RNA samples from leaves of NI and 48-h post-inoculation plantlets of each clone were sent to BGI for sequencing, following the company´s protocols. Threes different samples were used per treatment and six samples per clone.

### Bioinformatic Pipeline

Clean raw data from BGI were analyzed for quality using FastQC (Andrews, 2010). Due to the lack of a complete reference genome for chestnut, the *Quercus suber* draft genome version 1.0 was used (Ramos et al., 2018). Clean raw reads were aligned to the reference genome using STAR (Dobin et al., 2013), and transcriptome assembly and quantification were performed with Stringtie 2 (Kovaka et al., 2019).

### 2.2.1. Third level sub-heading

### Differential Gene Expression

DESeq2 within the R environment was used to identify differentially expressed genes (DEGs) between conditions (Love et al., 2014). Comparisons were made between PO11 and CS12 48-h post-inoculation samples versus their respective controls (NI samples). Benjamini and Hochberg's adjusted p-values to establish the False Discovery Rate (FDR), set at $p < 0.05$. Transcripts with a log2 fold change > 1 were considered DEGs.

### Pathway Enrichment Analysis

DEGs were analyzed using the Kobas tool with information from the Kyoto Encyclopedia of Genes and Genomes repository (Xie et al., 2011). Statistical enrichment of DEGs in the different pathways was calculated with the hypergeometric and the Fisher´s exact tests (p value < 0.05, corrected p value < 0.05).

## 3. RESULTS

The overall results of the transcriptomics analysis for CS12 and PO11 are represented in the Volcano plots (Figure 1). The analysis identified 807 differentially expressed genes (DEGs) in CS12 samples 48 h after inoculation and 732 DEGs in PO11 samples 48 h post-infection. The principal component analysis of the sequences revealed a clear separation between the genotypes and treatments (Figure 2).

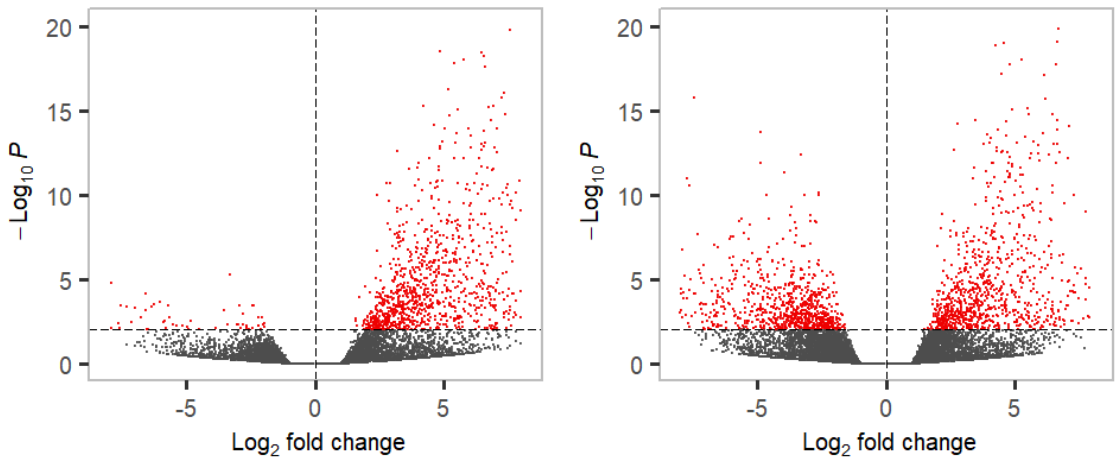


**Figure 1.** Overall results of the gene expression in the transcriptomics analysis of the CS12 and PO11 libraries, and identification of differentially expressed genes (DEGs). (A) Volcano plot showing the DEGs in CS12_NI samples (right, log2FC > 1) and CS12_48h samples (left, log2FC < 1) and the adjusted p value (<0.05). (B) Volcano plot showing the DEGs in PO11_NI samples (right, log2FC > 1) and PO11_48h samples (left, log2FC < 1) and the adjusted p value (<0.05). Red dots indicate DEGs for one condition or the other.

The differential gene expression analysis in the chestnut response to *P. cinnamomi* inoculation identified genes related to signal transduction, TFs and defense-related genes in response to the pathogen (Figure 3). The identified genes related to signal transduction include a range of serine/threonine-protein kinases, lectin-domain containing receptors, and receptor-like protein kinases, indicating a complex signaling network in the defense mechanisms. Additionally, TFs such as MYB, WRKY, NAC, and ethylene-responsive TF were detected, suggesting the activation of transcriptional regulation in response to the pathogen. Furthermore, the presence of defense-related genes like pathogenesis-related proteins, calcium-dependent protein kinases, and disease

resistance proteins highlights the activation of defense pathways against *P. cinnamomi* in chestnut species. These findings highlight the intricate molecular responses and signaling cascades involved in chestnut defense mechanisms against ink disease. These DEGs were upregulated in inoculated samples with the highest expression in PO11 shoots 48 h post-inoculation.

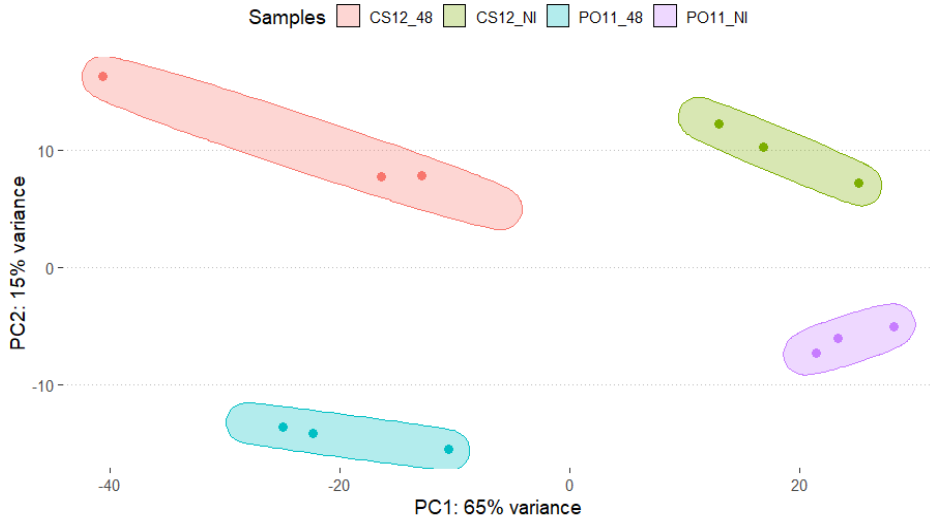


**Figure 2.** Principal component analysis of the sequencing results from the twelve libraries

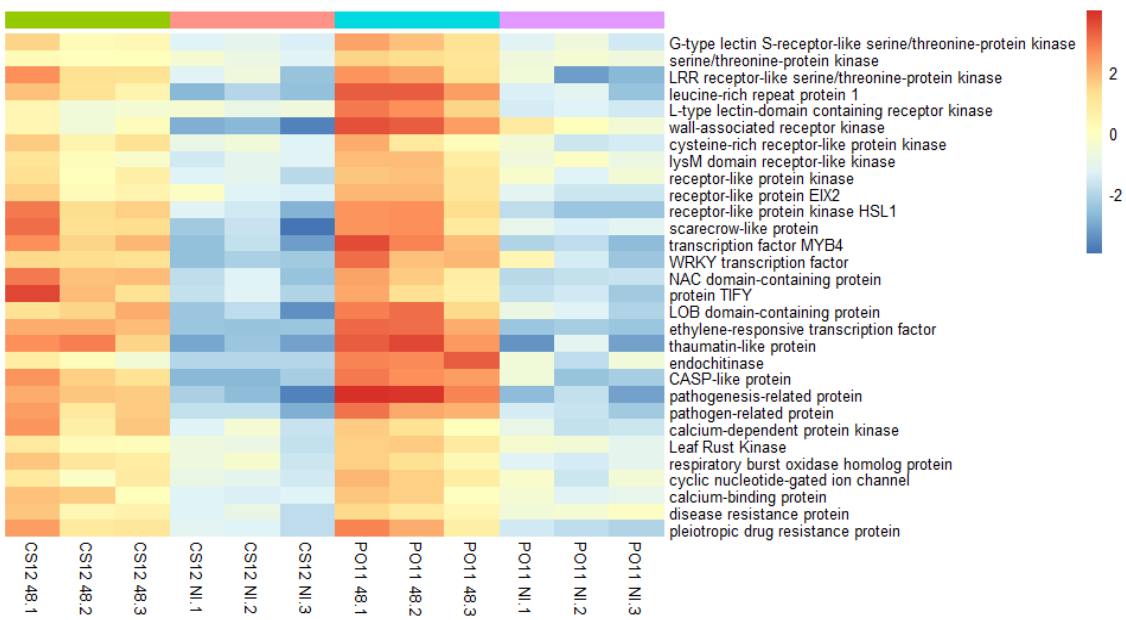


**Figure 3.** Heatmap of the expression of DEGs detected in the conditions. Level of expression is shown as the log2FC of the transformed count data from the transcriptomics analysis. Different columns in the figure represent different samples, and different rows represent different genes. The colors from blue to red indicate increasing levels of expression.

To gain further insight into the processes regulated by the identified DEGs, a KEGG pathways enrichment analysis was developed to further characterize the responses of CS12 and PO11 shoots to *P. cinnamomi* infection. A comparison of pathway enrichment in both CS12 and PO11 is shown in Figure 4.

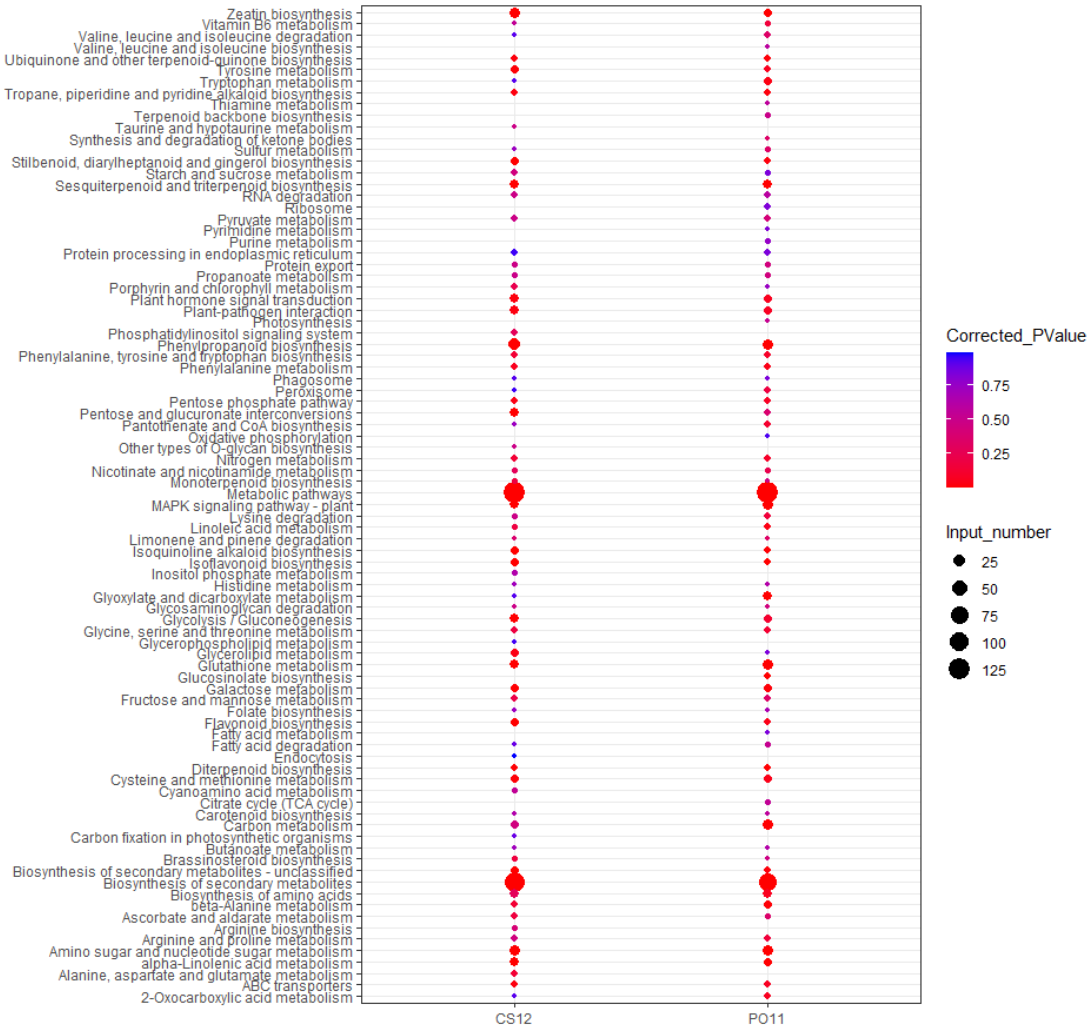


**Figure 4.** Comparison of enriched pathways in the KEGG analysis. The name of the pathway is shown on the Y axis, the size of the dots indicates the number of genes detected, and the color indicates the value for the corrected p value.

Various metabolic pathways were found to be enriched, including biosynthesis pathways like phenylpropanoid, flavonoid, and carotenoid, as well as metabolic pathways such as starch and sucrose, glycolysis/gluconeogenesis, and sugar metabolism. Additionally, pathways related to biotic stress response like MAPK signaling, plant hormone signal transduction, and plant-pathogen interaction were enriched. Furthermore, pathways involved in transport like ABC transporters, genetic information processing pathways like RNA degradation and cellular processes such as

those involving peroxisomes were also enriched. There are pathways enriched exclusively in PO11 and others enriched only in CS12, suggesting that the tolerant genotype may have a more robust and efficient defense response, while the sensitive genotype may rely more on the production of specific defense compounds. These findings indicate a complex molecular response of chestnut to *P. cinnamomi* infection (metabolic, signaling, and defense-related pathways), providing valuable information on the mechanisms involved in the defense and metabolic regulation of chestnut trees under biotic stress conditions.

## 4. DISCUSSION

Next Generation Sequencing (NGS) offers a means to study plant-pathogen interactions through transcriptomic analysis and to investigate plant response to biotic stresses. Therefore, in the current study the molecular responses of two chestnut genotypes, one susceptible and the other tolerant to *P. cinnamomi*, were evaluated 48 h after inoculation with the oomycete. Transcriptomic analysis of inoculated and control plants revealed significant insights into the molecular responses to infection in these chestnut trees. The identification of DEGs highlights the dynamic changes in gene expression that occur during the early stages of pathogen response and suggests that both broad-spectrum and specific immune responses are activated in response to the pathogen.

The enriched pathways identified through KEGG analysis highlight critical metabolic and biosynthetic processes that are activated in response to infection. Both CS12 and PO11 showed enrichment in metabolic pathways and the biosynthesis of secondary metabolites, reflecting the broad reprogramming of cellular metabolism to combat the pathogen (Camisón et al., 2019; Saiz-Fernandez et al., 2020). The presence of biosynthesis pathways such as phenylpropanoid, carotenoids, and flavonoid suggests that both genotypes are involved in the production of compounds with known roles in plant defense and signaling (Ramaroson et al., 2022; Sun et al., 2022). Interestingly, pathways involved in the metabolism of amino acids, such as phenylalanine, tyrosine, and tryptophan, were also enriched. These pathways are essential to the production of secondary metabolites, including phytoalexins, which are antimicrobial compounds synthesized in response to pathogen attack (Islam et al., 2018; Camisón et al 2019; Zhan et al 2022). The enrichment of these pathways indicates that both CS12 and PO11 are mobilizing these resources. The analysis also revealed significant enrichment of pathways related to biotic stress response, including the MAPK signaling pathway which plays a central role in transducing stress signals and activating defense mechanisms (Devendrakumar et al., 2018), and plant hormone signal transduction pathways that are involved in regulating growth, development, and stress responses (Pieterse et al., 2012). The plant-pathogen interaction pathway, which encompasses various molecular interactions between the host and the pathogen, was also significantly enriched, highlighting the activation of specific immune responses.

Receptors such as LRR and RLK, and other signaling components identified in this study, play crucial roles in pathogen recognition and the initiation of downstream defense responses. The detection of numerous TFs, such as MYB, WRKY, and NAC, involved in the regulation of the host response following pathogen perception, further supports the activation of a complex regulatory network for an effective defense response (Serrazina et al., 2015; Islam et al., 2018; Fernandes et al., 2022; Saiz-Fernández et al., 2022). The presence of pathogenesis-related proteins and other defense-related genes suggests that both genotypes are actively engaged in mitigating the effects of *P. cinnamomi* infection (Islam et al., 2018; Pavese et al., 2021; Saiz-Fernández et al., 2020). Additionally, transcriptome analysis has revealed DEGs involved in cell wall biosynthesis, reactive oxygen species, and hormones. The highest expression of those genes was detected in the most oomycete-tolerant genotype (PO11).

Early response to *P. cinnamomi* involves a coordinated activation of metabolic, biosynthetic, and signaling pathways that are essential for plant defense. Our findings contribute to the understanding of the molecular mechanisms underlying chestnut resistance to this pathogen and provide potential targets for breeding programs aimed at enhancing resistance. A wider analysis including more sampling times, the functional validation of identified genes and pathways would provide a more comprehensive view of the temporal changes in gene expression as well as confirm the role of those genes in plant defense. The analysis of downregulated genes in response to inoculation will also provide relevant information about their function. Additionally, further research into the signaling pathways and master regulators involved in the response to biotic stresses will enhance our understanding of plant-pathogen interactions.

## 5. CONCLUSIONS

In this study, candidate genes for ink disease resistance in *Castanea* were identified through RNA-seq analysis, providing valuable genomic resources for further studies on disease resistance mechanisms in chestnut trees. Differential gene expression patterns related to biotic stress and plant immune response were detected between *P. cinnamomi*-inoculated and -non-inoculated plantlets. Genes involved in plant defense responses (signal transduction, TFs and pathogenesis-related genes) were upregulated after *P. cinnamomi* inoculation, indicating recognition of pathogen attack. Overall, the highest expression of these genes, was detected in the PO11 resistant clone at 48 h post-inoculation. Candidate genes included wall-associated receptor kinases, LRR and RLK receptors or MYB and WRKY TFs.

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# *In vitro* conservation of *Zizyphus spina-christi* L. germplasm by using abscisic acid

**Doaa Elazab[1,2], Claudia Ruta[3] and Maurizio Lambardi[1]**

[1] *IBE-Institute of BioEconomy, National Research Council, 50019 Sesto Fiorentino (Florence), Italy*

[2] *Department of Pomology, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt*

[3] *Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti (Di.S.S.P.A.), University of Bari Aldo Moro, 70125 Bari, Italy*

doaa.elkassas@ibe.cnr.it

## Abstract

Jujube (*Zizyphus spina-christi* L.), also known as napq or sidr, belongs to the buckthorn family (Rhamnaceae). Zizyphus species are important fruit trees for several countries, such as Egypt, India and Gulf area, for the fleshy drupes rich in sugars and vitamins. Traditional tree improvement programs are time-consuming and difficult, and *in vitro* propagation methods provide an effective alternative. Slow growth storage is a technique that allows *in vitro* storage of shoots for period ranging from 6 months to 2 years, depending on the species, after which shoots can be returned to standard culture conditions and micropropagated when desired. In this study, slow growth storage of *Z. spina-christi* was conducted using 0, 7 or 9 mg/L of abscissic acid (ABA) in different media (MS, ½MS and N&N). Conservation was extended for up to 12 months, with observations on the quality of shoots performed every 3 months. Moreover, after each period of slow growth storage, the ABA-treated and untreated shoots were moved back to standard culture conditions and evaluated for their recovery of proliferation. After 12 months of storage, ABA treatment could maintain high survival percentage of shoots that recovered well its proliferation capacity. In particular, the main outcome of the present study is that, on full or half-strength MS medium added of 7 mg/L ABA, it is possible to achieve 12-month storage of nabq shoot cultures, maintaining a good quality level of material and an effective recovery in post-conservation. It has also been observed that the effect of ABA in slow growth storage is mediated by the formulation of the storage medium.

***Keywords:*** *Zizyphus spina-christi, jujube, slow growth storage, abscisic acid*

## 1. INTRODUCTION

Nabq (Christ's thorn jujube or sidr-indian jujube; *Zizyphus spina-christi* L.) belongs to the buckthorn family (Rhamnaceae). Nabq originated in China where it has been cultivated for more than 4,000 years and where there are over 400 cultivars. The fleshy drupes are rich in sugars and vitamins, and this fact has made *Zizyphus* species important fruit trees for many centuries. Leaves of *Z. spina-christi* are used in traditional medicine for abscesses, furuncles and swollen eye, the ash of its wood may

be used to treat snake bites (Abdel-Galil & El-Jissry, 1991). Nabq tree is traditionally grown as scattered trees in the Egyptian orchards as borders surrounding shrub or trees.

*In vitro* methods use less space and fewer pest inputs than conventional methods, and cultures are protected from pests and diseases, including viruses. One drawback of *in vitro* culture is the possibility of genetic instability due to somaclonal variation which can occur during the culture process. However, the storage of cultures under slow growth conditions, where the rate of cell division is reduced, may help to limit the frequency of such mutations.

Slow growth storage is a technique aimed to allow the conservation of plants *in vitro* for period ranging from 6 months to 2 years, depending on species. After the conservation period, when desired, the stored plants can be moved back to standard culture conditions and micropropagated rapidly (Perez-Tornero et al., 1999; Lambardi and Ozudogru, 2013). Some approaches can be used to achieve slow growth storage, in addition to the classical application of low temperature and darkness or low light intensity, among which the modification of the culture medium by using growth inhibitors. Growth inhibitors are synthetic or natural chemical substances that have the potential to alter plant structure or impact plant life processes directly by interfering with their hormonal balance. Natural growth retardants are, e.g., abscisic acid (ABA) and jasmonic acid (JA). ABA, in particular, is the hormone that is usually associated with major plant responses to stress. The aim of this research was to assess the effect of different concentrations of ABA (0, 7 and 9 mg/L) on slow growth storage under laboratory conditions of *Zizyphus* germplasms.

## 2. MATERIAL AND METHODS

### 2.1. Plant material and culture conditions for initiating micropropagation

Shoot tips of nabq (*Zizyphus spina-christi* L.) were collected from healthy and mature trees at the beginning of spring growth cycle. To clean the samples, they were washed under running tap water for one hr. The explants (1-cm length) were then surface sterilized by soaking in 1% sodium hypochlorite for 15 min, rinsed 3 times by sterilized distilled water for 5 min each, soaked in 70% ethanol for 30 sec, and finally rinsed again 3 times in sterilized distilled water before to be transferred to the culture medium.

For shoot initiation, the culture medium was the MS formulation (Murashige and Skoog, 1962). The pH was adjusted to 5.6-5.8 before autoclaving at 1.5 $kg/cm^2$ pressure and 120°C temperature for 20 min. For each jar, 10-15 mm-long shoot segments were inserted into the culture medium and the vessels were placed in incubation room at 23±2°C, under a photon flux density of 40 µmol $m^{-2}$ $sec^{-1}$ and a photoperiod of 16 hr/8 hr. After 45 days, proliferating shoots were obtained, then divided into 2-3 segments and cultured in 100 mL glass jars containing 25 mL of MS culture medium, supplemented with 30 g/L sucrose, 0.5 mg/L 6-benzylaminopurine (BAP) for further proliferation.

### 2.2. Slow growth storage experiment

To study the effect of different medium composition on nabq shoot culture during the storage for up to 12 months and subsequent recovery, MS medium at full- and half-strength concentrations (MSfs and MShs) and N&N medium (Nitsch and Nitsch, 1969) at full-strength were used with different concentrations of ABA: 0 (control), 7 and 9 mg/L.

Every 3 months during the storage period, the following data were recorded: (i) the % of shoot survival, considering alive shoots that had a green appearance with no browning, (ii) shoot proliferation, calculated as the number of shoots formed per each primary shoot, and (iii) the average shoot length (cm).

### 2.3. Shoot recovery in post-conservation

Every 3 months, 5 randomly-selected jars from each treatment were moved back to the above-described standard culture conditions and evaluated for shoot recovery after 6 weeks by calculating the same parameters of 2.2.

### 2.4. Statistical analysis

For each treatment, 45 jars (replicates) were used, and the experiment was repeated twice. The effects of the different treatments were evaluated by ANOVA, and the Tukey test used for mean separation at $p \leq 0.05$ significant level. All data were processed with statistical package SPSS v.16. (SPSS, 2007).

## 3. RESULTS

### 3.1. Effect of ABA on nabq shoot quality during the 12-month storage

#### 3.1.1. Effect of ABA on shoot survival percentage

As indicated in Table 1, ABA at 7 mg/L induced, after 12 months, a shoot survival of 55% and 20% for MSfs and MShs, respectively. It is noteworthy that the best shoot survival (60%) after 12 months of storage was achieved when the MSfs storage medium was added of 9 mg/L of ABA. No shoot survival after 12 months was obtained when the N&N medium was used, with a fast shoot decay registered in the last 3 months of conservation.

**Table 1.** Effect of ABA on survival percentage of nabq shoots during 12 months of storage in different media (control, ABA-free MS medium)

| **Months** | **3** | **6** | **9** | **12** |
|---|---|---|---|---|
| **Treatment** | **Full-strength MS** | | | |
| ABA, 7 mg/L | 76 b* | 72 a | 70 b | 55 ab |
| ABA, 9 mg/L | 96 a | 100 a | 90 a | 60 a |
| Control | 100 a | 88 a | 85 a | 16 b |

| | | | | |
|---|---|---|---|---|
| | **Half-strength MS** | | | |
| 7 ABA, 7 mg/L | 100 a | 100 a | 95 a | 20 a |
| 9 ABA, 9 mg/L | 87 a | 42 b | 40 b | 0 b |
| Control | 94 a | 91 b | 70 ab | 55 a |
| | **Full-strength N&N** | | | |
| ABA, 7 mg/L | 85 a | 80 a | 78 a | 0 a |
| ABA, 9 mg/L | 81 a | 100 a | 70 a | 0 a |
| Control | 94 a | 81 a | 80 a | 0 a |

*For each ABA/medium combination, different letters in each column indicate significant difference by ANOVA and Tukey test at $p \leq 0.05$ (percentage values have been previously transformed by arcsin$\sqrt{\%}$).

### 3.1.2. Effect of ABA formulation on shoot length (cm)

Table 2 shows that, at 9 months of storage, only 7 mg/L of ABA in MSfs medium was able to produce shorter shoots, in comparison with the control (ABA-free MS medium). After the full time of storage (12 months), this consistent effect of MSfs/ABA at 7 mg/L on shoot length was confirmed.

**Table 2.** Effect of ABA on shoot length (cm) of nabq shoots during 12 months of storage in different media (control, ABA-free MS medium)

| **Months** | **3** | **6** | **9** | **12** |
|---|---|---|---|---|
| **Treatment** | | **Full-strength MS** | | |
| ABA, 7 mg/L | 2.30 b* | 1.98 b | 2.36 b | 2.64 b |
| ABA, 9 mg/L | 3.07 a | 2.66 b | 3.82 a | 3.60 a |
| Control | 4.18 a | 3.86 a | 3.43 a | 3.90 a |
| | | **Half-strength MS** | | |
| ABA, 7 mg/L | 1.94 a | 1.83 a | 2.36 a | 2.54 a |
| ABA, 9 mg/L | 1.19 b | 1.36 b | 2.26 a | ---- |
| Control | 2.24 a | 1.52 ab | 2.26 a | 2.26 a |
| | | **Full-strength N&N** | | |
| ABA, 7 mg/L | 1.67 b | 1.85 a | 1.61 b | ---- |
| ABA, 9 mg/L | 2.61 a | 2.00 a | 2.62 a | ---- |
| Control | 1.66 b | 1.09 b | 1.38 b | ---- |

*For each ABA/medium combination, different letters in each column indicate significant difference by ANOVA and Tukey test at $p \leq 0.05$.

### 3.1.3. Effect of ABA on shoot proliferation rate during the storage period

Table 3 shows that ABA on MSfs medium was able to block shoot proliferation for the whole storage period, both at 7 (Fig. 1a) and 9 mg/L. A slightly higher proliferation rate was observed on MShf medium containing 7 mg/L ABA. In control jars, containing ABA-free MS medium, the proliferation rate ranged between 1.60 (Fig. 1b) and 2.80.

**Table 3.** Effect of ABA on shoot proliferation of nabq during 12 months of storage in different media (control, ABA-free MS medium)

| Months | 3 | 6 | 9 | 12 |
|---|---|---|---|---|
| **Treatment** | | **Full-strength MS** | | |
| ABA, 7 mg/L | 1.00 b* | 1.00 b | 1.00 b | 1.00 b |
| ABA, 9 mg/L | 1.20 b | 1.20 b | 1.00 b | 1.00 b |
| Control | 2.00 a | 2.80 a | 2.60 a | 2.60 a |
| | | **Half-strength MS** | | |
| ABA, 7 mg/L | 1.20 b | 1.20 a | 1.20 b | 1.20 b |
| ABA, 9 mg/L | 1.20 b | 1.20 a | 1.20 b | 0.00 c |
| Control | 2.40 a | 1.60 a | 1.60 a | 1.60 a |
| | | **Full-strength N&N** | | |
| ABA, 7 mg/L | 1.40 a | 1.20 b | 1.20 b | 0.00 a |
| ABA, 9 mg/L | 1.40 a | 1.20 b | 1.00 c | 0.00 a |
| Control | 1.40 a | 1.60 a | 1.60 a | 0.00 a |

*For each ABA/medium combination, different letters in each column indicate significant difference by ANOVA and Tukey test at $p \leq 0.05$.

## 3.2. Shoot recovery after slow growth storage in ABA

### 3.2.1. Effect of ABA on survival percentage during the recovery stage

ABA at 7 mg/L, used in MSfs, was largely the best treatment to induce the highest recovery of shoots that were preserved in slow growth storage for 9 and 12 months (Table 4). On the other hand, the same concentration of ABA in MShs produced a recovery of 50-60% of shoots from 6, 9 and 12 months of storage. When 7 mg/L ABA was coupled to N&N medium, the recovery of shoot culture from 12 months conservation was 50%. It is also interesting to note that shoots from the storage in ABA-free MS medium (control) had a recovery of 25% (MSfs) and 0% (MShs and N&N) if derived from 12 months of conservation.

**Table 4.** Effect of ABA on survival percentage of nabq shoots during the recovery stage (control, ABA-free MS medium)

| **Months in storage** | **3** | **6** | **9** | **12** |
|---|---|---|---|---|
| **Treatment** | | **Full-strength MS** | | |
| ABA, 7 mg/L | 75 ab* | 50 b | 100 a | 100 a |
| ABA, 9 mg/L | 50 b | 50 b | 60 b | 50 b |
| Control | 83 a | 75 a | 20 c | 25 b |
| | | **Half-strength MS** | | |
| ABA, 7 mg/L | 25 b | 50 a | 60 a | 50 a |
| ABA, 9 mg/L | 25 b | 75 a | 50 a | 0 b |
| Control | 75 a | 75 a | 20 b | 0 b |
| | | **Full-strength N&N** | | |
| ABA, 7 mg/L | 50 b | 50 b | 30 b | 50 a |
| ABA, 9 mg/L | 75 a | 100 a | 50 b | 0 b |
| Control | 60 ab | 100 a | 100 a | 0 b |

*For each ABA/medium combination, different letters in each column indicate significant difference by ANOVA and Tukey test at $p \leq 0.05$ (percentage values have been previously transformed by arcsin√%).

### 3.2.2. Effect of ABA on shoot length (cm) during the recovery stage

Regarding the length of the shoots after storage in the different combinations of ABA/medium formulation, a general trend of reduction of this parameter can be noted during the recovery, correlated with the increase in the storage time (Table 5). Moreover, in general, shoots from ABA treatments always showed less regrowth in post-storage compared to the control (ABA-free medium).

**Table 5.** Effect of ABA on length of nabq shoots during the recovery stage (control, ABA-free MS medium)

| **Months in storage** | **3** | **6** | **9** | **12** |
|---|---|---|---|---|
| **Treatment** | | **Full-strength MS** | | |
| ABA, 7 mg/L | 1.91 a* | 1.77 a | 1.40 b | 1.70 a |
| ABA, 9 mg/L | 2.23 a | 1.60 a | 1.88 ab | 0.60 b |
| Control | 2.45 a | 1.75 a | 2.20 a | 1.80 a |
| | | **Half-strength MS** | | |
| ABA, 7 mg/L | 1.20 b | 0.95 b | 0.66 b | .50 |
| ABA, 9 mg/L | 0.50 c | 1.30 b | 1.34 a | --- |
| Control | 2.46 a | 2.11 a | 0.91 ab | --- |

| | | | | |
|---|---|---|---|---|
| | **Full-strength N&N** | | | |
| ABA, 7 mg/L | 1.30 b | 1.33 a | 0.70 b | 0.95 |
| ABA, 9 mg/L | 2.10 a | 1.25 a | 0.52 b | --- |
| Control | 2.21 a | 1.54 a | 1.34 a | --- |

*For each ABA/medium combination, different letters in each column indicate significant difference by ANOVA and Tukey test at p≤0.05.

### 3.2.3. Effect of ABA on proliferation rate (shoots/explant) during recovery stage

As shown in Table 6, the addition of ABA at 7 mg/L in the MSfs storage medium for the whole period (12 months) allowed a very high proliferation rate during recovery stage (Fig. 1c), not obtained in any other combination or in the control (ABA-free medium; Fig. 1d). This value of proliferation rate was double, in comparison with the same ABA concentration in MShs and N&N media.

**Table 6.** Effect of ABA on proliferation rate of nabq shoots during the recovery stage (control, ABA-free MS medium)

| **Months in storage** | **3** | **6** | **9** | **12** |
|---|---|---|---|---|
| **Treatment** | **Full-strength MS** | | | |
| ABA, 7 mg/L | 2.00 a | 2.00 b | 2.00 a | 4.00 a |
| ABA, 9 mg/L | 2.00 a | 2.40 a | 1.20 b | 1.00 b |
| Control | 1.60 a | 1.20 c | 1.00 b | 0.20 c |
| | **Half-strength MS** | | | |
| ABA, 7 mg/L | 2.00 a | 2.40 a | 2.40 a | 2.00 a |
| ABA, 9 mg/L | 1.00 b | 1.00 b | 1.40 b | 0.00 b |
| Control | 1.80 a | 1.60 b | 2.00 ab | 0.00 b |
| | **Full-strength N&N** | | | |
| ABA, 7 mg/L | 2.40 a | 2.00 a | 1.80 a | 2.00 a |
| ABA, 9 mg/L | 2.60 a | 1.40 b | 1.40 a | 0.00 b |
| Control | 2.00 b | 2.20 a | 1.60 a | 0.00 b |

*For each ABA/medium combination, different letters in each column indicate significant difference by ANOVA and Tukey test at p≤0.05.

## 4. DISCUSSION

Data of the effect of ABA as growth retardant during conservation and recovery indicate that ABA at 7 mg/L induced best results, compared to ABA at 9 mg/L or control (non treated shoots). These results confirm the outcomes of previous reports. For

instance, Gopal et al. (2004) reported that ABA and sucrose were effective in promoting the conservation of potato germplasm. Similar observations were reported by Westcott (1981) who showed that ABA and mannitol extended intervals between subcultures of potato cultivars up to 12 months, with a survival of 60%; moreover, when ABA was combined with a reduced temperature regime, culture survival had a further increase up to 73%. The same Author observed also that, in the presence of ABA and mannitol, shoot cultures became stunted, with short internodes and poor root development. Watt et al. (2000) stored shoot cultures of *Eucalyptus grandis* for up to 10 months by either the addition of 10 mg/L ABA to the storage medium, or by the halving of nutrient supply (1/2MS) and removal of plant growth regulators.

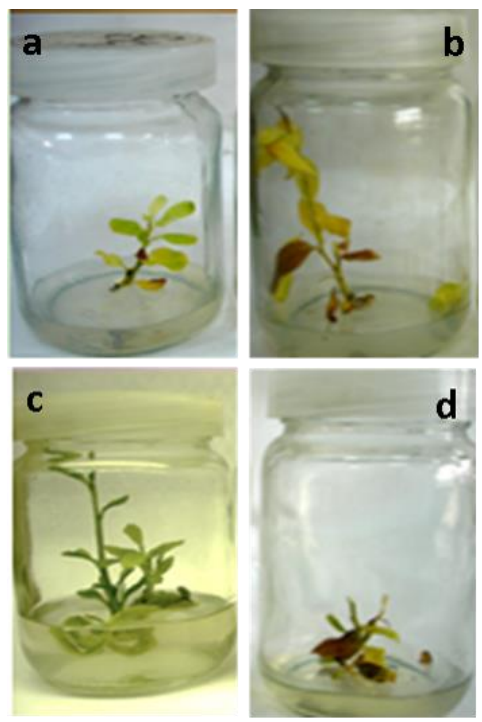


**Figure 1.** Effect of ABA in the storage medium on shoot proliferation: a) shoots after 12 months in MSfs medium added of 7 mg/L ABA; b) the same, but in ABA-free MSfs medium; c) shoots from 12 months in MSfs medium, added of 7 mg/L ABA, after 6 weeks on recovery medium; d) the same for shoots coming from the storage in ABA-free MSfs medium

## 5. CONCLUSIONS

The main outcome of the present study is that on MS medium, at full or half-strength, added of ABA at 7 mg/L, it is possible to achieve to a 12-month storage of nabq shoot cultures, maintaining a good quality level of material and an effective recovery in post-conservation. It has also been observed that the effect of ABA in slow growth storage is mediated by the formulation of the storage medium.

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# WORKING GROUP 3:

## Scaling up and automation

# Micropropagation of two commercial varieties of apple in bioreactors

**Simon Miranda[1], Mickael Malnoy[1], Stefano Piazza[1], Anxela Aldrey[2], Mª José Cernadas[2], Conchi Sánchez[2], Nieves Vidal[2]**

[1]*Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via Mach 1, 38098, San Michele all'Adige, Italy*

[2]*Dept. of Plant Production, Misión Biológica de Galicia (MBG), CSIC, Avda de Vigo s/n, 15705, Santiago de Compostela, Spain*

nieves@mbg.csic.es

## Abstract

The aim of this study was to apply new biotechnological tools to apple tree improvement. The commercial varieties 'Golden Delicious' and 'Royal Gala' were proliferated by temporary immersion and rooted by temporary or continuous immersion. For proliferation we used RITA® bioreactors, and the factors investigated were media type, cytokinin type and concentration, immersion frequency, supplementation with silver nitrate and the use of physical supports to hold explants in a vertical position (plastic slots and rockwool cubes). 'Golden Delicious' produced vigorous shoots in most of the treatments, whereas 'Royal Gala' was more prone to produce hyperhydric shoots and required more experimental work. Vigorous shoots cultivated in liquid medium were rooted in medium with auxin either in jars or in bioreactors (RITA® and Plantform™). Both apple varieties rooted successfully and were transferred to soilless media for acclimation. The researchers from the Fondazione Edmund Mach (FEM) provided the initial idea and the plant material, the researchers from Misión Biológica de Galicia (MBG) their expertise in woody plant propagation in bioreactors. Both groups participated in the design of the experiments, the strategies for solving problems, the discussion of the results and preparation of the manuscript for publication.

***Keywords:*** *'Golden Delicious', 'Royal Gala', hyperhydricity, rooting, temporary immersion.*

## 1. INTRODUCTION

/ Apples are the second most widely cultivated temperate fruit crop after bananas. Among the great cultivar diversity of apples, 'Golden Delicious' and ''Royal Gala'' are two major commercially important cultivars. Although *in vitro* propagation in semi solid medium (SSM) and gene editing by CRISPR/Cas9 are well established in these apple cultivars (Pompili et al., 2020; Miranda et al., 2023), the *in vitro* propagation conditions and delivery of editing components into apple require further optimization to improve the applicability of new breeding techniques.

Propagation in bioreactors can improve plant proliferation and physiological status, which can help implement novel technologies including nanoparticle delivery (Pérez-Caselles et al., 2023). This technology can be useful for sanitation, genetic transformation and general propagation improvement (Gupta et al., 2028). To the best of our knowledge the studies for apple micropropagation in bioreactors have been limited to M16, M9 rootstocks (Chakrabarty et al., 2002; Zhu et al., 2005; Kim et al., 2020) and *Malus sylvestris* native to Albania (Sota et al., 2021) with no reports of protocols based on liquid media for 'Golden Delicious' and 'Royal Gala'. This study is a collaboration between FEM (Italy) and MBG-CSIC (Spain), initiated by a STSM of COPYTREE. The main aim of the study was to investigate the culture conditions that can improve the growth of these cultivars in bioreactors and the potential application of nanoparticle delivery.

We used RITA® and Plantform™ bioreactors for multiplication and rooting stages of micropropagation, as these vessels have been used successfully with other woody plants (Vidal and Sánchez, 2019). The multiplication stage was carried out by temporary immersion (TIS). Adventitious rooting was induced by auxin treatment applying TIS in RITA®, TIS or continuous immersion (CIS) in Plantform™, as well as semisolid medium in jars. Our results demonstrated that 'Golden Delicious' and 'Royal Gala' can be cultured in bioreactors. 'Golden Delicious' proliferated more than 'Royal Gala' and showed less hyperhydricity. Both varieties responded well to rooting treatments and plants have been successfully acclimated.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Initial explants for proliferation in TIS consisted on apical shoots (15-20 mm) of apple shoot cultures from the varieties 'Golden Delicious' (GDE) and 'Royal Gala' (RGA). Both varieties had been previously established *in vitro* in semisolid medium in the FEM laboratories and maintained by periodic subcultures (Miranda et al., 2023). GDE was cultured in Murashige and Skoog medium plus vitamins (MS) (Murashige and Skoog, 1962) with 1.3 µM of indole-3-butyric acid (IBA), 0.6 µM of gibberellic acid, 100 mg/L myo-inositol and 30 g/L sucrose (GDE-PM). Cultures maintained in semisolid medium were supplemented with 4.4 µM of $N^6$-benzyladenine (BA) and 7.5 g/L agar. The proliferation medium for RGA consisted of MS with 220 µM of Fe-EDDHA and 30 g/L sucrose (RGA-PM), supplemented with 3.1 µM of BA and 7.5 g/L agar for semisolid cultures. Cultures were incubated under a 16 h photoperiod at 25 °C light/20 °C dark.

### 2.2. Methods

Proliferation in liquid medium was carried out in commercial RITA® (www.vitropic.fr) bioreactors prepared and operated as described for chestnut (Vidal et al., 2024). Twelve to sixteen explants were used in each RITA®, and were immersed for 90 s 3 or 6 times

per day. To test the effect of maintaining the explants in vertical position they were held between 1 $cm^3$ rockwool cubes or inserted in plastic slots in a custom made (in-house) holder (Figure 1a), whereas others were placed directly on the basket surface. For some of the experiments RITA® baskets were divided in 2 sections with aluminum foil to hold explants with or without support (Figure 1b).


**Figure 1.** Supports used to maintain the explants in vertical position. a) plastics slots, b) RITA® divided into two sections (cubes and no support).

The factors investigated were the media type (MS or MS with half-strength $NH_4NO_3$ and $KNO_3$ (MS ½ N), cytokinin type (BA, metaTopolin (mT), metaTopolin riboside (mTR)) and concentration, immersion frequency (3 and 6 immersions per day), supplementation with silver nitrate plus use of physical supports to hold explants in a vertical position (plastic slots and rockwool cubes).

Rooting was performed with half-strength MS supplemented with 4.9 µM of IBA and 30 g/L sucrose. We used jars (with 7 g/L of agar), RITA® with plastic slots, as well as Plantform™ (www.plantform.se). Plantform™ were used by temporary immersion (TIS) as described for chestnut proliferation (Vidal et al., 2024) or by continuous immersion (CIS) as described for rooting of plum (Gago et al., 2022b). In both cases we used rockwool cubes of 1 or 2 $cm^3$. Shoots (≥ 25 mm long) were placed between 1 $cm^3$ cubes or inserted in 2 $cm^3$ cubes. Shoots rooted in Plantform™ operated by TIS were immersed 3 times per day for 90 s, with 12 additional aerations per day, whereas those rooted in Plantform™ operated by CIS were aerated 15 times per day for 90 s. After 6 weeks, the rooted shoots were transferred to plug trays filled with a peat:perlite (3:1). Plantlets were acclimatized for 4 weeks in a phytotron before being transferred to the greenhouse.

***Data recording and Statistical Analysis***

The parameters analyzed were: (a) the number of shoots longer than 15 mm per explant, differentiating between normal (N) and hyperhydric shoots (H); (b) the length of the longest shoot per explant; (c) multiplication coefficient, defined as the number of nodal

segments useful for multiplication obtained per initial explant; (d) the number of rootable shoots obtained per initial explant, defined as non-hyperhydric shoots longer than 20 mm and with an active apex, (e) rooting and acclimatization percentage.

The data were analysed by Levene's test (to verify the homogeneity of variance) and subjected to analysis of variance (ANOVA) followed by comparison of group means (Tukey-b test). When the requirements for ANOVA were not met a Chi-square test was used. Statistical analyses were performed using SPSS 26.0 (IBM).

## 3. RESULTS

Preliminary experiments indicated that the use of a physical support produced significant differences in hyperhydricity and proliferation of 'Golden Delicious' cultured in RITA®, with p=0.021 for the percentage of hyperhydric shoots and p=0.029 for the multiplication coefficient. More proliferation and less hyperhydricity was obtained with supports, without significant differences between rockwool cubes and plastic slots (Table 1). As a practical consideration, in next experiments with both varieties we used rockwool cubes in preference to plastic slots due to easier handle.

**Table 1.** Effect of support material on the hyperhydricity and proliferation capacity of apical shoots of 'Golden Delicious' cultured in RITA bioreactors for 8 weeks in GDE-PM with BA 4.4 µM. Shoots were immersed 6 times per day for 90 s. Means ± standard error were calculated from 2 replicates, each with 4 shoots per treatment. Different letters indicate significant differences in relation to the support (Chi-square, test $p < 0.05$). HS: percentage of the new shoots showing hyperhydricity; MC: multiplication coefficient.

| Support | HS (%) | MC |
|---|---|---|
| none | 26.3 a | 3.7 ± 0.8 b |
| cubes | 9.4 b | 6.1 ± 0.9 a |
| slots | 11 b | 6.6 ± 1.4 a |

The treatments used in Table 1 for GDE were applied to 'Royal Gala' cultured with the medium reported for this variety (RGA-PM supplemented with BA 3.1 µM). Although the BA concentration was lower than for GDE, most of the shoots were highly hyperhydric and not suitable for further multiplication. This problem persisted even when the frequency of immersion was reduced from 6 to 3 per day, as shoots for proliferation could not be obtained in those conditions.

Figure 2 shows the effect of BA and the support on the quality and proliferation of GDE. This variety can be propagated without support, but with rockwool cubes longer shoots can be obtained, as well as higher proliferation and more rootable shoots. To decrease the BA concentration didn't affect positively any of the studied parameters, although the leaves were larger (data not shown).

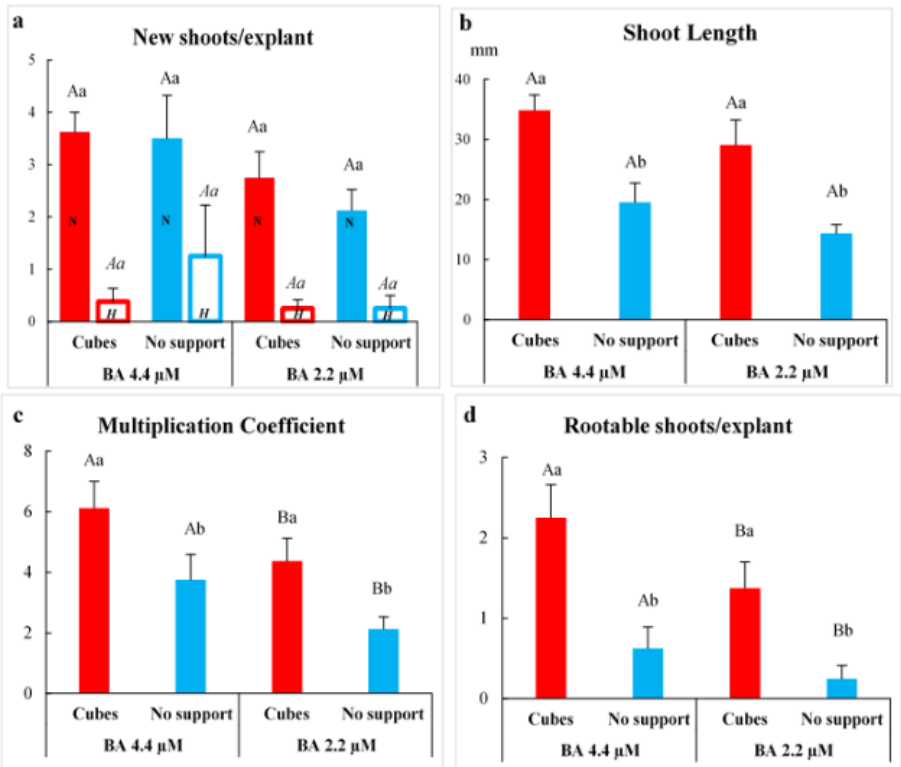


**Figure 2.** Effect of BA concentration and the use of a support on the growth of 'Golden Delicious' cultured in RITA bioreactors for 8 weeks in GDE-PM with BA 2.2 or 4.4 µM. Shoots were immersed 6 times per day for 90 s. a) Number of normal (N, solid columns) and *Hyperhydric* (*H*, empty columns) shoots, b) Shoot length of the longest shoot per explant, c) Multiplication Coefficient, d) Number of rootable shoots per explant. Means ± standard error were calculated from 2 replicates, each with 6 shoots per treatment. Different uppercase letters indicate significant differences between BA supplementation and different lowercase letters indicate significant differences between use of a support ($p<0.05$). In a) the differences regarding the number of normal shoots are presented with normal font and the differences regarding the number of *hyperhydric* shoots in *italics*.

The main limitation for the growth of 'Royal Gala' in bioreactors was the high proportion of hyperhydric shoots. We approached this problem by: i) reducing the N of the medium (substitution of MS with MS N ½), ii) lowering the cytokinin dose (1.55 µM instead of 3.1 µM), iii) evaluating other cytokinins besides BA (mT and mTR), iv) adding $AgNO_3$ (30 µM) combined or not with rockwool cubes. Results are shown in Figure 3.

These results indicate reduction of N in the medium was positively correlated with the reduction in hyperhydricity, as some of the treatments produced mostly non-hyperhydric shoots. Silver nitrate didn't show a clear effect on hyperhydricity, whereas the use of a support alone ("$Ag^-$ $Cubes^+$" treatment) increased proliferation and the production of rootable shoots. This effect was observed with all the cytokinins, although it was more noticeable in the case of BA. Interestingly, mTR produced the longest shoots and shoots treated with mT showed less hyperhydricity. Good quality shoots obtained from jars and RITAs at the end of the proliferation stage were selected for rooting experiments (Figure 4).

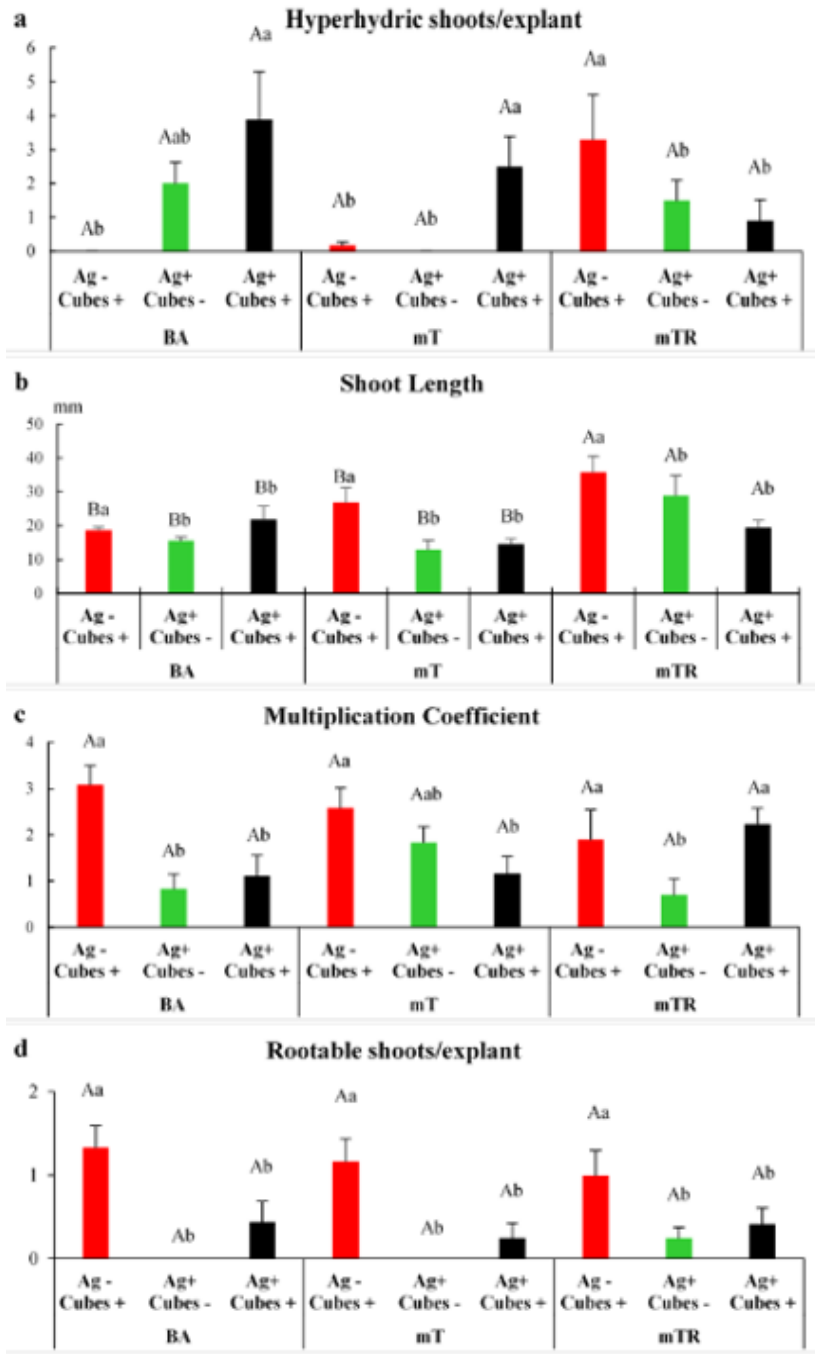


**Fig. 3.** Effect of cytokinin type, support and $AgNO_3$ (30 µM) on the growth of 'Royal Gala' cultured in RITA bioreactors for 8 weeks in RGA-PM (substituting MS with MS N ½). Cytokinins were applied at 1.5 µM. Shoots were immersed 6 times per day for 90 s. a) Number of hyperhydric shoots per explant, b) Shoot length of the longest shoot per explant, c) Multiplication Coefficient, d) Number of rootable shoots per explant. Means ± standard error were calculated from 2 replicates, each with 6 shoots per treatment. Different uppercase letters indicate significant differences between cytokinins, and different lowercase letters between the treatments combining presence (+) or absence (-) of support (*cubes*) and silver nitrate (*Ag*) at $p < 0.05$.

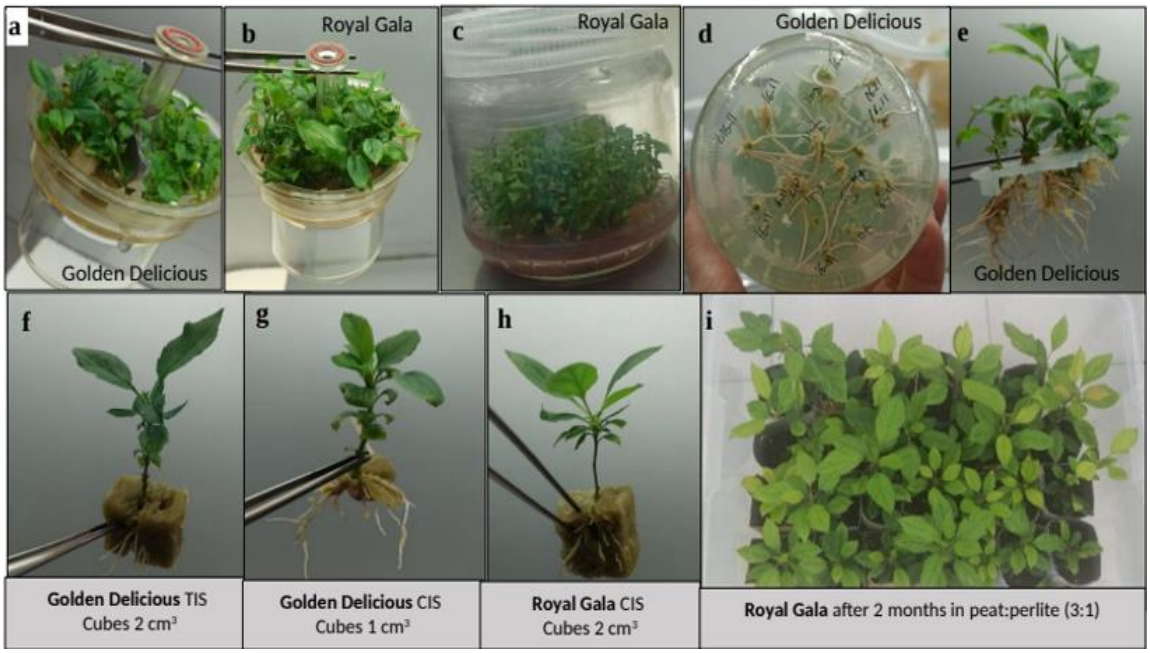


**Figure 4.** a-c) Shoots at the end of the proliferation stage that were used for rooting. d) Shoots rooted in jars. e) Shoots rooted in RITA. f-h) Shoots rooted in Plantform by TIS (f) and CIS (g, h). i) 'Royal Gala' plantlets after 2 months of acclimation.

Both apple varieties rooted more than 75% in all treatments (Figure 4d-h), irrespective of the system used for multiplication and rooting (jars or bioreactors). Results of rooting experiments carried out in Plantform are shown in Table 2. 'Golden Delicious' shoot rooting was 100% in Plantform using TIS with shoots placed between 1 $cm^3$ cubes. In 'Royal Gala' 100% rooting occurred in CIS with shoots inserted in 2 $cm^3$ cubes. Rooted shoots were successfully transferred to soilless media for acclimation (Figure 4i).

**Table 2**. Effect of culture system (TIS or CIS) and support material size (1 or 2 $cm^3$ rockwool cubes) on the rooting response of 'Golden Delicious' and 'Royal Gala' shoots cultured in bioreactors on half-strength MS supplemented with 4.9 µM of IBA and 30 g/L sucrose. In TIS shoots were immersed 3 times per day for 90 s and aerated 12 times per day for the same 90 s, in CIS shoots were aerated 15 times a day. TIS: Temporary immersion, CIS: Continuous immersion. Rooting percentages were recorded after 6 weeks and correspond to 20 shoots per treatment.

| | **TIS** | | **CIS** | |
|---|---|---|---|---|
| Cube size | 1 $cm^3$ | 2 $cm^3$ | 1 $cm^3$ | 2 $cm^3$ |
| **'Golden Delicious'** | 100% | 90% | 70% | 90% |
| **'Royal Gala'** | 75% | 95% | 85% | 100% |

## 4. DISCUSSION

In this study we investigated some variables affecting the propagation of the apple varieties 'Golden Delicious' and 'Royal Gala' in liquid medium in bioreactors, for both proliferation and rooting.

The proliferation step was carried out using temporary immersion in RITA®. We detected some marked differences between the two varieties even though they are closely related genetically. 'Golden Delicious' having a multiplication coefficient of 6 in the best treatment was more productive than 'Royal Gala'. 'Royal Gala' was severely affected by hyperhydricity, and in the best treatments only achieved a multiplication coefficient of 3. Hyperhydricity is one of the most important challenges affecting micropropagation of woody plants in bioreactors (Vidal and Sánchez, 2019) including apple rootstocks (Zhu et al., 2005; Chakrabarty et al., 2008), and frequently the degree of susceptibility is related to the genotype, as reported for pear (Lotfi et al., 2020). 'Golden Delicious' also developed some hyperhydric shoots, but this could be effectively controlled with the use of a support to maintain the explants in a vertical position, as reported for chestnut (Vidal et al., 2015). Decreasing the concentration of cytokinin added to the medium also reduced hyperhydricity in GDE, but this strategy was less effective as the proliferation and the number of rootable shoots were significantly reduced as well.

Other strategies that can be found in the literature for controlling hyperhydricity include: a) the reduction of the frequency of immersions which showed good results with juvenile oaks (Gatti et al., 2017) and pear (Lofti et al., 2020), b) the use of lower concentrations of ammonium and cytokinin, recommended for aloe in semisolid medium (Ivanova and van Staden, 2008), c) silver nitrate, which controlled hyperhydricity in *Dianthus chinensis* and *Antirrhinum majus* in semisolid medium (Gao et al., 2017; Lee et al., 2023), and d) the substitution of BA for other cytokinins as mT and its derivatives, which was effective for pear in bioreactors (Lotfi et al., 2020). In 'Royal Gala' reducing the immersion frequency from 6 to 3 immersions per day alone didn't have a positive effect on the development of normal shoots. In our study, the best results with this variety were obtained using rockwool cubes as a support and the concurrent reduction of both the cytokinin and the nitrogen of the medium (Figure 3a). The use of cytokinins other than BA seems to be promising, as all of them produced shoots suitable for proliferation and rooting (Figure 3c). The longest shoots were obtained with mTR (Figure 3b) and mT was the treatment with the least hyperhydricity (Figure 3a). However, the treatment with silver nitrate did not have clear effects in the case of RGA in the concentrations it was used. While shoot production was higher in GDE than in RGA, in both cases enough shoots were produced to allow root initiation comparisons to be made.

We used different strategies for rooting, as all of them could provide different insights or advantages depending on the subsequent utilization of the rooted shoots: the

treatment with semisolid medium allows visualisation of the moment when and how the roots emerge without disturbing the shoots, to use RITA® with plastic slots enables a rapid and easy change of medium for other biotechnological treatments, as well as to increase the uptake of active substances due to the forced ventilation. This later characteristic is shared by the Plantform™ bioreactors when operated by TIS, with the additional benefit that they can hold a higher number of explants. Finally, shoots inserted in 2 $cm^3$ rockwool cubes in Plantform™ bioreactors when operated by TIS or CIS are easier to handle without causing root damage if the purpose is to transfer them to soilless media for acclimation and large-scale plant production. The use of fibrous or porous support materials for rooting has been recommended as a simple and cost effective means of micropropagation (Newel et al., 2003; Dutta Gupta and Prasad 2006) and was beneficial for other woody plants such as American and European chestnut (Maner and Merkle, 2010; Cuenca et al., 2017; Gago et al., 2022a), peach (Adelberg et al., 2021) and plum (Gago et al., 2022b). In our case we obtained high rooting rates with all the treatments and the plantlets were successfully acclimated.

## 5. CONCLUSIONS

In this study we demonstrated the feasibility of micropropagating ‘Golden Delicious’ and ‘Royal Gala’ in liquid medium in RITA® bioreactors. The two varieties reacted differently to liquid medium: ‘Golden Delicious’ produced vigorous shoots in most conditions whereas ‘Royal Gala’ showed more hyperhydricity and less multiplication capacity. Future research will evaluate different cytokinins as well as the roles of silver and nitrogen on apple propagation. The use of Plantform™ bioreactors and aeration regime will be also investigated during the proliferation stage of ‘Golden Delicious’ and ‘Royal Gala’. This is the first report in the micropropagation of these two commercial fruiting varieties in temporary immersion. By using bioreactors, we obtained successful results for rooting and acclimation for both of them, whereas the multiplication phase in ‘Royal Gala’ has to be improved before being more widely applicable.

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# Overcoming challenges for micropropagation of *Prunus domestica* cv. Tropojane in various TIS bioreactor systems

**Brunilda Çuko[1], Ledina Shkëmbi[1], Efigjeni Kongjika[2], Valbona Sota[1,2]**

[1]*Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Boulevard "Zog I" Tirana 1001, Albania*

[2]*Biotechnology & Genetics Scientific Research Unit, Section of Natural and Technical Sciences, Academy of Sciences of Albania, "Murat Toptani" Promenade, Tirana 1000, Albania*

*cuko.brunilda@gmail.com*

## Abstract

*Prunus domestica* cv. Tropojane is an important autochthonous fruit crop in Albania, and it is widely used for its well-known values in the food industry. Beyond the traditional propagation methods, efficient methodologies and protocols have recently been established for the *in vitro* micropropagation of this fruit species using conventional micropropagation methods. However, the new approaches in the field, such as temporary immersion systems, bring new perspectives to the large-scale micropropagation of *P. domestica*. This study presents some strategies and methodologies for overcoming the challenges faced during the micropropagation of *P. domestica* cv. Tropojane in the Plantform and SETIS bioreactors and comparison with the cultivation in semi-solid medium. Some cultural practices to decrease contamination rate were optimized. Plum shoots grown in a gelled nutrient medium were isolated at 1-1.5 cm in length, and placed in the specific containers of bioreactors. Previously, the liquid nutrient medium was placed in the respective containers and sterilized inside the bioreactor to avoid culture contamination by reducing the manipulation time in the laminar flow. Different immersion frequencies were tested since this is considered an essential factor which affect the hyperhydricity and shoot tip necrosis rates. Optimization needs to be done regarding the type and concentrations of cytokinins in the nutrient media and types of basal media to achieve a high regeneration rate of lateral shoots, with reduced symptoms of oxidative stress and plantlets necrosis.

***Keywords:*** *plum, immersion frequency, hyperhydricity, shoot tip necrosis*

## 1. INTRODUCTION

*Prunus domestica* L., also known as the European plum is a fruit-bearing tree of significant economic importance due to its nutritious and versatile fruits. It is one of the most widely cultivated and economically significant fruit trees globally, even though it is believed to have originated in the region spanning the Caucasus, the Caspian Sea, and the Balkans. Even in Albania, plum trees are widely cultivated (Osmani *et al.,* 1996). Also, some autochthonous plum cultivars are considered an integral part of Albania's

agricultural heritage, offering unique genetic resources and contributing to the region's biodiversity. These cultivars are valued not only for their adaptability and resilience but also for their distinctive flavors and use in traditional Albanian cuisine. The object of this study is an autochthonous cultivar of plum called "Tropojane", well-known for its high yield and excellent fruit quality. The fruits are medium to large, with an oval to oblong shape and a deep purple to blue-black skin. The flesh is golden yellow, firm, and juicy, with a sweet and slightly tart flavor. "Tropojane" plums are popular for fresh consumption and processing into prunes, jams, and other preserves. Due to these specific characteristics and the national and international market needs, it is essential to establish efficient strategies for mass propagation of this autochthonous cultivar of plum.

*In vitro* micropropagation has emerged as a powerful alternative for the clonal propagation of *Prunus domestica*, offering a suitable method to provide fruit plant growers with sufficient, true–to-type, disease-free planting materials (Hammerschlag & Bauchan, 1985; Kassaye & Bekele, 2015). These techniques and methodologies based on the *in vitro* micropropagation of plants have continuously been improved in order to gain a mass production of plants at low costs. Therefore, new systems, such as temporary immersion systems (TIS), were developed to address some of the limitations of traditional liquid and solid culture systems, such as poor gas exchange and hyperhydricity (Teisson & Alvard, 1995). These systems, which are considered a promising alternative to the traditional culture system in gelled media, have significantly improved the efficiency and quality of micropropagated plants (Sota *et al.,* 2021; Vidal & Sánchez, 2019; Takayama & Akita, 1994). However, together with their advantages, like any technology, TIS comes with its own set of limitations and challenges. The most noted limitations of TIS bioreactors are related to contamination risk and physiological issues such as hyperhydricity and vitrification. Each TIS may require specific optimization of immersion frequency, duration, and growth regulator concentrations. These parameters can vary widely depending on the plant species and even the specific cultivar being propagated (Georgiev *et al.,* 2014; Etienne & Berthouly, 2002).

This paper presents some strategies used to overcome the challenges during the *in vitro* micropropagation of *Prunus domestica* L. cv. Tropojane in TIS bioreactors (Plantform™ and SETIS™). The aim is to optimize protocols for the efficient propagation of this species in temporary immersion systems.

## 2. MATERIALS AND METHODS

***Plant material*:** Stabilized *in vitro* culture of *Prunus domestica* L. cv. Tropojane originated from meristem shoot tips culture were used to isolate the plant material for the TIS bioreactor (Plantform™ and SETIS™) micropropagation and for the tests done in gelled nutrient medium. Virus-free shoots of 1 – 1.5 cm in length were selected and placed directly the bioreactor containers since there was no need for surface sterilization of explants.

***Nutrient medium:*** Liquid and gelled nutrient media were used for the micropropagation of *Prunus domestica* L. cv. Tropojane plants. LP (Long & Preece, 2004) was used as basal medium. For the semi-solid medium, it was supplemented with 0.7% (w/v) agar.

In all cases, the basal medium was supplemented with 1 mg/L 6-benzylaminopurine (BAP), 0.1 mg/L α-naphthalene acetic acid (NAA) and 3% (w/v) sucrose. The pH was adjusted at 5.5-5.7 before autoclaving and then the media were sterilized in 121°C for 20 min at 1.05 kg/cm$^2$ (15-20 psi).

***Inoculation:*** In the aseptic conditions of laminar flow, the shoots from the previous culture were prepared by cutting the ends and removing the brown leaves to be propagated in Plantform and SETIS bioreactor. The same procedure was done for the explants which will be cultivated in jars with a gelled nutrient medium. After that, 500 mL of liquid nutrient medium was added to the container of the Plantform bioreactor, and 1000 mL of nutrient medium was used for the SETIS bioreactor. In some tests, the same amount of liquid nutrient medium was previously added to the containers and sterilized inside the bioreactors. Then, the explants weight was measured (approximate 10 g for Plantform and 16 g for SETIS) and they were horizontally placed in the specific container of each bioreactor. The bioreactors were completely closed and isolated with transparent cellophane paper.

***Culture conditions:*** The cultivation phase for all the cultures was done under the controlled physic parameters of growth chamber (23 ± 2°C, 16 / 8 h photoperiod. and 40 - 45 µmol/m$^2$·s light intensity). For the micropropagation of plum in bioreactors three different immersion time were tested (i) 2 min / 6 h; (ii) 1 min / 8 h; (iii) 1 min / 12 h, at the same ventilation time and frequency (15 min / 6 h).

***Evaluation method:*** The evaluation of *Prunus domestica* L. cv. Tropojane micropropagation in temporary immersion bioreactors was based on visual and morphometric parameters such as uniformity, color, shape of the obtained plants, level of culture contamination and Relative Growth Rate (RGR). These parameters, evaluated in 15 replications on Plantform and 10 replications on SETIS were compared with those obtained from the cultivation of plum in conventional *in vitro* methods in gelled nutrient medium.

## 3. RESULTS

**Risk of contamination:** A few days after the inoculation in both bioreactors, spots of contamination were detected. The contamination was present in nutrient medium, containers walls and explants surface. Based on visual evaluation, the contamination was both fungal and bacterial. The bacterial contamination appeared after 15-20 days of cultivation when the nutrient medium changed its color from light pink to orange/brown (Fig. 1.).


**Figure 1. a)** Fungal contamination, **b)** Bacterial contamination of cultures in Plantform, and **c)** SETIS

Generally, a high level of contamination (62%) was detected during the micropropagation of plums in bioreactors. Comparing the level of contamination in different bioreactors, resulted that the explants micropropagated in Plantform have a higher risk of contamination (Fig. 2.).

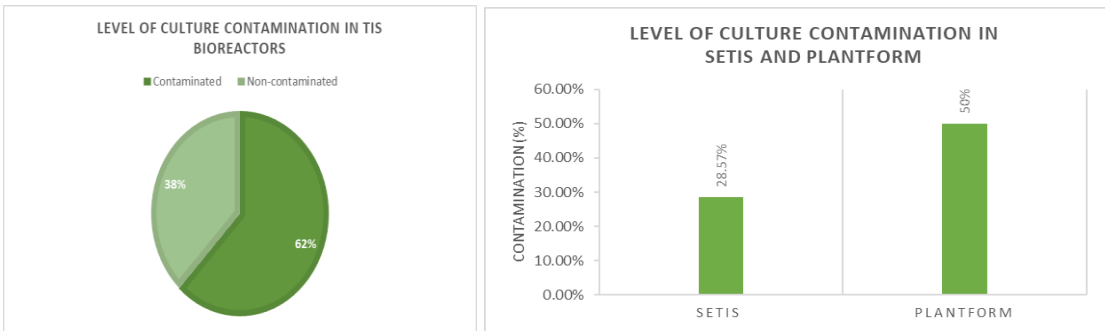


**Figure 2.** Level of culture contamination in SETIS and Plantform bioreactors

**Plant quality:** The cultivation of *Prunus domestica* L. cv. Tropojane in different times of immersion in both bioreactors by using LP nutrient medium (1 mg/L BAP + 0.1 mg/L NAA) produced explants with significant differences in color and shape of leaves, presence of hyperhydric explants, length, and form of new shoots (Fig. 3).

The influence of the immersion frequency: The relative growth rate (RGR) was evaluated for the explants of *Prunus domestica* L. cv. Tropojane, testing different immersion frequencies. Our results showed that reducing the immersion time to 1 min every 12 hr gave better RGR values in comparison to the other immersion periods tested. On the other hand, even the quality of explants in terms of hyperhydricty, normal color and leaf shape was better on this treatment.

Differences between cultivation systems: The relative growth rate (RGR) was evaluated for the plants of *Prunus domestica* L. cv. Tropojane cultivated in SETIS and Plantform bioreactors and gelled nutrient medium. The first tests showed that relative growth rate was higher in Plantform after 21 days of cultivation on LP nutrient medium. There was observed a slight difference on the RGR values between cultivation on semi-solid medium and SETIS bioreactor, but not a significant one (Fig. 5.).

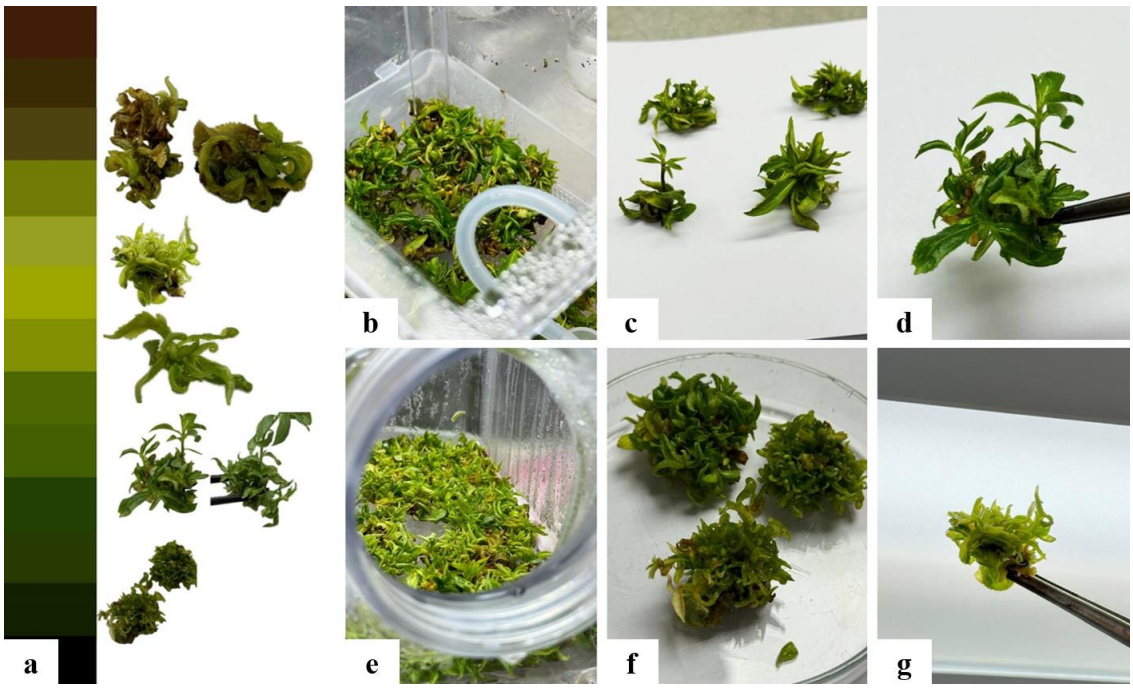


**Figure 3. a)** Differences in explants obtained during the micropropagation of *Prunus domestica* L. cv. Tropojane in Plantform **(b, c**, d) and SETIS **(e, f, g)** bioreactors

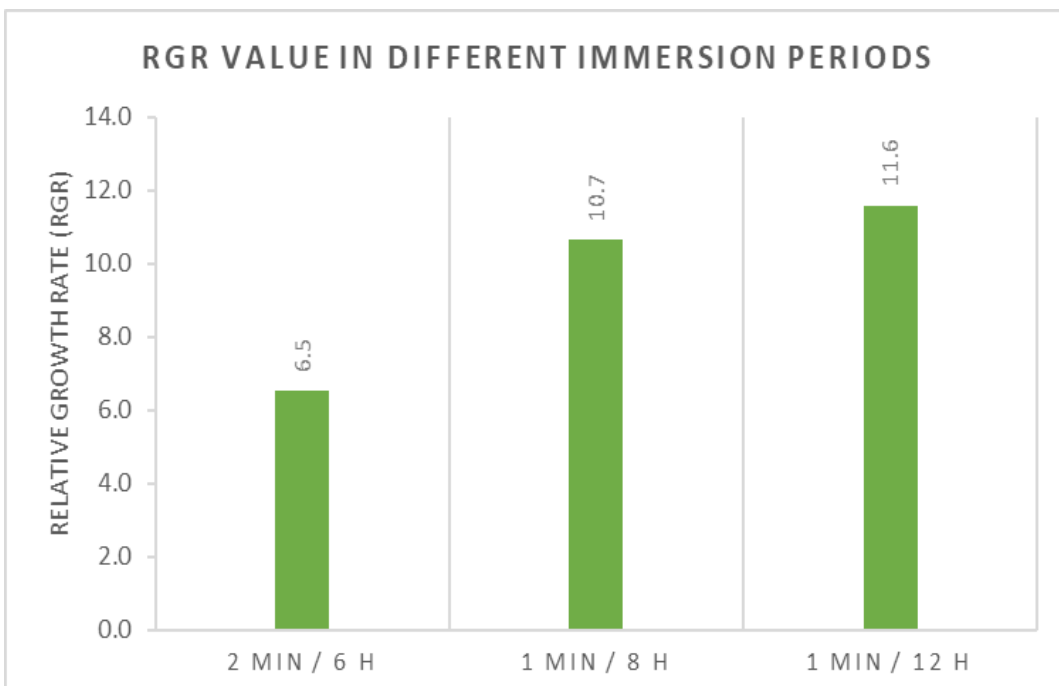


**Figure 4.** The influence of different immersion periods on the RGR values after 21 days of cultivation in LP nutrient medium (average value in both bioreactors)

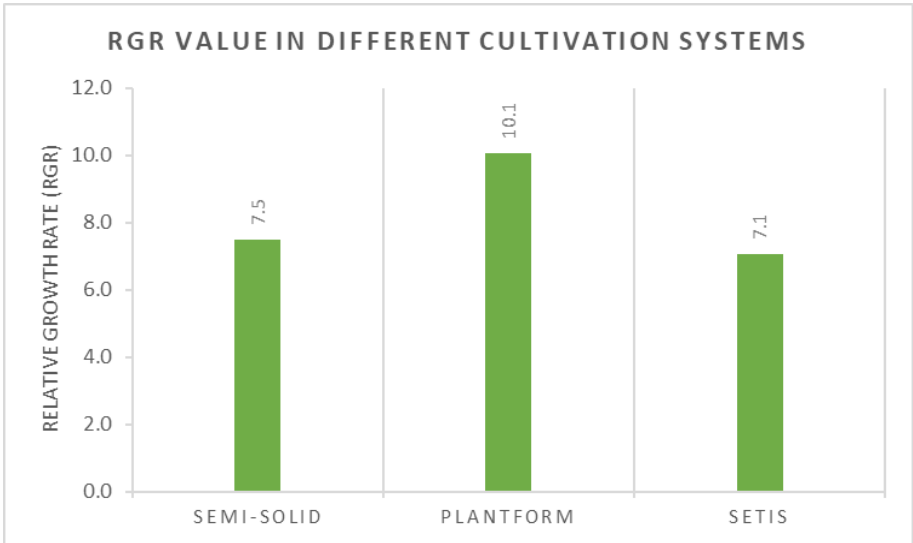


**Figure 5.** RGR values for 21 days of cultivation in LP nutrient medium in different cultivation systems

The growth dynamic in TIS systems was also evaluated. It showed that in the first days of cultivation of plants in bioreactors, the speed of growth was higher, and after that there was noticed a reduction of growth intensity (Fig. 6.). This phenomena is observed on both SETIS and Plantform bioreactors. Since the time of cultivation, negatively affects the RGR value, it was concluded that a specific time of cultivation should be determined and used for the evaluation and comparison of all the tests. On the other hand, testing the frequency of the changing of the nutrient medium should be another parameter to be defined, which might keep a high value of RGR during all cultivation periods.

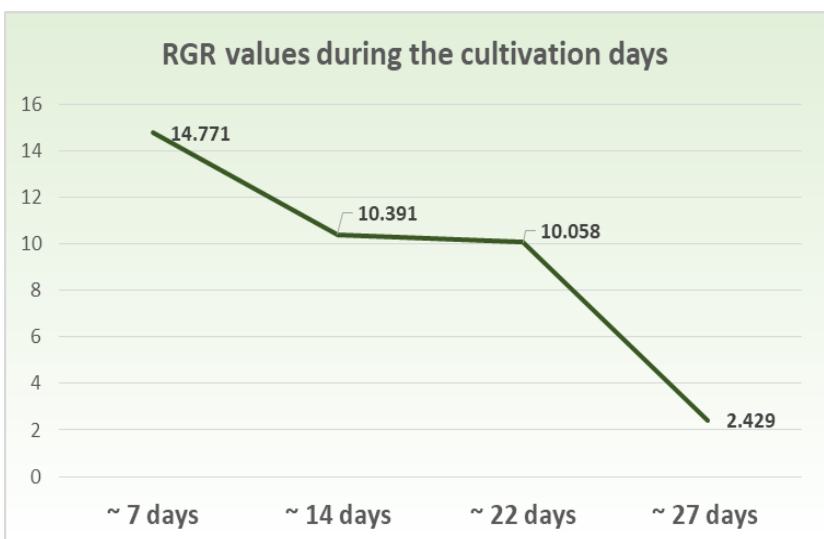


**Figure 6.** RGR values during cultivation in TIS bioreactors (average value on both bioreactors)

## 4. DISCUSSION

The contamination of *in vitro* cultures is a significant problem detected in temporary immersion systems, and in conventional techniques of *in vitro* micropropagation of plants. The level of contamination, as supported by other studies (Cassells, 2001; Etienne & Berthouly, 2002; George, *et al.,* 2008; Kane, 2011) affects the cost production of plants because at the same container are propagated a considerable number of plants which all will be lost.

All the variables in our study seem to have a role in the morphology of plants regenerated in TIS systems. The basal solution used, the time of immersion and that of ventilation, and the duration of the culture have a great influence in this regard. Since the initial plant material used for micropropagation of *Prunus domestica* L. cv. Tropojane was taken from stabilized cultures, the contamination in these cases could not be significantly related to the species and type of explant used. Our study showed a high level of contamination in TIS bioreactors, but it was noticed that the sterilization of nutrient medium inside the specific container of each bioreactor reduces the risk of contamination. In addition, improving practical skills during the manipulation in laminar reduces the cost of hand labor and the contamination risk.

Another challenge faced during the micropropagation of *Prunus domestica* L. cv. Tropojane in TIS bioreactors is the plant quality in terms of hyperhydricity, shoots malformation, non-uniformity of plants, and shoot tip necrosis. The hyperhydric plants with thick and translucent leaves were frequently presented during the micropropagation in both bioreactors but the strategy followed in our tests by reducing the immersion time to 1 min / 12 h resulted to be effective. However, the reduction of immersion time should be moderated because very short immersion periods may not provide sufficient hydration and nutrients, potentially leading to other growth issues (Ziv, 1991). The differences in plant quality noted during micropropagation of plants in different nutrient media showed that the mineral and hormonal composition of the nutrient medium might affect the hyperhydricity of the plants in bioreactors (Mehrotra *et. al.,* 2007; Polivanova & Bedarev, 2022). On the other hand, previous studies report that high relative humidity within culture vessels and hyperhydricity are associated with shoot tip necrosis (Teixeira da Silva, *et al.,* 2020; Bairu, *et al.,* 2009). Our results showed that LP nutrient medium was more effective for the micropropagation of plums based on shoot tip necrosis incidence. Other authors who claim that the cause of this phenomena is related to nutrient deficiency and imbalance support this result. De Block (1990) found that shoot tip necrosis was linked to $Ca^{2+}$ deficiency and associated with the use of Woody Plant Medium (WPM). The MS nutrient medium has almost the same $Ca^{2+}$ content. Since the $Ca^{2+}$ concentration in LP nutrient medium is higher, these prove that our results align with the above mentioned theory. Subsequent objectives in our experimental platform should continue with the optimization of all the above

influencing factors, to obtain normal plants with reduced signs of hyperhydricity and shoot tip necrosis.

Many studies promote the temporary immersion system for mass propagation of plants, because of the great advantages they bring in the direction of reducing the overall cost. The time of manipulation of the explants, the lack of agar in the nutrient medium, the number of explants that can be obtained for a short period, are advantages of such systems. Of course, micropropagation protocols in TIS systems are not universal and require specific optimization in relation to the cultivated plant species. In our tests we tried the *in vitro* micropropagation of Prunus domestica L. cv. Tropojane in SETIS and Plantform bioreactors, which is a highly recalcitrant plant species. Based on the growth dynamic, we noticed that the cultivation period in TIS bioreactors is a crucial parameter, which significantly affects the RGR level. After 21 days on *in vitro* cultivation of plants, the growth level significantly reduces due to nutrient medium consumption and it is necessary to replace it or to pass to another subculture phase. On the other hand, especially optimizing determining an optimal immersion and ventilation frequency is crucial to achieve the desired results.

## 5. CONCLUSIONS

The temporary immersion bioreactors (SETIS and Plantform) offer a great potential for the micropropagation of many plant species. However, our first tests for the micropropagation of *Prunus domestica* L. cv. Tropojane show that some parameters must be better optimized which directly affect the plant quality, and biomass production of plants. The preliminary results presented in this paper confirmed the factors affecting the effectiveness of plant micropropagation in TIS bioreactors and the strategies proposed and followed in our laboratory in order to overcome the present limitations of TIS bioreactors were effective. However, further studies need to be done in this direction to optimize protocols for the *in vitro* micropropagation of *Prunus domestica* L. cv. Tropojane in SETIS and Plantform bioreactors.

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# Temporary Immersion System and addition of silver nanoparticles to eliminate pathogens in apricot (*Prunus armeniaca* L.)

**Pérez-Caselles C., Martín-Valmaseda M., Alfosea-Simón F.J., Alburquerque N., Burgos L.**

*Fruit Biotechnology Group. Department of Plant Breeding, CEBAS-CSIC, Campus Universitario de Espinardo, Edificio Nº25, 30100 Murcia, Spain*

burgos@cebas.csic.es

## Abstract

Temporary Immersion Systems (TIS) are necessary to micropropagate apricot in liquid medium and paramount to apply silver nanoparticles (AgNPs) since their availability to the plant is restricted in semisolid medium. AgNPs have been described as antimicrobial agents and, in our laboratory, we have tested their effect on micropropagted shoots of Plum pox virus (PPV)-infected 'Canino' and Hop Stunt Viroid (HSVd)-infected 'Mirlo Rojo'. The aim of this work is to produce PPV- and/or HSVd-free apricot plants under *in vitro* conditions. AgNPs have been added to the liquid shoot multiplication medium in TIS at different concentrations (0, 25, 50, 75, and 100 mg/L). After eight weeks of culture with the AgNPs, meristems were isolated and cultured on appropriate media for meristem multiplication. The viability of the meristems was evaluated and the presence/absence of the pathogens was analyzed by RT-PCR in the established shoots. In both cultivars the highest viability of isolated meristems was observed at 0 mg/L AgNPs (83% approximately). This percentage significantly decreased when meristems were isolated from shoots treated with AgNPs, although not significant differences were found between AgNPs treatments. Regarding the elimination of PPV, free-virus 'Canino' shoots were produced in all treatments. Maximum virus elimination rates (75%, percentage of clean plants regarding the number of shoots established from surviving meristems) were achieved with the application of 75 mg/L of AgNPs in TIS. However, HSVd-free 'Mirlo Rojo' plants were not obtained from any of the treatments.

***Keywords:*** *liquid medium, plum pox virus, meristem culture, virus-free plants*

## 1. INTRODUCTION

Cultivated species of *Prunus* genus are affected by viruses and viroids associated with vegetative propagation techniques. Rubio et al. 2017 (indicated that 44 viruses and three viroids have been described in the main cultivated *Prunus* species like peach, plum, apricot, almond, sweet and sour cherry. *Prunus* yields and fruit quality can be negatively affected by these pathogens.

All these results indicate the need for efficient methodologies to produce virus- and viroid-free *Prunus* trees for the new plantations after removing infected trees from

traditional plantings. Production of virus-free plants is necessary to control viral diseases, import novel cultivars from other countries, exchange breeding materials between countries or regions, and preserve plant germplasm.

*In vitro* techniques represent the most successful approaches for virus eradication. Biotechnological approaches have been used in different species to produce virus- and viroid-free plants. *In vitro* culture combined with chemotherapy, thermotherapy or cryotherapy have produced clean plants in different species (Barba et al., 2017; Wang et al., 2018). The use of metallic nanoparticles, in particular silver nanoparticles (AgNPs), is widely applied in medicine due to their antimicrobial, viricidal, and anticancer activities, among others (Al-Radadi and Abu-Dief, 2022; Sharma et al., 2015; Wang and Shao, 2017). This has generated a great interest in the application of nanoparticles to agriculture, especially as antiviral agents.

In our laboratory, 'Canino' shoots infected with PPV (Ca-PPV) and 'Mirlo Rojo' shoots infected with HSVd (MR-HSVd) are maintained by micropropagation. These *in vitro* shoots constitute excellent apricot model plants to study the possible effect of AgNPs on those pathogens (Pérez-Caselles et al., 2022). The aim of this work was to apply different concentrations of a commercial formulation of AgNPs (Argovit$^{TM}$ -7) in order to study the interaction between AgNPs and PPV or HSVd under *in vitro* conditions.

## 2. MATERIAL AND METHODS

Ca-PPV and Mr-HSVd shoots were cultured in a Temporary Immersion System (TIS) using 4 L Plantform$^{TM}$ bioreactors (Welander et al. 2014) and 0.22 µm air filters. The cultivation conditions and the liquid medium (developed for AgNPs addition) were described by Pérez-Caselles et al. (2022). Briefly, shoots were immersed for two minutes every six hours and aerated for three minutes every three hours. Explants were subcultured every four weeks on fresh medium (cultivation cycle). Thirty shoots (approximately 1.5 cm) and 500 mL of liquid media (LM) were used in each bioreactor. Shoots of both cultivars were exposed to 20 different treatments: addition of 25, 50, 75, and 100 mg L-1 AgNP plus a control without AgNP addition during four or eight weeks of each cultivar (Ca-PPV and Mr-HSVd). Apical meristems were rescued from control (0 mg L-1) and treated shoots at the end of each exposure time. Isolated meristems were individually identified as a potentially pathogen-free independent line.

PCR for the detection of PPV and HSVd in 'Canino' and 'Mirlo Rojo' shoots, respectively, was performed 12 and 24 weeks after meristem rescue using GoTaq® Green Master Mix (Promega). The oligonucleotides used for pathogens detection were:

**For PPV detection:**

PPV-F (5'-CAATAAAGCCATTGTTGGATC-3')

PPV-R (5'- CTCTGTGTCCTCTTCTTGTG-3')

**For HSVd detection:**

HSVd-F (5'-AATTCTCGAGTTGCCGCAACA-3')

HSVd-R (5'-CAGGGGCTCAAGAGAGGATC-3').

PCR products were observed by an electrophoretic analysis using Green Safe (NZYTech, Lisboa, Portugal).

## 3. RESULTS

Survival of untreated MR-HSVd and Ca-PPV meristems was similar and around 83%. Exposure to Argovit™-7 caused a significant decrease ($p<0.001$) in the survival of rescued meristems, ranging from 15% to 42%. No significant differences were found between concentrations of the Argovit™-7 treatment nor between times exposed to the treatment (four or eight weeks) in the survival of treated meristems.

Argovit™-7 treatments were not effective in eliminating the viroid (HSVd) and all 'Mirlo Rojo' plants were infected after meristem rescue and shoot development. However, 4 or 8 weeks of culture in the presence of Argovit™-7 produced Canino PPV-free lines in all treatments. A logistic regression of the effect of the different treatment concentrations on the production of PPV-free plants was highly significant. The 75 mg $L^{-1}$ treatment had the best efficiency after both four and eight weeks of exposure to the treatments, with 73.7% and 75.0% PPV-free plants, respectively.

PPV-free plants were rooted 44 weeks after meristem recovery and then were acclimatized to greenhouse conditions. Finally, an *ex vitro* evaluation was carried out with these plants and all lines remained clean and PPV was not detected.

## 4. DISCUSSION

It is of vital importance to produce virus-free plants in order to effectively manage viral diseases, introduce new germplasm, share breeding resources across countries or regions, and preserve plants genetic diversity (Wang et al., 2018). *In vitro* techniques represent the most successful approaches for virus eradication. Meristem culture or rescue was the methodology traditionally used to produce virus-free plants since meristems are plant tissues where viruses are not found or they are at very low concentrations (Hosokawa, 2008; Kumar and Reddy, 2011). However, some viruses and viroids, among which those studied here are included, are able to invade meristematic regions quickly and, therefore, decrease or eliminate the efficiency of the meristem culture methodology (Polák et al., 2008). Here, we have analyzed if the pathogens were present in plants produced after meristems from Ca-PPV or MR-HSVd were rescued and cultured. Virus or viroid-free plants were never obtained with this methodology which means that additional treatments, combined with meristem rescue and culture, are necessary.

Silver nanoparticles have a long history of general use as an antiseptic and disinfectant. In this work, for the first time, AgNPs are reported to eliminate PPV from apricots although they were not useful in obtaining viroid-free apricot plants. Argovit™-7 produced PPV-free Canino plants at all tested concentrations with a maximum efficiency of 75% after exposure to the 75 mg $L^{-1}$.

Limiting the virus spread would be helpful to obtain virus-free shoot apical meristems. Our results support this hypothesis since PPV was found in leaves of treated Ca-PPV by RT-PCR and Transmission Electron Microscopy. Despite the uneven distribution described for PPV *in vivo* (Glasa and Candresse, 2008), it quickly spread in plant tissues *in vitro* (Clemente-Moreno et al., 2011). The interference of AgNPs with processes in the virus life cycle has probably led to a slower pathogen spread, avoiding the infection of the meristematic cells.

To the best of our knowledge, AgNPs have never been applied before to produce virus-free plants. However, AgNP application have clear advantages over other techniques found in the literature. For instance, thermotherapy with similar cleaning efficiencies (Koubouris et al., 2007), produces damages to the plants, growth inhibition, wilting and necrosis. Additionally, requires specific equipment and energy consumption. Cryotherapy usually is related to very low surviving rates and requires skilled personnel and very specific species- or even cultivar-dependent protocols. Application of chemotherapy is straightforward, as nanoparticles, but is much more expensive and antiviral compounds affect shoot viability.

## 5. CONCLUSIONS

To the best of our knowledge, this constitutes the first report on the use of silver nanoparticles combined with meristem culture to obtain virus-free plants. A high efficiency (ranging from 73.8% to 75%) of PPV-free meristems was achieved after the treatment of infected apricot plants with 75 mg $L^{-1}$ of Argovit™-7. Similar efficiencies of PPV-free *Prunus* plants have been reported using cryotherapy or thermotherapy but application of AgNPs is straightforward. Antiviral treatments are much more expensive and also may cause detrimental effects on plants. Further studies are needed to fully understand the action mechanism and the effect of other Argovit™ formulations on different pathogens.

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# Somaclonal variation in *in vitro* culture

**Yıldız Aka Kaçar**

*Department of Horticulture, Faculty of Agriculture, Çukurova University, 01330 Adana, Türkiye*

ykacar@cu.edu.tr

## Abstract

Somaclonal variation encompasses the genetic and epigenetic changes that occur in plants reproduced via *in vitro* techniques, differing from the donor plant. These changes are expressed in various ways, including morphological, cytological, biochemical, and genetic or epigenetic alterations at the DNA level. Such variations often challenge the scalability of traditional clonal propagation methods, such as those involving temporary immersion systems, by presenting considerable obstacles. Influential factors like genotype and ploidy, the balance of plant growth regulators, explant origin, culture duration, and the composition of macro and microelements used *in vitro*, together with physiological stress, are instrumental in inducing somaclonal variation. Assessment methods include phenotypic evaluations, examinations of chromosome numbers and structures, protein analysis, and direct DNA assessment. Notably, molecular markers have proven highly effective in detecting somaclonal variations. These markers are crucial for assessing genetic stability or changes in plants undergoing micropropagation, prolonged preservation, or cryopreservation *in vitro*, as well as in plants derived from prolonged culture conditions or after multiple subcultures. This review aims to provide a detailed exploration of somaclonal variation during the *in vitro* propagation of woody plants, examining the causes of somaclone formation, associated challenges, detection methodologies, and potential innovative solutions to address these issues.

***Keywords:*** *in vitro culture, genetic stability, somaclonal variations, DNA markers*

## 1. INTRODUCTION

Plant tissue culture involves the cultivation of plant tissues in synthetic nutrient media under aseptic conditions, distinctively removed from their natural growing environments. The development of a specialized tissue culture protocol for each plant species is essential when culturing explants *in vitro*. This adaptation requires significant alterations in the plant's physiological, anatomical, and metabolic characteristics to support its growth and replication within the culture medium (Ranghoo-Sanmukhiya, 2020).

Micropropagation stands as a crucial application of plant tissue culture, presenting a viable and scalable method for propagating genetically uniform plants with enhanced phytosanitary standards in limited spaces. Beyond its role in mass propagation, plant

tissue culture serves as a foundational tool in biotechnological applications, facilitating research in developmental biology, secondary metabolite production, bioenergy, cryopreservation, and the development of transgenic and genome-editing techniques (Hesami et al., 2023).

The micropropagation of woody plants and fruit crops has proven commercially successful within the scope of *in vitro* culture applications. A primary concern in the micropropagation of perennial plants is maintaining genetic fidelity to the donor plant. In this regard, somaclonal variation manifests at various levels—including morphological, cytochemical, biochemical, and molecular dimensions—in plants propagated through micropropagation (Leva et al., 2012). Preserving genetic integrity during tissue culture is critical, as the regeneration of plants under controlled conditions can introduce unexpected and undesirable variations in traits. Addressing and mitigating somaclonal variation is imperative for the successful clonal reproduction of plants through micropropagation. This review explores somaclonal variation as a frequent phenomenon during the *in vitro* propagation of woody plants, offering an extensive analysis of its effects and strategies for its reduction.

## 2. SOMACLONAL VARIATION

Somaclonal variation refers to the genetic or epigenetic changes that emerge in plants grown under *in vitro* conditions, which differentiate them from their original donor plants. These variations are inheritable, propagated through meiosis, and are typically irreversible. It is essential to understand the concept of somaclonal variation in the context of agriculture, as it involves heritable mutations that remain in a plant population even after transplantation to field conditions.

Some variations are temporary and can revert to their initial state under different cultural conditions due to epigenetic and physiological influences, these changes are non-heritable and reversible. In contrast, permanent and inheritable modifications indicate the presence of "de novo" genomic alterations. The term 'somaclonal variation' was initially introduced by Larkin and Scowcroft in 1981 to describe plant variants emerging from any type of cell or tissue culture. Over time, this term has come to be universally applied to all tissue culture-derived variants, although more specific terms such as protoclonal, gametoclonal, and mericlonal variation are used to describe variants from protoplast, anther, and meristem cultures, respectively (Bairu et al., 2011).

Genetic alterations can occur at the chromosomal level, including changes in chromosome number (such as aneuploidy or polyploidy) or structure (including deletions, duplications, insertions, and translocations), or at the DNA sequence level, typically resulting in point mutations. Significant epigenetic changes include gene amplifications and alterations in normal DNA and histone methylation patterns. Thus, it is crucial to utilize methods that evaluate somaclonal variation to ensure the

identification of true-to-type plants following the *in vitro* propagation of a genotype (Duta-Cornescu et al., 2023).

According to the literature, somaclonal variation can affect specific traits or span the entire genome of a plant. This variation also serves as a critical source of genetic diversity for crop improvement, offering the potential to select novel variants that may display improved disease resistance, quality, or yield (Emaldi et al., 2004; Mehta et al., 2000; Leva et al., 2012).

### 2.1. Factors affecting somaclonal variation

Numerous factors are acknowledged as influencing somaclonal variation. These encompass the regeneration methodologies, the type and origin of the explant, the duration of culture or the frequency of subculturing, the constituents of the culture medium, the conditions of the culture environment, the impact of hormones or plant growth regulators applied *in vitro*, the fidelity and adaptability of the genotype, and the activity of transposable elements (Deepthi, 2016). Despite comprehensive investigations, the specific mechanisms that lead to somaclonal variation are not fully understood, particularly in the case of perennial woody plants (Skirvin et al., 1994; Leva et al., 2012).

#### 2.1.1. Regeneration systems

The structure of cellular organization is critical in enhancing plant development. Disruptions in cellular control can lead to somaclonal variations within the context of tissue culture. The ranking of regeneration systems, ordered by their genetic stability from most to least stable, includes micropropagation using pre-existing structures such as shoot apices or nodal explants; shoots generated adventitiously; somatic embryogenesis; and organogenesis originating from callus, cellular, and protoplast cultures (Leva et al., 2012).

Although the direct morphogenesis of plant structures from meristematic cultures, avoiding any callus intermediate, reduces the potential for genetic instability, the stabilizing influence of meristems can sometimes be compromised *in vitro* cultures (Bairu et al., 2011).

#### 2.1.2. Explant and explant source

The choice of plant tissue utilized in micropropagation plays a vital role in maintaining genetic integrity. Employing meristematic tissues such as pericycle, procambium, and cambium for initiating tissue culture significantly minimizes the risk of genetic anomalies. In contrast, more differentiated tissues like roots, leaves, and stems tend to yield a higher frequency of variants, mainly due to the involvement of a callus phase, as opposed to explants derived directly from pre-existing meristems (Leva et al., 2012).

Additionally, using explants from a single donor plant increases the potential for culture-induced variation (Kunitake et al., 1994). This highlights the significance of the

donor plant's genetic make-up and the consistency of its genome across all tissues. Somaclonal variation may also stem from somatic mutations inherent in the tissues of the donor plant. To detect pre-existing somaclonal variations, somatic embryos produced in the initial regeneration cycle can be subjected to a subsequent cycle of *in vitro* regeneration to observe any variability in tissues with pre-existing differences. These variations are expected to be more pronounced in the first generation of somaclonal offspring than in the second, with anomalies in the latter potentially being eliminated or stabilized (Skirvin, 1994; Leva et al., 2012).

Genomic alterations such as endopolyploidy, polyteny, and the expansion or contraction of DNA sequences are common during somatic differentiation in plant growth and development. Thus, variations in the frequency and characteristics of somaclonal variation are expected when regeneration is based on tissues from various origins. During the processes of differentiation and redifferentiation, both qualitative and quantitative genomic changes may occur, with certain DNA sequences being amplified or eliminated as part of these cellular alterations. These genomic variations are closely associated with the source of the tissue and the regeneration system utilized (Pasqual et al., 2014).

### 2.1.3. The composition of the culture medium

The composition of hormonal elements in the culture medium plays a pivotal role in influencing somaclonal variation. There is substantial evidence suggesting that varying types and concentrations of plant growth regulators significantly affect the occurrence of somaclonal variation across different plant species. Imbalanced ratios of auxins to cytokinins may lead to polyploidy, while normal ploidy levels are often observed under low concentrations or in the absence of these regulators. Additionally, rapid and disorganized growth induced by hormonal imbalances can contribute to somaclonal variation (Leva et al., 2012).

A high concentration of 2,4-D (2,4-dichlorophenoxyacetic acid) is known to contribute to chromosomal instability. While the mutagenic effects of growth regulators can sometimes appear contradictory, often these effects are indirectly caused by promoting rapid and disorganized cellular growth. Furthermore, the specific types and concentrations of growth regulators can influence the variation in plants regenerated by selectively stimulating cells of certain ploidy levels. The disturbance of the cell cycle by exogenously applied chemicals is considered a primary mechanism triggering variability in tissue culture. In particular, cytokinins such as BAP (Benzyl Amino Purine) and kinetin are significant in inducing genetic variability (Deepthi, 2016).

The type and concentration of growth hormones, particularly synthetic ones in plant culture media, are closely linked to somaclonal variation. The addition of auxins to unorganized callus cultures or cell suspensions notably increases genetic variability by elevating the rate of DNA methylation (LoSchiavo et al., 1989).

### 2.1.4. Culture environment

The tissue culture's physical settings, including temperature, lighting, medium agitation rate, and osmolarity, can induce somaclonal variations. The disruption of the standard cell cycle within tissue cultures hampers proper cell division before DNA replication, leading to chromosomal breakages. Such disruptions result in genomic anomalies such as deletions, duplications, inversions, and translocations, contributing to *in vitro* abnormalities.

### 2.1.5. Subculture number

The conditions of *in vitro* culture and rapid tissue multiplication can compromise genetic stability, potentially leading to somaclonal variations. These variations are often linked to the increased number of subcultures and prolonged culture durations (Deepthi, 2016). Limiting the number of subcultures during micropropagation is advised to decrease somaclonal variation. It is noteworthy that the variation among regenerated plants tends to escalate with the culture's age.

### 2.1.6. Genotype

Genotype plays a critical role in the occurrence of somaclonal variation. The *in vitro* culture conditions exert considerable stress on plant cells, which may trigger mutagenic processes. Genomic responses to stress-induced variations differ, highlighting a genotypic component to somaclonal variation. Variations in genetic stability are connected to changes in genetic makeup, with certain genomic regions, such as repetitive DNA sequences, exhibiting instability during cultivation (Leva et al., 2012). This understanding could greatly benefit plant breeding and conservation efforts, acknowledging the interplay between genotype and culture environment.

## 2.2. Detection of somaclonal variation

The impact of somaclonal variation is significant, especially in fruit crops and woody plants with lengthy life cycles. It is crucial to evaluate the performance of micropropagated plants after they mature from their juvenile stage in field conditions. While somaclonal variation has been extensively studied in herbaceous plants, limited research has been conducted on temperate perennial fruit crops (Leva et al., 2012).

Somaclonal variants are a major concern in any micropropagation system. They can be identified using a variety of methods, broadly classified into morphological, biochemical and molecular techniques (Bairu et al., 2011).

### 2.2.1. Morphological Screening and Morphological Marker

Visual inspection during the acclimatization phase in greenhouses or nurseries is effective for identifying potential off-types and is generally adequate for commercial objectives. The use of morphological markers aids in this detection process. Historically, morphological characteristics have been instrumental in classifying plant

species, genera, and families. Variants are readily identifiable through features such as variations in plant stature, leaf morphology, pigmentation abnormalities, height, branching patterns, leaf margins, stem thickness, and flowering behaviors. Nonetheless, environmental factors can significantly influence these morphological traits, potentially obscuring the actual genetic makeup of the plant (Mandal et al., 2001). Moreover, the number of morphological markers available for phenotypic traits is limited, they are subject to developmental regulation, and are highly susceptible to environmental influences (Cloutier and Landry, 1994). Due to these limitations and associated cost considerations, this method is not considered ideal for commercial applications (Israeli et al., 1995; Bairu et al., 2011).

### 2.2.2. Molecular Markers

Molecular markers, identifiable DNA sequences or proteins located on chromosomes or within organelles, detect polymorphisms in genetically diverse individuals. These markers, encompassing proteins, genes, gene fragments, or sequences in non-coding regions, are pivotal in various genetic applications. They are employed in estimating genetic diversity, determining genetic identity, creating linkage maps, identifying trait-associated markers, and selecting desirable traits in breeding programs. In tissue culture, molecular markers are extensively used to assess the extent of somaclonal variation occurring during *in vitro* culture (Aka Kaçar et al., 2015). These markers are stable, inheritable variations measurable by appropriate methods and linked to specific traits. Molecular markers include both biochemical markers/isozymes and DNA-based markers.

- ***Biochemical Markers***

Isozymes represent various forms of enzymes that, despite slight differences in their amino acid sequences, perform identical biochemical functions. The polymorphisms of isozymes can be discerned through separation techniques such as electrophoresis gels, where they typically exhibit codominant behavior. Initially, isozymes served as the primary molecular markers in plant genetics. However, their relatively scarce presence in plant genomes has led to their gradual replacement by more plentiful and practicable methodologies (Aka Kaçar et al., 2015).

- ***DNA Based Markers***

Molecular techniques are indispensable for assessing the genetic fidelity of plants propagated via *in vitro* micropropagation. Chromosomal variations, including changes in number and structure, as well as more nuanced DNA sequence alterations, can occur in tissue culture-derived plants (Gostimsky et al., 2005). Although visible morphological variations appear less frequently than DNA-level changes (Evans et al., 1984), it is crucial to explore potential molecular variations to ascertain the scope and specifics of deviations from the true-to-type cloned plants (Cloutier and Landry, 1994; Bairu et al., 2011). Molecular marker systems are extensively used to examine genetic

stability or somaclonal variation in plants that have undergone long-term preservation or cryopreservation under *in vitro* conditions, as well as in plants from prolonged culture or after multiple subcultures. These markers also facilitate quality control in tissue-cultured plants, molecular breeding *in vitro*, and the identification of genes that influence tissue culture responsiveness (Aka Kaçar et al., 2015).

Various molecular techniques enable the detection of genetic discrepancies between source plants and somaclones. These methods predominantly rely on different DNA markers, which are phenotypically neutral and unaffected by developmental stages or environmental conditions. The most frequently employed DNA markers are inter simple sequence repeats (ISSR), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellites (SSR), amplified fragment length polymorphism (AFLP), and start codon-targeted (SCoT) polymorphism. Many researchers opt for a combination of markers to leverage the strengths and mitigate the limitations of each method in assessing genetic variation (Duta-Cornescu et al., 2023).

### 2.3. Advantage of somaclonal variation

Tissue culture techniques provide abundant and unique sources of variability, holding significant potential for crop improvement without the need for mutations or hybridizations. Somaclonal variation, in particular, can be effectively utilized to produce disease-resistant clones, providing a simpler, quicker, and more cost-effective alternative to traditional breeding approaches. This method is especially beneficial for perennial species with extended vegetative periods, as it allows for high-frequency variations that are advantageous over traditional mutagenesis. It is well-suited to plant species with restricted genetic diversity. Moreover, the exploitation of somaclonal variations does not necessitate knowledge of the species' genome. This approach can also lead to the development of varieties that produce higher levels of valuable metabolites with phototherapeutic properties (Duta-Cornescu et al., 2023).

## 3. CONCLUSIONS

Somaclonal variation, both heritable and non-heritable, poses a significant challenge in tissue culture. The origin of such somatic deviations can be linked to various factors, highlighting the importance of recognizing that somaclonal variation is generally undesirable, particularly in plants propagated clonally. Therefore, it is vital to identify and address somaclonal variation at early stages. Although various methods are available for detecting somaclonal variation, molecular markers and next-generation sequencing techniques have become essential tools for ensuring genetic stability in cultured plants. Despite its potential drawbacks, somaclonal variation offers opportunities in plant breeding for the creation of new, potentially enhanced plant varieties.

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# Endophytic bacteria in date palm tissue culture: Impact on the large scale micropropagation using RITA bioreactors and remedies

**Ameni Nasri[1], Emna Baklouti[1], Emna Graja[1], Ahlem Ben Ahmed[1], Hazar Akrimi[1], Riadh Drira[1], Alain Rival[2], 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] Cirad - DGDRS, Jakarta, Indonesia.*

*lotfifki@yahoo.fr*

## Abstract

Endophytic bacteria expression during *in vitro* propagation of date palm is a major constraint which obstructs large scale propagation schemes using temporary immersion systems. This study has been designed to analyze the consequences of the anarchic proliferation of endophytic bacteria during date palm micropropagation and to develop a strategy to minimize culture losses. Endophytic bacteria expression was generally detected when cultures were under stress conditions such as temperature fluctuation and delayed subculture. Embryogenic cultures and bud clusters growing in RITA® bioreactors were seriously affected by the anarchic proliferation of endophytic bacteria. They slowly turned yellow and brown. Although some part of the tissue continued to form new shoot buds and somatic embryos, within 2 weeks all the tissues necrosed and it became impossible to continue cultures any longer. Four strains of endophytic bacteria were identified through molecular and biochemical characterization. Only juvenile leaves could be used to establish relatively clean *in vitro* tissue cultures. Immaturity of vascular tissue in these explants may explain the low number of contaminated cultures. Defining physico-chemical conditions hampering bacterial growth, without affecting plant cell proliferation can be a promising way to overcome anarchic proliferation of endophytic bacteria. To sum up, we can conclude that endophytes have negative consequences on *in vitro* tissue cultures when they become out of plant cell control.

***Keywords:*** *Date palm, endophytic bacteria, large scale micropropagation, RITA® bioreactor*

## 1. INTRODUCTION

The use of innovative techniques presents a powerful tool for enhancing the efficiency and scalability of plant micropropagation. Temporary immersion bioreactors are an improvement over traditional liquid-phase bioreactors, designed to overcome issues like hyperhydricity. They provide a periodic immersion of *in vitro*-cultures in nutrient media, which enhances aeration and nutrient uptake.

The automation of bioreactors and uniform growth conditions systems makes them particularly suitable for large-scale commercial production. However, the control of

challenges like initial investment costs, technical expertise, and customization of growth conditions is necessary. Temporary immersion systems (TIS) are a well-known type of bioreactor used in plant micropropagation. TIS enhances the advantages of liquid cultures to improve the growth of *in vitro*-cultures (Ozudogru et al., 2002). Furthermore, the forced aeration in the vessel creates an environment with a high gas exchange, which improves the growth and development of *in vitro* cultures (Abahmane, 2020). While TIS bioreactors offer significant advantages for plant micropropagation, TIS present several disadvantages such as high initial costs, technical complexity, contamination risks and especially endophytic bacteria contamination (Murthy et al., 2023).

Endophytic bacteria are microorganisms that live within plant tissues without causing harm and can provide various benefits to their host plants (Hallman et al., 1997). The integration of endophytic bacteria into plant micropropagation protocols presents a valuable strategy to enhance growth, rooting, stress tolerance, and reduce contamination. The diversity of these bacteria can establish great relationship between them and plants. Their capacities to live and to associate with varieties of plants give them the opportunity to growth and to multiply rapidly. Generally, endophytic bacteria are not harmful to plants (Hallman et al., 1997); however, they may affect plants culture growth parameters either *in vivo* or *in vitro* conditions and they can act as 'vitro-pathogens' in some stress situations (Khan et al., 2020). In fact, endophytic bacteria can establish a serious problem in plant tissue culture especially for large scale propagation (Şeker et al., 2017). Alternatively, they may induce hormone-mediated modification of the *in vitro* response (Singh, 2018), which puts into question the reproducibility of tissue culture protocols. The majority of endophytic bacteria are classed as Plant Promoting Growth Bacteria "PGPB". They can induce some positive effects on growth parameters of plants (germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth) (Li et al., 2021).

Our objectives are to contribute to the study of the date palm microbiome by isolating and characterizing endophytic bacteria from multiple bud clusters of date palms grown in a temporary immersion bioreactor system (RITA®). Additionally, we aim to identify endogenous contaminants using biochemical and molecular methods.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

Multiple bud clusters of date palm (*Phoenix dactylifera* L.) were cultivated in room culture of Plant Laboratory-Faculty of Sciences of Sfax.

### 2.2. Methods

#### 2.2.1. Shoots multiplication

The multiplication of multiple bud clusters was described by Fki et al. (2011). Multiple bud cluster was cultivated in MS liquid medium supplemented with 50 $g.L^{-1}$ sucrose. At

this stage, we used RITA® bioreactors for the temporary immersion of cultures in liquid medium. The RITA® vessel is made of two compartments: explants are placed in the upper compartment, and the lower one holds the liquid medium. Six bud clusters per bioreactor were cultivated using 200 mL of MS medium supplemented with 50 g $L^{-1}$ sucrose. The immersion cycle was 5 min every 24 h and clusters were subcultured once every 4 months.

### 2.2.2. Bacterial isolation

Bacteria were isolated from multiple bud cultures of date palm cultivated in RITA® bioreactors. Bacterial colonies were cultivated in different medium such as nutrient agar (1% beef extract, 2% yeast extract, 5% peptone, 5% NaCl, 10% agar (w/v)), Bacteria screening medium (Viss et al., 1991) and trypticase soy broth (Doyle et al., 1968). Petri plates were incubated 7 days at 30°C in the dark. Bacterial colonies were distinguished through visual observations of colony morphology and picked after 72 hours of incubation at 25°C and subsequently transferred to the same medium. Following successive subcultures, pure colonies were obtained and stored at -80°C in 20 % glycerol solution and fresh cultures were established prior to biochemical and molecular identification.

### 2.2.3. MALDI-TOF mass spectrometry analysis

Fresh culture of endophytic bacteria was washed twice with 1 mL of water, cells were lysed with 40 L of a formic acid (35%): acetonitrile (50%) mixture and the suspension were centrifuged at 25,000 × g for 15 min. One microliter of the supernatant was deposited onto a stainless steel MALDI-TOF target plate (Bruker-Daltonics, Braunschweig, Germany). One microliter of -cyano-4-hydroxycinnamic acid (CHCA) 10 mg/mL in acetonitrile 50% and trifluoroacetic acid 2.5% was added to the dried samples and allowed to dry under air. Mass spectra were performed by an Auto flex II MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Automated spectra acquisition was done using the Auto Execute tool of Flex Control 3.0 (Bruker-Daltonics) with fuzzy control of laser intensity. The raw data were converted into a peak list using Flex Analysis 3.0 software (Bruker Daltonics, Braunschweig, Germany).

### 2.2.4. DNA extraction of bacteria and Polymerase chain reaction amplification and sequencing of 16S rDNA of endophyte bacteria

For DNA extraction, one or two colonies of a pure culture are diluted in 180 ml of sterile lysis buffer (50mM NaOH and 0.25% SDS) and the mixture is homogenized by vertexing for 2 minutes. 180 µLof Tris buffer (10 mM and pH: 8) is added to the bacterial suspension. Then, the suspension is centrifuged at 12000 rpm at 4 ° C for 10 min. The supernatant, containing bacterial DNA, is recovered and stored at 4°C. The 16S DNA of the bacterial isolates was amplified using universal primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r-Y (GGYTACCTTGTTACGACTT) (Thomas and Graham, 1952). Each 24µl PCR contained 100 ng of template DNA, 1×

reaction buffer (Nitrogen), 100 µM dNTP, 10 pmol of each primer and 1 U of DNA polymerase (Nitrogen). Thermal cycling conditions were: initial denaturation at 94 °C for 5 min followed by 33 cycles of 94 °C for 30 s, 52 °C for 40 s, 72 °C for 60 s and a final extension step for 5 min at 72 °C.

Sequencing process was made at DSMZ institute, Braunschweig, Germany. The results are then processed and edited using Bio Edit software. Sequencing results were compared with existing sequences using Basic Local Alignment Search Tool program on National Centre for Biotechnology Information site (www.ncbi.nlm.nih.gov) to obtain the homology.

## 3. RESULTS

The transfer of some multiple bud cultures to RITA® bioreactor stimulated the proliferation of endophytic bacteria which can be easily observed after two to three subcultures (Fig 1). The contaminations appeared more frequently on bud clusters that were initiated on leaves having a high degree of differentiation. Although some part of the tissue continued to form new shoot buds and somatic embryos, within 2 weeks necrosis affected all the tissues and it became impossible to maintain the cultures any longer.

**Figure 1.** Appearance of endophytic bacteria contamination around multiple bud clusters of date palm. Bar = 1cm

Phenotypic identification of bacteria was based typically on simple morphologic tests like as the appearance of the colonies by microscopy. Our experiments revealed the presence of five bacterial isolates. All the isolates were identified by MALDI-TOF

(score > 2,000) and 16S DNA sequencing. A perfect match between two techniques used was observed for the identification of isolated strains. Therefore, it can be concluded, at this stage, that MALDI-TOF mass spectrometry is well established as an accurate and cost-effective method for bacterial species identification (Table 1).

**Table 1.** Morphological, biochemical and molecular identification of endophytic bacteria isolated from multiple bud clusters of date palm

| Isolates references. | Morphological aspect | Bacterial identity by MALDI-TOF | Bacterial identity by 16S rDNA sequencing | Isolates reference | Percentage of identity |
|---|---|---|---|---|---|
| **DI1** | White-Irregular | *Achromobacter xylosoxidans* | *Ochromobacter sp.* | MT165557.1 | 90% |
| **DI2** | White-Irregular | *Pseudomonas korensis* | *Pseudomonas fluorescens* | KT350501.1 | 99% |
| **DI3** | Yellow-round | *Mycobacterium sp.* | *Mycobacterium gordonae* | MH144561.1 | 100% |
| **DI4** | White-round | *Tsukomurella pseudopumae* | *Tsukomurella pseudopumae* | MT525264.1 | 100% |
| **DI5** | Yellow-round | *Paenibacillus favisporus* | *Paenibacillus favisporus* | MT448940.1 | 99% |

## 4. DISCUSSION

This study highlights a significant challenge in the *in vitro* propagation of date palm using temporary immersion systems: the anarchic proliferation of endophytic bacteria. This uncontrolled bacterial growth is particularly appeared under stress conditions such as temperature fluctuations and delayed subcultures, leading to severe consequences for the embryogenic cultures and bud clusters in RITA® Bioreactors. The tissues affected by endophytic bacteria exhibited yellowing, browning, and eventual necrosis within two weeks. In this case, our previous investigations showed that some endophytic bacteria strains don't affect the growth of structured *in vitro* plants (Data not published).

In this study, *M. gordonae* was found to be the dominant group in multiple bud clusters of date palm. This finding is in agreement with previously reported results showing that they are frequent plant tissue colonizers in several plant species. Bacteria of this genus have already been described as widespread contaminants in ornamental plant crops (Pa et al., 2020), as well as endophytes of wheat (Conn and Franco, 2004), rice (Koskimäki

et al., 2010). The second dominant group of endophytes identified in multiple bud cultures of date palm was *Paenibacillus*. This genus has been identified in many species such as corn (Larkin and Fravel, 1998) and rice (Steinberg et al., 1997). These Bacteria directly stimulate plant growth by producing indole 3-acetic acid (IAA) and other phytohormones, solubilizing phosphorus that can be assimilated by plant roots and by fixing atmospheric nitrogen (Lrich et al., 2008).

The use of such antibiotics seems to be a solution for disinfecting certain lines of embryogenic cells (Ewald et al., 2000). However, many authors have noted that antibiotics are often not effective when used in tissue culture (Mukhtar et al., 2020). According to our preliminary experiments on the effects of various antibiotics, the addition of cefotaxime (250 mg/L) to the culture medium clearly has bacteriostatic effects rather than bactericidal ones.

The use of juvenile explants generally leads to the differentiation of embryogenic callus devoid of endophytic contamination. Furthermore, it is well-established that meristematic tissues, composed of undifferentiated embryonic cells, do not contain bacteria or viruses. The benefits of using young explants are due to from two key factors:

- The disinfectant can easily reach the tissues' superficial internal structure;
- The juvenile explants' conductive tissues have not yet reached the final stage of differentiation. Similar results were shown in Banana, with the possibility of establishment of healthy *in vitro* plants from juvenile explants (Singh et al., 2011).

## 5. CONCLUSION

In conclusion, while endophytes pose a risk to *in vitro* tissue cultures when they escape plant cell control, strategic measures to manage the bacterial environment can significantly enhance the success rates of date palm micropropagation. Future research should focus on optimizing these conditions to ensure an efficient large-scale propagation protocol of date palm.

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# *WORKING GROUP 4:*

# Technological risk assessment, public acceptance, legislation, and commercialization

# The impact of regulatory oversight on the development and adoptıon of plant bıotechnology

**Selim Cetiner**

*Sabancı University, 34956 Istanbul, Turkey*

cetiner@sabanciuniv.edu

## Abstract

It is a fact that molecular techniques are providing important possibilities in boosting the agricultural production. However, the perceived negative effects on human health and environment of insect resistant and herbicide tolerant plant varieties, developed during the last 30 years through modern biotechnology methods or genetic engineering techniques, are the subject of heated debates. Recently, genome editing or New Breeding Techniques are facing the same problem, and possibilities offered by these new technologies are being questioned from many different standpoints. Especially in the EU and in many of the developing countries, possible adverse effects of transgenic crops on human health and environment are quite controversial issues. It cannot be denied that these arguments are based on ideological, sentimental, personal and economic choices rather than hard scientific facts. Despite these negative public perceptions, several national, regional and international regulations are in place to address these issues. However, it is still not possible to say that a complete consensus is reached in the international community. For instance, the biosafety legislation of the USA is quite different from that of the EU, where even the existing regulations are still not interpreted and implemented in a harmonious way by the member states. The presentation will address the prospects and constraints of plant biotechnology from the perspective of regulatory oversight developed by national, regional and international institutions.

***Keywords:*** *genome editing, New Breeding Techniques, biosafety, public perception*

## 1. INTRODUCTION

Modern molecular biology along with plant tissue techniques, also called plant biotechnology, offers important opportunities for increasing agricultural production. Here, the development and propagation of high yielding and high quality plant varieties resistant to biotic and abiotic stress conditions are important in the implementation of sustainable agricultural production. In the development of these plants, it is necessary to focus not only on transgenic plants obtained through genetic transformation, but also on other molecular plant breeding techniques as well as somaclonal variation. However, in parallel with technological advances, it is necessary to make legal regulations on biosafety both during the development of these techniques and products in the

laboratory and their release into the environment, and to train competent people to implement this legislation. Although often ignored by the practitioners, it is also important to consider various implications of regulations on intellectual property rights related to plant biotechnology.

The increasing global population demands more resources, including food, water, and energy, leading to intensified environmental stress and competition for resources. Along with population growth, the degradation of ecosystems due to human activities such as deforestation, pollution, and overfishing reduces biodiversity and the ability of ecosystems to provide essential services like clean air and water, soil fertility, and climate regulation. The warming of the planet due to greenhouse gas emissions causes significant changes in weather patterns, sea level rise, and increased frequency of extreme weather events, impacting ecosystems and human societies. Also, the depletion of natural resources, including water, arable land, and fossil fuels, poses a threat to economic stability and development. Together, these four pressures also called "the Quadruple Squeeze" create a complex and urgent challenge for sustainable development and require coordinated global action to mitigate their impacts and ensure a viable future for the planet and humanity (Rockström & Karlberg, 2010).

Sustainable development which was coined first in the Brundtland Report in 1987, is a concept that refers to meeting the needs of the present without compromising the ability of future generations to meet their own needs. Basically, it aims to balance three core elements namely economic growth, social inclusion and environmental protection. The goal of sustainable development is to create a harmonious relationship between these elements, ensuring long-term health and stability for both human societies and the natural environment. It is often encapsulated in frameworks such as the United Nations' Sustainable Development Goals (SDGs), which outline specific targets to be achieved by 2030. However, it is worth noting that these were originally detailed in Agenda 21 as a comprehensive action plan by the United Nations to promote sustainable development globally. It was adopted at the Earth Summit (United Nations Conference on Environment and Development) held in Rio de Janeiro, Brazil, in 1992. Chapter 16 of Agenda 21, "Environmentally sound management of modern biotechnology", makes two important points:

1) Biotechnology can make important contributions to sustainable food, feed and fiber production, meeting water shortages, health care and environmental protection;
2) Due to the lack of experience in modern genetic modification and biosafety, there is a need to develop and apply internationally recognized principles for risk assessment and management.

Chapter 16 also provides a roadmap for international action and cooperation to ensure the availability and safe use of biotechnology for all.

These two concepts of Agenda 21 - making biotechnology accessible to developing countries and international cooperation on risk identification and management - are reflected in the 1992 Treaty on Biological Diversity. This convention, which has been signed by over 190 countries, aims to conserve biological diversity, ensure the sustainable use of its components and ensure the fair and equitable sharing of the benefits derived from gene resources. Articles 8 and 19 of the Convention on Biodiversity deal with biotechnology and biosafety.

Paragraph g. of Article 8 on "Conservation of biodiversity *in situ*" requires Member States to establish and operate a National Biosafety System: "Establish or maintain the necessary means for the regulation, management and control of living genetically modified organisms that are likely to have an adverse environmental impact, including the conservation and sustainable use of biological diversity."

The first paragraph of Article 19, "The use of biotechnology and the sharing of its benefits", requires signatories to the Convention, particularly developing countries, which are home to a significant proportion of genetic resources, to take the necessary measures to develop and participate in biotechnological research with other Member States. The second paragraph requires the countries developing the technology to take all measures to ensure that developing countries in particular have fair and equitable access to biotechnology products derived from genetic resources. The third paragraph of this article proposes to establish a protocol to prevent potential adverse effects of the transfer and use of living organisms of biotechnology (LMO=GMO) between countries.

## 2. BIOSAFETY REGULATIONS

The decision to establish such a protocol was taken by the member countries at the Conference of Member States held in Jakarta in 1995, and the negotiations that started in 1996 led to the Cartagena Protocol on Biosafety in 2000. The Protocol, signed by some 150 countries, entered into force on September 11, 2003. The overall objective of the Protocol is to "...contribute to ensuring an adequate level of protection for the safe transport, handling and use of living modified organisms (LMOs) derived from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking into account the risks to human health and with a particular focus on transboundary movements." However when we consider the objectives of the Agenda 21 in 1992 and the outcomes of the Cartagena Protocol, we see that the benefits of biotechnology for sustainable development have been completely forgotten; the whole emphasis is on identifying and preventing risks, taking into account the "precautionary principle". The "precautionary principle" simply means that just because something has not been proven to be harmful does not mean that it will not be harmful.

The examples of the United States and the European Union are worth mentioning. However, first it would be better to briefly remind the developments that led to the need for biosafety regulations. About 50 years ago, scientists demonstrated that genetic

engineering methods could be used to transfer genes into microorganisms, plant and animal cells to modify and improve desired traits, and that this could be done much more precisely and quickly than conventional breeding methods. Shortly thereafter, Paul Berg, one of the scientists who made this discovery, and his colleagues wrote to the journals "Nature" and "Science" in 1974, asking for a one-year moratorium until the safety and ethical aspects of this new technology could be established. In this context, the scientists who came together at the conference held in Asilomar, California on 24-27 February 1975 declared that these technological products should be handled and evaluated individually, that the organisms studied should be divided into risk groups, and that autoregulation would be useful.

While research and discussions continued, the issue was taken up as an international technical problem by the OECD, and as a result of many technical discussions by experts, the "Recombinant DNA Safety Considerations" document, also known as the "Blue Book", was published in 1986. The main message in the book is one that opponents of the technology will not like: "there is no scientific justification for separate regulation of the use of recombinant DNA organisms (GMOs)". Nevertheless, the Blue Book has been the underlying principle of all national and international biosafety regulations to date (Schiemann, 2006).

Around the same time, an *ad hoc* committee was established by the directive of the President of the United States, and the document titled "Coordinated Framework for the Regulation of Biotechnology" prepared by this commission was published in the official gazette (Federal Register) in June 1986 and entered into force. According to this regulation, the assessment and management of the risks that genetically modified organisms may pose to human health and the environment will be carried out by the Department of Agriculture (USDA), the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) in coordination within the framework of the duties specified in their founding laws (Kingsbury, 1988.)

While the US preferred to use existing laws and institutions for regulating biotechnology, EU countries have prepared a new legislation for the regulation of biotechnology products. Accordingly, after long discussions, Directives EC 90/219 on the contained use of genetically modified organisms and EC90/220 on the deliberate release of GMOs into the environment entered into force. Subsequently, the EU has both amended these and drafted a series of other regulations, notably EC 1829/3003 and 1830/2003. The EU also established European Food Safety Authority that was responsible for the risk assessment of genetically modified organisms and their products.

Meanwhile, the Atlantic divide, i.e. the different approaches of the US and EU countries to modern biotechnology products, has started to emerge. To put it very briefly, the US prefers to look at the end product, while the EU prefers to look at the technique by which that product is obtained. In other words, it is not how a product is made, but what it is and

how it will affect human and environmental health that is at the forefront in the US, while EU rules subject products obtained through genetic engineering to a separate evaluation.

This fundamental difference in the formulation and implementation of biosafety legislation seems to have directly affected the cultivation areas of genetically modified organisms. GMO cultivation areas have exceeded 200 million hectares in the United States of America, Canada, Argentina, Brazil, Australia, China, India and other countries that are hospitable to the cultivation of transgenic crops. While in Europe only a few countries like Spain and Portugal allow the cultivation of only one approved corn variety in a limited area, over 100 GMOs are approved for importation for food, feed and processing the by the EU from GMO growing countries (Klümper & Qaim, 2014).

Here it's important to note that in agreement with the global scientific community European researchers also declared in many occasions that "Modern gene technologies, also called biotechnology, offer important opportunities to increase agricultural production to ensure adequate and balanced nutrition for the rapidly growing world population. Each GMO is different from the other. GMOs, which are handled separately and subjected to scientific risk analyses and whose production and consumption are permitted as a result of these analyses, are at least as safe as their classical equivalents...". Therefore, this dilemma in the EU is result of the personal and political preferences that was reflected in the rules and regulations as well as the bureaucratic decision making processes at the EU level. Perhaps the most important impact of the restrictive biosafety regulations in the EU was on research and development of genetic improvement of plants. Many of the pioneering research on transgenic plants in the European institutions have been either cancelled or moved to the United States and elsewhere. While *de facto* moratorium on GMOs continues in the EU.

## 3. SUSTAINABLE INTENSIFICATION

Below are the Farm to Fork Targets that form the heart of the Green Deal of the European Commission:

- Reduce the amount of pesticides used in agricultural production by 50% by 2030.
- Reduce the amount of chemical fertilizers used in production by 20% by 2030.
- Reduce chemical fertilizer leaching by 50% without reducing soil fertility.
- Reduce the amount of antibiotics used in livestock and aquaculture by 50%.
- Dedicate at least 25% of agricultural land to organic farming.
- Reduce food losses at the retail chain and consumer level by 50% per capita.
- Supporting €10 billion for Research and Innovation projects to help achieve these.

These are indeed very important for sustainable development that requires sustainable intensification in agricultural production. However, organic production, which is seen as a priority of the green agriculture policy, does not give way to chemical fertilizers and

synthetic pesticides, as well as GMOs and new plant varieties obtained through genome editing. Considering that organic agriculture yields are 30-50 % less than the conventional agriculture, sustainable intensification of agriculture elsewhere is of prime importance. However, meeting the food demands of increasing human population will lead deforestation in other parts of the globe. Results of a meta study shows that American and Canadian farmers, who adopt and apply all technological developments, including GMOs, rather than organic agriculture, achieve higher yields per unit area and their production is greener and more environmentally friendly (Sutherland et al., 2021) .

In the last 10 years, new knowledge in the field of molecular biology and its reflection on technology has made new plant breeding techniques such as genome editing possible. This has made it possible to develop new plant varieties that are resistant to diseases and pests, more drought tolerant and/or have improved nutrient content. The difference of this new technology from the previous GMO technology can be summarized as changing the genetic structure of the plant varieties themselves, i.e. obtaining them without transferring a foreign gene from another organism. Of course, one should not expect a miracle such as the emergence of a feature that does not already exist in the genome. It should not be forgotten that genome editing only seeks to achieve results by reactivating and/or silencing genes present in the plant genome that have been silenced in the classical breeding process for yield increase. For a successful breeding program, the entire genome map of the plant, the gene structure of the character being studied and the control sequences must be determined. Likewise, gene transfer and plant tissue culture protocols suitable for CRISPR-Cas editing of the plant in question must also be optimized (Movahedi et al., 2023).

Biosafety legislation approaches to genome-edited plants vary among countries. as well. For example, countries such as Canada and the US take a more science-based approach. In other words, they determine the risk analysis and level by looking at the characteristics of the developed plant, not the method used. Currently, several new plant varieties obtained as a result of genome editing are considered outside the scope of GMO biosafety legislation and have been put on the market. In the European Union, however, a new proposal by the Commission passed by the European Parliament by a small margin is far away from paving way to the new plant varieties developed by genome editing. Although, it proposes to separate at least some of the varieties from transgenics and therefore from GMO legislation, it still bans their use in organic agriculture, requires labelling and does not allow patenting. It is still unknown if this proposal will be accepted by the Council and even so how these will be implemented. Although anti-biotechnology NGOs in EU countries continue to demand that products developed using genome editing methods be subject to GMO legislation, it seems inevitable that this opposition will hinder plant breeding activities in Europe. Nevertheless, public opinion polls show that public opinion is more favorable to genome editing, although it varies between countries.

## 4. INTELLECTUAL PROPERTY RIGHTS

In addition to biosafety regulations, there is yet another group of legal framework that is even more complicated than the biosafety legislation. Patents and plant variety protection are important tools for protecting intellectual property and are designed to encourage innovation and investment in plant breeding by granting breeders exclusive rights to their new plant varieties. There are some differences between the US and the EU intellectual property protection system regarding the plant varieties, the principles are similar around the globe. Yet, the CRISPR patents that are used in plant genome editing or new breeding techniques are much more complicated that the plant variety protection. Therefore, amendments by the European Parliament on the new proposal of the European Commission mentioned above regarding the regulation of the New Genomic Techniques require no patenting on these new plant varieties. Considering the patent landscape with over 11000 patents over genome editing techniques, commercialization of these newly developed plants will be rather difficult even though they will be kept exempt from the GMO legislation (Martin-Laffon, 2019).

## 5. CONCLUSIONS

Obtaining the highest agricultural production from existing agricultural areas without further depleting the world's natural resources and minimizing the negative impacts of agriculture on the environment requires the development and use of technologies that will maximize the genetic potential of plants. To put it more succinctly, sustainable intensification will be possible through the rational use of all modern agricultural technologies, especially modern plant biotechnology. For this, however, it is essential that the regulations are designed based on scientific facts rather than personal and political preferences. The experience in the past 50 years showed that those countries that embraced modern plant biotechnology products had achieved a good level of sustainable intensification in agriculture while also advancing in scientific development of new breeding techniques.

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# Micropropagation of some *Anthurium* varieties for commercial *in vitro* production

**Yeşim Yalçın Mendi[1,3], Yıldız Aka Kaçar[1,3], Belgin Biçen[2], Melike Cengiz[1] and Çağlar Yıldız[3]**

[1]*Department of Horticulture, Faculty of Agriculture, University of Çukurova Adana, Türkiye*

[2]*YK Technopark Company, Adana, Türkiye*

[3]*Department of Biotechnology, University of Çukurova Adana, Türkiye*

*ymendi@gmail.com*

## Abstract

Turkey has an advantageous position for the cultivation of ornamental plants for reasons such as favourable conditions, proximity to markets and cheap labour. Biotechnological methods, especially plant tissue cultures and molecular techniques, come to the fore to support breeders and producers in meeting the demands of the next century in the ornamental plants sector. Anthurium plant is an important species in the world cut flower trade. In addition to classical production methods, biotechnological methods are used to meet the demand of Anthurium, which is an indoor plant. Within the scope of the present study, micropropagation and rooting studies were carried out using tissue culture in the commercially important Anthurium, ''Debby'' and ''Lilian'' genotypes. In the use of Murashige and Skoog (MS) medium (1/2) and 20% sucrose, 0.1 mg $L^{-1}$ 1-Naphthylacetic acid (NAA) was constant in every medium and different concentrations/combinations with 6-Benzylaminopurine (BAP) and $N^6$-furfuryladenine (Kinetin) were tried. The best micropropagation result was the combination of NAA (0.1 mg $L^{-1}$) and BAP (0.5 mg $L^{-1}$) for Debby, and the combination of NAA (0.1 mg $L^{-1}$) and Kinetin (1.0 mg $L^{-1}$) for Lilian. In rooting experiments, different concentrations of NAA and Indole-3-butyric acid (IBA) were tried in the medium containing 1/2 MS, and the best rooting results for both genotypes were obtained from 2 mg $L^{-1}$ IBA. The results of micropropagation and rooting have been found to be successful in both genotypes.

***Keywords:*** *Anthurium, in vitro, micropropagation, MS, BAP, IBA*

## 1. INTRODUCTION

*Anthurium andreaum* is a highly valued ornamental plant worldwide and is marketed as cut flowers or potted ornamentals due to its attractive and long-lasting inflorescences (Venkata et al, 2014; Teixeira da Silva et al, 2015). Seed propagation of Anthurium leads to genetic divergence due to high heterozygosity, affecting quality, yield and time to first flowering in commercial plantations (Jahan et al., 2009; Winarto 2010). Additionally, seeds exhibit short-term viability (2-3 days after harvest) and a low germination rate (20-

30%) (Jahan et al, 2009; Gantait et al, 2012). Due to the slow vegetative development of Anthurium, asexual propagation through the shoot or stem part is time-consuming (Cardoso and Habermann, 2014). For the above reasons, it is necessary to develop a clonal propagation method for large numbers of commercial propagations in a short time (Cardoso and Habermann, 2014; Teixeira da Silva et al, 2015).

In order to meet the demand for Anthurium, which is an indoor plant, biotechnological methods are used in addition to classical production methods. Biotechnological methods, especially plant tissue cultures and molecular techniques, come to the forefront in order to support growers and producers in meeting the demands of the next century in the ornamental plants sector. Moreover, micropropagation being a useful alternative for the propagation of Anthurium, is a rapid method suitable for application in small areas to produce pathogen-free plants (Teixeira da Silva et al, 2015; Martínez-Estrada et al, 2016). In this study, it was aimed to investigate the *in vitro* micropropagation possibilities of Anthurium plant and to develop appropriate protocols.

## 2. MATERIALS AND METHODS

### 2.1. Plant Material and Explant Sterilization

As a plant materials, 'Debby' and 'Lilian' genotypes were used for this study. Some problems were encountered in determining the correct explant type as the starting material and optimizing the surface sterilization protocol, and they were resolved by using different experiments. Initially, the basal parts of 7-8 cm sized Anthurium seedlings were used as explants. Sterilization of these tissues has been extremely difficult. Then, shoot tips of approximately 0.1-0.2 cm in size were isolated from these seedlings and used as starting material (Figure 1). Then, experiments were conducted with different agents for surface sterilization (Figure 2). Different surface sterilization protocols were applied to shoot tips (Table 1).

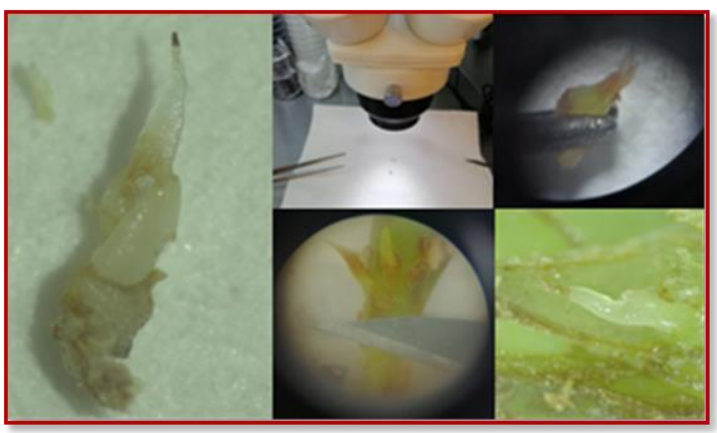

**Figure 1.** Shoot tips of approximately 0.1-0.2 cm in size were isolated from these seedlings and used as starting material

**Figure 2.** Sterilization of Anthurium explants

**Table 1.** Different surface sterilization protocols were applied to shoot tips

| Sterilization Number 1. | Sterilization Number 2. | Sterilization Number 3. | Sterilization Number 4. | Sterilization Number 5. |
|---|---|---|---|---|
| 1. Washing in tap water for 1 hour | 1. Washing in tap water for 30 minutes | 1. Washing in tap water for 1 hour | 1. Washing in tap water for 1 hour | 1. Washing in tap water for 30 minutes |
| 2. Soaking in polysorbate 20 (10 ml/L$^{-1}$) for 30 minutes | 2. Soaking in Polysorbate 20 (5 mg/L$^{-1}$) for 30 minutes | 2. Soaking in Polysorbate 20 (5 mg/L$^{-1}$) for 30 minutes | 2. Keeping it in dishwashing detergent in the sterile cabinet | 2. Soaking in Thiram (250 mg/L$^{-1}$) + Streptomycin Sulfate for 10 minutes (50 mg/L$^{-1}$) |
| 3. Fosetyl Al. (250 mg/L)+Streptomycin Sulfate (50 mg/L$^{-1}$)+ Soaking in chlorhexidine (100 ml/L$^{-1}$) for 10 minutes | 3. Soaking in Streptomycin Sulfate (50 mg/L$^{-1}$) for 10 minutes | 3. Soaking in Thiram (250 mg/L$^{-1}$) + Streptomycin Sulfate for 10 minutes (50 mg/L$^{-1}$) | 3. Soaking in 1 % Sodium hypochlorite for 60 minutes in a sterile cabinet | 3. Soaking in 0.1 g/L$^{-1}$ $HgCl_2$ + Tween-20 for 40 minutes |
| 4. Soaking in 0.1% g/L$^{-1}$ $HgCl_2$ + Tween-20 for 40 minutes | 4. Soaking in 0.1 g/L$^{-1}$ $HgCl_2$ + Tween-20 for 40 minutes | 4. Soaking in 1 % Sodium hypochlorite for 30 minutes in a sterile cabinet | 4. Soaking in 3 % Hydrogen peroxide for 5 minutes | 4. Soaking in 70 % Ethyl Alcohol in a sterile cabinet for 3 minutes |
| 5. Soaking in 70 % Ethyl Alcohol in a sterile cabinet for 3 minutes | 5. Soaking in 70 % Ethyl Alcohol in a sterile cabinet for 3 minutes | 5. Rinsing with pure water | 5. Rinsing with pure water | 5. Soaking in 20 % Soidum hypochlorite + Tween-20 for 20 minutes |
| 6. Soaking in 20 % Sodium hypochlorite + Tween-20 for 20 minutes | 6. Soaking in 20 % Soidum hypochlorite + Tween-20 for 20 minutes | | | 6. Soaking in 20 % Soidum hypochlorite + Tween-20 for 10 minutes |
| 7. Rinsing with pure water | 7. Rinsing with pure water | | | 7. Rinsing with pure wter |

## 2.2. Micropagation and Rooting

After selection of correct explant type and determination of successful sterilization trials were established. Different concentrations/combinations of BAP and Kinetin were used in 1/2 MS medium and 20% sucrose, keeping 0.1 mg $L^{-1}$ NAA constant in each medium (Table 2).

**Table 2.** Hormone combination of micropropagation medium

| Medium Number | Medium | NAA (mg $L^{-1}$) | BAP (mg $L^{-1}$) | Kinetin (mg $L^{-1}$) |
|---|---|---|---|---|
| Control | MS (1/2) | 0 | 0 | 0 |
| 1 | MS (1/2) | 0.1 | 0 | 0.5 |
| 2 | MS (1/2) | 0.1 | 0 | 1.0 |
| 3 | MS (1/2) | 0.1 | 0.5 | 0 |
| 4 | MS (1/2) | 0.1 | 1.0 | 0 |
| 5 | MS (1/2) | 0.1 | 0 | 0 |

Thus, *in vitro* proliferation experiments were completed by rooting the proliferated plantlets. In the rooting experiments, different concentrations of NAA and IBA were used in the medium containing 1/2 MS (Table 3).

### 2.3. Acclimatization

Plantlets well-developed in solid medium were removed and roots of the plant material were gently washed under running tap water and plunged in a liquid including 50% (w/v) of a 2.5 g $l^{-1}$ fungicide (Captan 50WP, Fruit&Ornamental, NY, USA) for 10-15 s and afterward moved to plastic viols (7 cm × 7 cm with and length) including peat and perlite (2:1, v/v). Then, Anthurium plants were replaced in a controlled greenhouse under natural light at 95-98% relative humidity and 22-24 °C.

**Table 3.** Hormone combination of rooting medium

| Medium Number | Medium | NAA (mg $L^{-1}$) | IBA (mg $L^{-1}$) |
|---|---|---|---|
| 1 | MS (1/2) | 0.5 | 0 |
| 2 | MS (1/2) | 1.0 | 0 |
| 3 | MS (1/2) | 2.0 | 0 |
| 4 | MS (1/2) | 0 | 0.5 |
| 5 | MS (1/2) | 0 | 1.0 |
| 6 | MS (1/2) | 0 | 2.0 |

### 2.4. Data Analyses

Fifty explants were used in each application for surface sterilization. Survival rates (%) were observed. According to the propagation and rooting experiments, fifty explants were also used for each procedure. Tests were repeated three times for the propagation phase. The parameters examined in the study for propagation were plant height (cm)

and propagation coefficient (plant/plant). For the rooting experiment, root length (cm), number of roots/plant, plant length (cm) were examined. All quantitative data calculated as percentage value were subjected to arcsine transformation before variance analysis. All data were expressed as means, and analysis of variance was performed. Means detected the statistically different were separated by least significant difference test (LSD) to evaluate differences among different solid medium in Anthurium. Statistical analysis was performed using JMP® software (SAS Institute, Cary, NC) ver. 13.01.

## 3. RESULTS

### 3.1. Sterilization Data Results (%)

When the sterilization (%) data of Debby and Lilian genotypes were examined, no statistical difference was observed between the genotypes. When the interaction was examined, sterilization protocol Number-1 for Debby variety (74.19%), and sterilization protocol Number-2 for Lilian variety (58.09%) were found as the most successful sterilization protocols (Table 4).

**Table 4.** Sterilization (%) results of Debby and Lilian genotypes

| Genotype | Sterilization Number 1. | Sterilization Number 2. |
|---|---|---|
| Debby | 74.19(60.24)a | 43.71(41.27)ab |
| Lilian | 58.09(49.67)ab | 34.54(35.57)b |

$LSD_{GENOTYPE}$: N.S., $LSD_{STERILIZATION}$: N.S., $LSD_{GENOTYPE*STERILIZATION}$ : 25,54*

*Statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$.

### 3.2. Micropropagation Data Results

When the results were examined, a statistically significant difference was found between genotypes and nutrient medium in terms of propagation coefficient, and the best results were obtained in Medium-2 nutrient medium (5 plantlet/plant) for the Lilian genotype (Figure 3) and in Medium-3 nutrient medium (4,6 plantlet/plant) for the Debby genotype (Figure 4). While the interaction was found to be insignificant, Medium-5 and control medium gave the same result. When the propagation coefficient of the Lilian genotype was examined, it was determined that it performed better compared to the Debby genotype (Table 5).

**Table 5.** Multiplication rate (%) results for Debby and Lilian genotypes (plantlet/plant)

| Genotype | Control | Medium-1 | Medium-2 | Medium-3 | Medium-4 | Medium-5 |
|---|---|---|---|---|---|---|
| Debby | 2.3de | 3bcde | 4abcd | **4.6ab** | 2.6cde | 2e |
| Lilian | 3bcde | 4abcd | **5a** | 4.3abc | 4.6ab | 3.6abcde |

L

$SD_{GENOTYPE}$: 0,79**, $LSD_{MEDIUM}$: 1,37*, $LSD_{MEDIUM*GENOTYPE}$: N.S.

Statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$.

**Figure 3.** Multiplication stage of Lilian genotype

**Figure 4.** Multiplication stage of Debby genotype

### 3.2.1. Plant Length Data Results

When the results were examined, no statistical difference was observed between genotypes. The best nutrient medium in terms of plant length were determined as control, Medium-1 and Medium-2, respectively. Looking at the interaction, the best plant length result was observed from the Medium-2 (5,5 cm) for Lilian genotype and Medium-1 (5 cm) for the Debby genotype (Table 6).

**Table 6.** Plant length results for Debby and Lilian genotypes (cm)

| Genotype | Control | Medium-1 | Medium-2 | Medium-3 | Medium-4 | Medium-5 |
|---|---|---|---|---|---|---|
| Debby | 4.23bcd | **5ab** | 3.5cd | 4bcd | 3.23d | 4.16bcd |
| Lilian | 4.66abc | 4.33abcd | **5.5a** | 3.66cd | 3.16d | 3.83bcd |

$LSD_{GENOTYPE}$: N.S., $LSD_{MEDIUM}$: 0.86*, $LSD_{MEDIUM*GENOTYPE}$: 1,22*

Statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$.

### 3.3. Rooting Data Results

### 3.3.1. Number of Roots/Plant

When the results were examined, it was determined that the difference between both genotypes and media was statistically significant. No significant difference was observed in the interaction between genotypes and media. In terms of root number, Lilian genotype was more prominent than Debby and the best root number was obtained in Medium-6 (9,66 and 6,03 roots/plant, respectively) nutrient medium (Figure 5, Figure 6). It was observed that the high concentration of IBA (2 $mg/L^{-1}$) positively promoted root growth in both genotypes (Table 7).

**Figure 5.** Rooting stage of Lilium genotype

**Figure 6.** Rooting stage of Debby genotype

**Table 7.** Number of roots/plant for Debby and Lilian genotypes

| Genotype | Medium-1 | Medium-2 | Medium-3 | Medium-4 | Medium-5 | Medium-6 |
|---|---|---|---|---|---|---|
| Debby | 3d | 4.33bcd | 3.66cd | 3d | 4cd | **6.03b** |
| Lilian | 5bc | 5.33bc | 3.66cd | 3.66cd | 4.33bcd | **9.66a** |

$LSD_{GENOTYPE}$: 0,72**, $LSD_{MEDIUM}$: 1,25***, $LSD_{MEDIUM*GENOTYPE}$: N.S.

Statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$.

### 3.3.2. Root Length/Plant (cm) Results

When the results regarding root length were examined, it was determined that the difference between both genotypes and media was statistically significant. No significant difference was observed in the interaction between genotypes and media. In terms of root number, Lilian genotype (6,20 cm) was more prominent than Debby (4,8 cm) and the best root length was determined in Medium-6 nutrient medium. It was observed that this medium, in which IBA (2.0 $mg/L^{-1}$) was used at high concentration, positively promoted root growth (Table 8).

**Table 8.** Root length/plant for Debby and Lilian genotypes

| Genotype | Medium-1 | Medium-2 | Medium-3 | Medium-4 | Medium-5 | Medium-6 |
|---|---|---|---|---|---|---|
| Debby | 1.76fg | 2.32e | 2.46e | 1.56g | 2.16ef | **4.80b** |
| Lilian | 3.25d | 4.04c | 3.53d | 3.33d | 3.60cd | **6.20a** |

$LSD_{GENOTYPE}$: 0,21***, $LSD_{MEDIUM}$: 0,37***, $LSD_{MEDIUM*GENOTYPE}$: N.S.

Statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$.

### 3.3.3. Plant Length (cm) Results

According to the results regarding plant length were examined, it was determined that the difference between both genotypes and media was statistically significant. No significant difference was observed in the interaction between genotypes and media. In terms of the number of roots, Lilian genotype (4,10 cm) was more prominent than Debby genotype (3,32 cm) and the best plant length among the medium was found to be Medium-6 nutrient medium. The other medium were statistically in the same group and it was observed that the high concentration of IBA (2.0 $mg/L^{-1}$) had a positive effect on the development of plant length growth especially in Lilian genotype (Table 9).

**Table 9.** Plant length (cm) for Debby and Lilian genotypes

| Genotype | Medium-1 | Medium-2 | Medium-3 | Medium-4 | Medium-5 | Medium-6 |
|---|---|---|---|---|---|---|
| Debby | 3.12b | 3.13b | 3.16b | 3b | 3.13b | **3.32b** |
| Lilian | 3.10b | 3.36b | 3.33b | 3.03b | 3.33b | **4.10a** |

$LSD_{GENOTYPE}$: 0,17***, $LSD_{MEDIUM}$: 0,30***, $LSD_{MEDIUM*GENOTYPE}$: N.S.

Statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$.

### 3.4. Acclimatization

As a result of *in vitro* rooting experiments, plantlets containing shoots and roots were obtained. It was transferred to plastic vials. The plants were successfully acclimatized in the greenhouse. Survival rate was found as 80% in both genotypes (Figure 7).


**Figure 7.** Acclimatization of plantlets a:Dipping plants in fungicide, b:Opening a planting hole, c:Planting stage, d:Lilian genotype, e:Debby genotype, f: Mature plants

## 4. CONCLUSION

In conclusion, in the study for the *Anthurium andreaum*, some problems were encountered in determining the correct explant type as the starting material and optimizing the surface sterilization protocol, and they were resolved by using different experiments. Initially, the basal parts of 7-8 cm sized Anthurium seedlings were used as explants. Sterilization of these tissues has been extremely difficult. Then, shoot tips of approximately 0.1-0.2 cm in size were isolated from these seedlings and used as starting material. Meristems were isolated under microscope to get healty plants. Micropropagation and rooting experiments were established with developing shoots. Thus, healthy plantlets were obtained. To improve the quality and multiplication rate of Anthurium plantlets, it is suggested to use bioreactor systems in addition to solid culture for *in vitro* propagation. This can also enhance propagation and rooting performance.

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# Strategic innovations in *in vitro* woody crop production: balancing technical excellence with market and stakeholder dynamics

**Buhara Yücesan[1,2], Ahmet Tigrel[2]**

*[1]Dept. Of Seed Science and Technology, Faculty of Agriculture, Bolu Abant Izzet Baysal University, Bolu, Türkiye (ibuhara@yahoo.com)*

*[2]Xplant R&D Co., Dudullu OSB, DES:113-27, Ümraniye, Istanbul, Türkiye*

*ibuhara@yahoo.com*

## Abstract

Plant factories, leveraging vertical farming and plant tissue culture, offer significant advantages over traditional and semi-open greenhouse systems for large-scale crop production. This study examines the critical factors for successful *in vitro* crop production, emphasizing the balance between internal technical requirements and external market and stakeholder influences. Internally, the focus is on academic research, protocol development, and the application of innovative methodologies to optimize culture media and enhance plant regeneration processes. Externally, we address the realities faced by stakeholders and investors, including labor costs, energy consumption, market volatility, order cancellations, and logistics. Particularly, we explore the strategy for *in vitro* regeneration of woody crops, considering short-term production of rootstocks and long-term strategies for green foliage crops. The selection of starting materials is guided by market demand, highlighting the importance of strategic planning in tissue culture systems. This research presents a comprehensive risk assessment of plant cloning, aiming to stimulate technological awareness and acceptance among stakeholders. By analyzing labor efficiency and its correlation with regeneration rates, we propose a model for sustainable, cost-effective crop production. With the global horticulture market becoming increasingly competitive, particularly in Asia where costs are driven down, this study underscores the need for innovative approaches to maintain profitability in woody crop production. By aligning tissue culture practices with market demands and stakeholder expectations, we aim to contribute to the advancement of commercial *in vitro* crop production.

***Keywords*****:** *in vitro* regeneration, stakeholder engagement, market dynamics, sustainable agriculture.

## 1. OVERVIEW OF *IN VITRO* WOODY CROP PRODUCTION

*In vitro* woody crop production, a subset of micropropagation, involves the cultivation of woody plants such as trees and shrubs in a controlled, sterile environment using tissue culture techniques. This method begins with small tissue segments, known as explants, which are cultured on nutrient media under specific conditions to produce new plants. The

significance of this technique lies in its numerous advantages over traditional propagation methods, including the ability to produce large quantities of plants rapidly, maintain disease-free stock, conserve rare or endangered species, and enable year-round production independent of natural growing seasons (Minocha and Jain 2000). The *in vitro* approach is particularly valuable for species that are difficult to propagate through conventional methods, ensuring the production of uniform and high-quality plants. This method also plays a crucial role in the commercial propagation of forestry plants, fruit crops, and ornamental species, providing a reliable and efficient means to meet market demands and support conservation efforts (Broggini et al. 2024; Abdouli 2024; Cvjetkovic 2024).

The primary objective of this chapter is to explore the critical factors that influence the successful *in vitro* production of woody crops. The research aims to balance the internal technical requirements of tissue culture with the external market and stakeholder dynamics to develop sustainable and cost-effective strategies for large-scale production. Internally, the study focuses on optimizing protocol development, culture media, and plant regeneration processes. By advancing these technical methodologies, the goal is to enhance the efficiency and effectiveness of *in vitro* propagation systems. This involves rigorous academic research and the application of innovative approaches to address challenges such as genetic stability and scalability. Externally, the study examines the practical realities faced by stakeholders and investors, including labor costs, energy consumption, market volatility, and logistical challenges. By understanding these external factors, the research aims to align tissue culture practices with market demands and stakeholder expectations, ensuring the economic viability and profitability of *in vitro* woody crop production. Through a comprehensive risk assessment of plant cloning, this report seeks to stimulate technological awareness and acceptance among stakeholders. By proposing a model for sustainable, cost-effective crop production, the research contributes to the advancement of commercial *in vitro* crop production, particularly in a competitive global market.

## 2. ADVANTAGES OF PLANT FACTORIES

Plant factories, which leverage advanced techniques such as vertical farming and plant tissue culture, offer numerous advantages over traditional and semi-open greenhouse systems (Tigrel et al. 2022).. Here, we detail these advantages, highlighting how they contribute to the efficiency, sustainability, and productivity of crop production. Plant factories utilize vertical farming techniques, where crops are grown in stacked layers to maximize space efficiency. This approach allows for a significantly higher yield per square meter, making it particularly advantageous in urban environments where space is limited. By optimizing the use of vertical space, plant factories can produce a large number of plants in a relatively small area, enhancing overall productivity (Kozai 2020).

One of the primary benefits of plant factories is their ability to facilitate year-round production. Unlike traditional farming, which is subject to seasonal variations and weather conditions, plant factories provide a controlled environment that supports

continuous and predictable production cycles (Tigrel et al. 2022). This capability ensures a steady supply of crops to meet market demands, regardless of the time of year. Thus, Plant factories offer precise control over various growth conditions, including temperature, light, humidity, and nutrient supply. By fine-tuning these parameters, plant factories can create the ideal environment for each plant species, leading to enhanced growth rates and higher yields. This level of control also allows for the cultivation of plants with specific desirable traits, such as increased nutritional content or disease resistance. For the resource efficiency, the controlled environment of plant factories enables more efficient use of resources. For example, water usage can be significantly reduced through recycling and precision irrigation systems. Nutrient delivery can be optimized to ensure that plants receive exactly what they need, minimizing waste. Additionally, the controlled conditions can reduce or eliminate the need for pesticides, contributing to safer and more sustainable crop production practices. Therefore, plants grown in plant factories are typically free from pathogens due to the sterile conditions in which they are cultivated. This disease-free status is crucial for maintaining healthy crop production and can significantly reduce losses due to disease outbreaks. The ability to consistently produce disease-free plants is particularly valuable for the propagation of high-value crops and for the conservation of rare or endangered species (Kozai 2018).

Plant factories contribute to sustainable agriculture by enabling the cultivation of crops in a manner that reduces the environmental footprint. The efficient use of resources, elimination of pesticide use, and ability to produce crops in urban settings close to the point of consumption all contribute to a more sustainable agricultural system (Table 1). This approach aligns with global efforts to enhance food security and reduce the impact of agricultural practices on the environment.

**Table 1.** The diverse applications of plant factories across various sectors of agriculture:

| **Sector** | **Application** |
|---|---|
| Forestry | Production of high-quality, disease-free forestry plants for reforestation and commercial wood production. |
| Fruit Production | Particularly useful for fruit species where seed propagation does not yield true-to-type plants, such as apples, cherries, and walnuts. |
| Ornamental Horticulture | Enables the mass production of ornamental trees and shrubs with desirable traits, meeting the demands of the horticultural market. |

As seen in Table 1. plant tissue culture systems can also be evaluated as a form of plant factory. These systems use sterile environments to grow plants from small tissue segments, offering many of the same advantages as other plant factory techniques. By combining plant tissue culture with the controlled environments of plant factories, it is possible to further enhance the efficiency, sustainability, and productivity of crop production.

## 3. INTERNAL TECHNICAL REQUIREMENTS

The success of *in vitro* woody crop production is heavily dependent on the optimization of internal technical requirements. This section focuses on the importance of academic research and protocol development, the advances in innovative methodologies, and the technical challenges that need to be addressed.

### 3.1. Academic Research and Protocol Development

Academic research plays a crucial role in the optimization of protocols for *in vitro* woody crop production. The development of effective protocols involves meticulous experimentation and analysis to determine the best practices for plant tissue culture. This includes selecting the appropriate explants, optimizing the culture media, and refining the conditions for plant regeneration. Numerous studies have demonstrated the importance of these factors in successful tissue culture systems. The choice of explants, which are small tissue segments used for cultivation, is critical for the success of tissue culture. Factors such as the age of the plant, the type of tissue, and the physiological state of the explant can significantly impact the efficiency of the culture process. For instance, juvenile tissues are often preferred due to their higher regenerative capacity and lower contamination rates (Tigrel et al., 2022). The modification of culture media is another area where academic research has made significant contributions. The use of novel plant growth regulators, such as nanoparticles and biostimulants, has been shown to enhance the establishment and growth of explants. These advancements help in developing media that provide the necessary nutrients and hormonal balance for optimal plant growth. Additionally, studies have explored the benefits of incorporating vitamins, amino acids, and other supplements into the culture media to improve plant vigor and development.

Furthermore, refining the physical conditions of tissue culture, such as light intensity, photoperiod, temperature, and humidity, is essential for maximizing growth and minimizing stress responses. Techniques like micropropagation, somatic embryogenesis, and organogenesis have been optimized through continuous research, ensuring high success rates and scalability of tissue culture systems. The integration of automated systems for monitoring and controlling these conditions has also been explored, aiming to increase efficiency and consistency in plant production.

### 3.2. Innovative Methodologies

Advances in innovative methodologies have greatly improved the efficiency and effectiveness of *in vitro* woody crop production. One of the key innovations is the use of novel plant growth regulators, which have been found to significantly enhance the *in vitro* establishment of plants. These growth regulators, including various biostimulants and nanoparticles, help in promoting cell division, elongation, and differentiation, leading to improved plant regeneration rates. Another important innovation is the development of cost-effective media components for mass production. In Copytree Cost action (2024) Jaroslav Nisler presented his researches on novel compounds responsible for cytokinin

oxidase/dehydrogenase (CKX), that protects isoprenoid structure of cytokinins from oxidation. This is good news for the recalcitrance of the crops in tissue culture systems (Murvanidze et al., 2021; Nisler, 2024). Researchers have explored the use of alternative media components that are less expensive yet effective in supporting plant growth. This includes the utilization of organic derivatives and natural extracts, which not only reduce costs but also improve the sustainability of the tissue culture process. In addition to these advancements, the use of plant-fungus co-culture systems has shown promise in enhancing the growth and development of woody crops. Such systems involve the symbiotic association between plants and beneficial fungi, which can improve nutrient uptake, stress tolerance, and overall plant health.

### 3.3. Technical Challenges

Despite the advancements in academic research and innovative methodologies, several technical challenges remain in the field of *in vitro* woody crop production. One of the primary challenges is optimizing culture conditions to ensure the consistent growth and development of plants. This involves maintaining the right temperature, light intensity, humidity, and nutrient levels throughout the culture process. In this respect, Figure 1 presents a comprehensive Plant Factory Model designed for *in vitro* plant propagation. It highlights the critical internal and external factors that contribute to efficient and sustainable plant production. The internal factors include the maintenance of stock plants, explant preparation, explant selection, and the use of regeneration medium. These steps ensure a continuous supply of high-quality explants ready for propagation. Culture vessels are used to hold the explants and regeneration medium, while the plant canopy is managed to ensure proper light and nutrient distribution. Energy-efficient LED lights provide the necessary light spectrum, and even temperature distribution is maintained throughout the cultivation spaces to promote uniform plant growth (Uraisami, 2018). External factors such as cost management and quality management are also crucial. Effective cost management optimizes the economic viability of the plant factory, while quality management ensures the production of high-quality plants that meet market standards. This integrated approach underscores the importance of balancing technical excellence with economic and quality considerations to achieve successful and sustainable *in vitro* plant propagation.

Genetic stability is another critical challenge. Over time, plants cultured *in vitro* may undergo genetic variations, which can affect their growth, morphology, and overall quality. Ensuring genetic stability requires careful monitoring and the use of techniques such as somatic embryogenesis and molecular markers to detect and prevent genetic drift. Scalability is also a major concern for commercial applications. While laboratory-scale tissue culture techniques are well-established, scaling up these processes for large-scale production presents significant difficulties. This includes the need for large volumes of sterile culture media, the management of contamination risks, and the automation of labor-intensive processes.

## 4. EXTERNAL MARKET AND STAKEHOLDER INFLUENCES

Plant factories, leveraging advanced techniques such as vertical farming and plant tissue culture, must navigate a variety of external market and stakeholder influences to be successful on a commercial scale. These influences encompass economic factors, regulatory considerations, market demand, and stakeholder expectations. One of the significant economic challenges for plant factories is the high initial investment required for setup and the ongoing operational costs, particularly energy consumption. The use of artificial lighting, such as LED systems, is essential for maintaining optimal growth conditions but contributes to substantial electricity expenses. Innovations in energy-efficient lighting solutions and the integration of renewable energy sources can help mitigate these costs.

Based on our commercial scale applications and experiences, labor costs are another critical factor. Skilled workers are needed for the propagation, maintenance, and monitoring of plants within the factory. However, the high cost of labor can be offset by automating various processes, including planting, harvesting, and environmental control. Automation technologies, such as robotic systems and wireless sensor networks, can reduce the reliance on manual labor and increase efficiency. *In vitro* plant propagation in tissue culture systems is influenced by dynamic factors such as cultivar selection and market demands. For commercial labs and plant factories, the propagation strategies vary significantly between horticultural crops, ornamental plants, forestry and fruit trees.

Fruit trees require precise timing for planting, necessitating careful scheduling to ensure sustainable propagation. Conversely, ornamental plants, especially indoor varieties like succulents, leafy plants, and orchids, can be propagated throughout the year based on market demand, although this can increase workforce costs. Successful propagation involves maintaining a suitable micropropagation stock of at least 10,000 plants per variety as mother plants. This ensures a continuous supply for customer-specific products and aligns with market trends. The commercial propagation process must account for seasonal demands for fruit crops and continuous supply for ornamental plants.

Key steps in commercial horticultural plant propagation include:

- Literature surveys and research to optimize protocols for explant and media selection.
- Establishing protocols based on customer needs and market demands.
- Ensuring cost efficiency, which heavily depends on labor costs and the financial status of the operating country.

Labor efficiency is critical, with plant cutters playing a vital role in maintaining the propagation cycle (see Model study below). In countries like China, Thailand, and India, tissue culture labs produce value-added horticultural crops at competitive prices, enhancing their marketability (Tigrel et al., 2022). Post-pandemic trends show an increase in the auctioning of rare tissue culture plants through social media, highlighting the growing importance and commercial potential of micropropagation. This sales strategy underscores the significance of micropropagation in meeting the rising demand for high-quality, sustainable plant products.

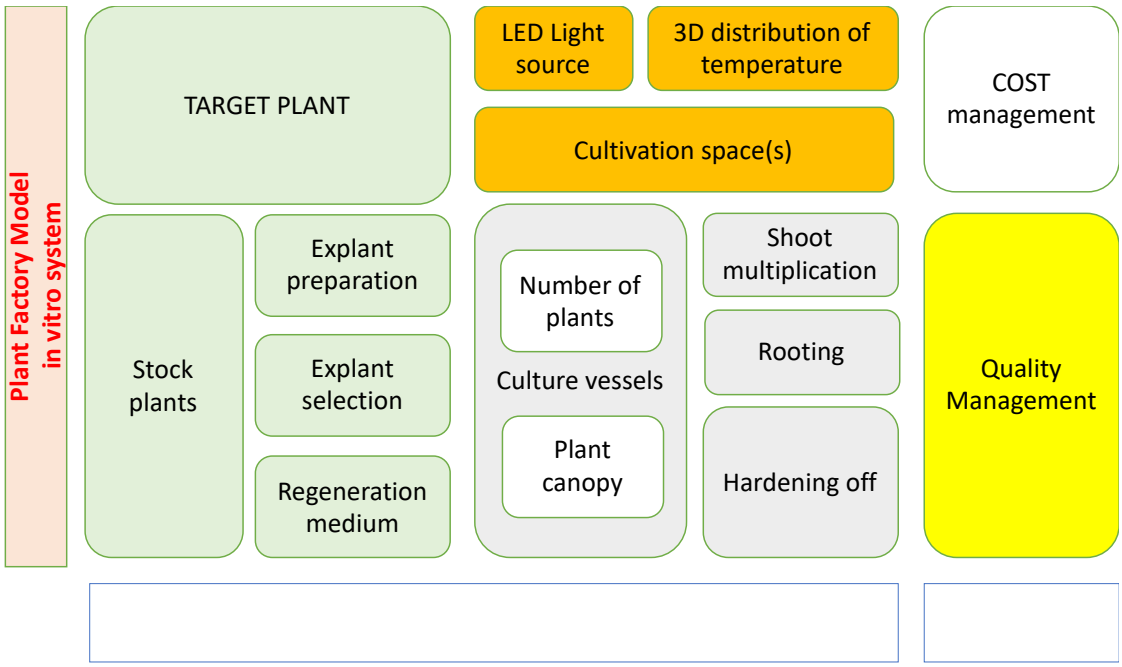


**Figure 1.** A comprehensive Plant Factory Model for *in vitro* plant propagation systems, integrating both internal and external factors crucial for efficient and sustainable plant production (*adopted from Tigrel et al. 2022*)

## 5. STRATEGIC PLANNING FOR *IN VITRO* WOODY CROP PRODUCTION

### 5.1. Short-Term Production of Rootstocks

The short-term production of rootstocks through *in vitro* clonal woody propagation is a strategic method to rapidly generate high-quality, uniform plant material essential for grafting in horticulture and plantation crops. This approach leverages tissue culture techniques to produce genetically identical plants from a single parent, particularly beneficial for woody species that are difficult to propagate traditionally. *In vitro* clonal propagation ensures that rootstocks are disease-free and genetically uniform, which is crucial for successful grafting (Bayhan and Yücesan, 2024). The process involves the use of novel plant growth regulators (PGRs) to enhance micropropagation efficiency and rootstock establishment. Key PGRs include meta-topolin, an aromatic cytokinin that promotes shoot proliferation and enhances overall growth, and jasmonates, which support rooting and shoot proliferation. Brassinosteroids enhance root and shoot growth, improve stress tolerance, and increase plant vigor, while salicylic acid improves shoot and root development and stress tolerance. Paclobutrazol, a triazole compound, controls plant growth and enhances root development. Strategic planning for *in vitro* cloning involves optimizing culture conditions, selecting appropriate PGRs, ensuring genetic fidelity, and developing efficient grafting techniques to produce high-quality grafted plants (Chugh and Kumar, 2022). This

integration significantly improves the short-term production of rootstocks, providing a reliable source of high-quality planting material for horticulture and plantation crops.

### 5.2. Long-Term Strategies for Green Foliage and timber Crops

Long-term strategies for *in vitro* plant production focus on sustainable practices that ensure the economic viability and environmental responsibility of green foliage and timber crops. These strategies involve adapting to market trends by emphasizing the use of renewable resources and minimizing environmental impacts. Current market trends indicate a growing demand for green foliage crops due to their environmental and aesthetic benefits, which necessitates aligning production strategies to maintain market relevance. To support these efforts, continuous research and development are critical for improving crop varieties and enhancing their resilience to environmental stresses, ensuring long-term sustainability. A notable example of such strategic efforts is the "Wood of Value (Wertholz)" project, which highlights the importance of forest tree breeding in enhancing the quality and economic value of timber (Eisold et al., 2024). This project focuses on breeding forest trees that provide high-quality wood, emphasizing genetic improvement and sustainable forest management practices. By integrating findings from such initiatives, *in vitro* propagation strategies can be refined to produce timber crops that meet high market standards and sustainability goals. Selecting the appropriate starting materials is a fundamental aspect of aligning production with market demand and consumer preferences. This selection process prioritizes plant varieties that exhibit desirable traits such as disease resistance, fast growth, and high market value. Market research plays a crucial role in guiding this selection, ensuring that the chosen varieties meet consumer expectations and can be effectively marketed. Based on our commercial experiences, tissue culture applications are highly suitable for year-round production of green foliage crops. For woody crops, house plants such as Ficus varieties (*Ficus benjamina, Ficus elastica*) and Musa species are excellent candidates for *in vitro* propagation. Other suitable house plants include Dracaena, Schefflera, Philodendron, and various species of Sansevieria (snake plant). These plants benefit from tissue culture techniques due to their high demand for consistent quality, disease-free status, and rapid multiplication.

## 6. RISK ASSESSMENT IN PLANT CLONING

### 6.1. Technological Risks

Technological risks in plant cloning encompass several critical issues that can impact the success and efficiency of tissue culture systems. First, large-scale production increases the risk of contamination. Automation in tissue culture systems can streamline processes, but it also means that a single contamination event can quickly spread, affecting large volumes of production. Rigorous monitoring and maintaining aseptic conditions are crucial to minimize this risk (Wibowo et al., 2022). Second, somatic

variation, or somaclonal variation, is another significant risk associated with repeated subcultivation under artificial light systems. Over time, genetic and epigenetic changes can accumulate, leading to undesirable phenotypic variations among cloned plants. This variation can compromise the uniformity and quality of the clonal plants, making it essential to monitor and limit the number of subcultivations (Kacar, 2024). Third, vitrification, or hyperhydration, is a common problem in tissue culture systems. It occurs when plants become overly hydrated, resulting in translucent, brittle tissues that are often non-viable. This condition can lead to substantial losses if not promptly addressed (Bayhan and Yücesan, 2024). The key to preventing vitrification lies in timely adjustments to the culture conditions, such as modifying humidity, light intensity, and nutrient media composition, to ensure the plants maintain a healthy balance of water and nutrients (Saez et al., 2012; Polivanova and Bedarev, 2022).

### 6.2. Market Risks

Market risks in plant cloning include volatility in consumer demand and the complexities of global trade policies. The demand for specific plant varieties can fluctuate due to changing consumer preferences and economic conditions. Trend topic crops, such as those with unique aesthetic or environmental benefits, are often favored for propagation. However, the growing parameters for these crops must be cost-effective not only in tissue culture systems but also in greenhouses for the final product. Asian countries, benefiting from lower labor costs, often provide cheaper options for *in vitro* plants and generally export plants ex-agar (without further acclimatization) to keep prices low. This practice necessitates a focus on the quality of plant material and packaging to ensure that the plants can survive transport and acclimatize successfully upon arrival. Integrating high-quality standards in both production and packaging is essential to meet market expectations and reduce post-shipping losses, thereby maintaining competitive market positioning (Hamdan et al., 2022).

### 6.3. Operational Risks

Operational risks encompass issues related to labor availability, cost variability, and energy price fluctuations. The labor-intensive nature of plant cloning, especially in tissue culture techniques, requires skilled personnel, which can be challenging to source consistently. Additionally, the skills and morale of the workforce play a significant role in operational efficiency. Providing regular breaks, conducting regular meetings for discussing techniques, especially when introducing new crops, and ensuring a positive work environment can significantly improve productivity and reduce errors. Moreover, the costs associated with labor, energy, and other inputs can vary significantly, affecting the overall cost-efficiency of plant cloning operations (Fig. 2). Implementing automation technologies and optimizing resource use can help address these operational challenges, ensuring more stable and predictable production processes. Training and retaining skilled workers, along with creating a supportive and engaging work

environment, are crucial for maintaining high standards in production and mitigating operational risks. By understanding and addressing these technological, market, and operational risks, the plant cloning industry can better navigate the complexities of producing high-quality clonal plants sustainably and profitably (Fig. 3).

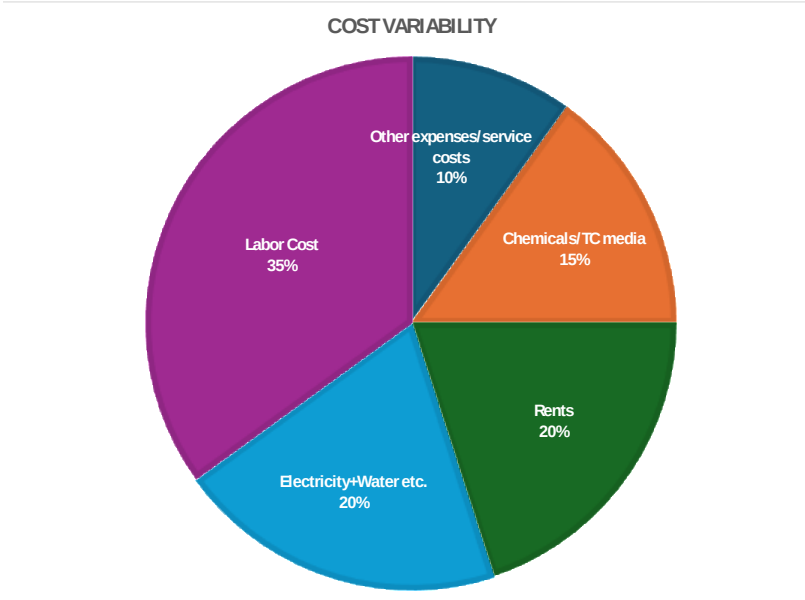


**Figure 2.** Pie chart for cost variability in a tissue culture systems including labor costs, chemicals for growth and regeneration media, operational bills, rents, and other expenses *(data provided from Xplant Co., Istanbul)*

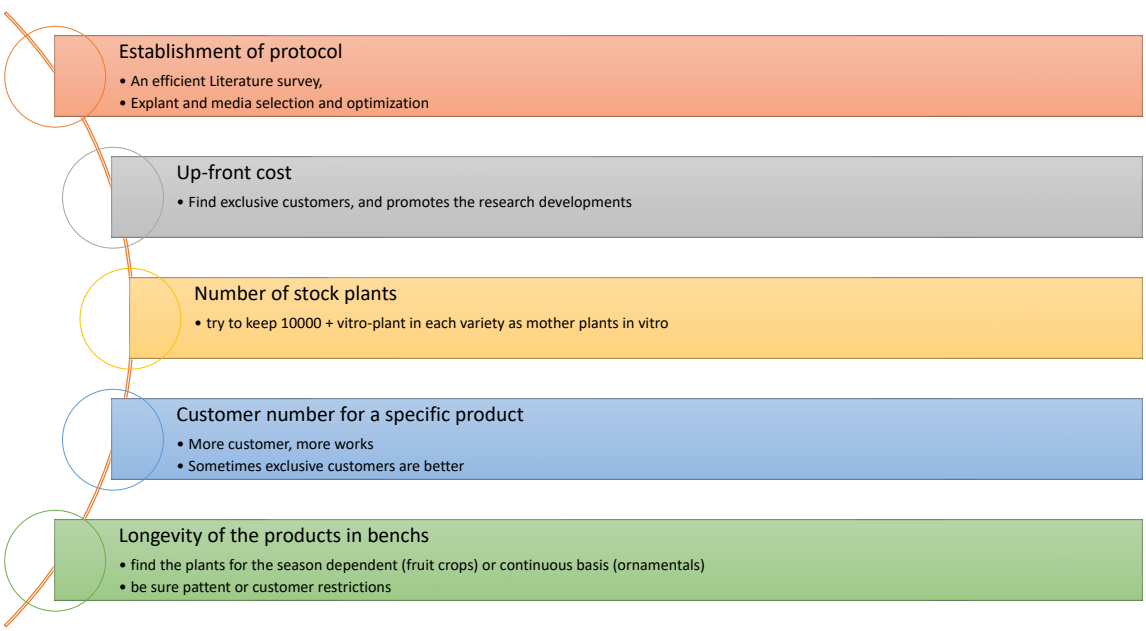


**Figure 3.** A business model for a sustainable woody crop production *in vitro* (*adopted from Tigrel et al. 2022*).

## 7. LABOR EFFICIENCY AND REGENERATION RATES

### A model Study Optimized Workflow Design for Synchronous Propagation of 10,000 Plants

This model ensures the synchronous propagation and shipment of 10,000 *in vitro* plants every 8 weeks, with an additional 10,000 plants ready for the next cycle (see Fig. 3 as well). By maintaining a consistent daily workflow, implementing quality control measures, and optimizing labor and resources, regular and reliable plant shipments are achievable. This approach ensures all plants are of uniform size and quality at the time of shipment. Utilizing a regeneration factor where each plant gives rise to five new plants, the model guarantees that 10,000 plants are prepared for shipment every 8 weeks while another 10,000 are ready for the subsequent cycle.

**Resources and Labor:**

- **Labor Force**: Calculated based on the workload
- **Daily Media Preparation**: 15 liters

**Media and Plant Distribution:**

- 1 liter of media = 12 jars
- Each jar contains a maximum of 10 plants
- Each plant regenerates into 5 new plants over 8 weeks

**Calculations:**

1. **Initial Requirements:**
   - To ship 10,000 plants and have another 10,000 ready for the next cycle, we need to start with a total of 4,000 plants, as each plant will regenerate into 5 plants (4,000 plants x 5 = 20,000 plants).
2. **Media and Jar Requirements:**
   - Total plants needed = 20,000 (10,000 for shipment + 10,000 for next cycle)
   - Each jar can hold 10 plants, so we need 20,000/10 = 2,000 jars.
   - Each liter of media fills 12 jars, so we need 2,000/12 ≈ 167 liters of media over 8 weeks.

## Weekly Breakdown:

**Plant Cutting and Preparation:**

- **Target Plants per Day**: Calculate based on 8-week cycle to reach 20,000 plants.
- **Daily Media Usage**: 15 × 12 = 180 jars.
- **Daily Plant Capacity**: 180 × 10 = 1,800 plants.

**8-Week Cycle:**

1. **Total Plants Needed**:
   - Starting plants: 4,000 (regenerating into 20,000 after 8 weeks).
   - Total plants required to handle every 8 weeks = 20,000.
2. **Daily Requirement**:
   - Total plants to be managed per day: 20,000/(8×5) = 500 plants per day.
   - Daily cutting should not exceed 500 plants to meet the target.
3. **Labor Requirements**:
   - If each cutter can handle 500 plants per day, only one cutter is needed to meet the target.
4. **Total Media Preparation**:
   - Total media prepared per day: 15 liters.
   - Each liter of media supports 1800 plants over the 8-week cycle.

**Daily Workflow:**

**Plant Cutting and Preparation:**

1. **Plant Cutting**:
   - 1 plant cutter handles approximately 500 plants daily.
2. **Media Preparation**:
   - 1 media preparer handles 15 liters daily.

**Weekly Workflow:**

1. **Weekly Plants Cut**:
   - Total plants cut per week: 500 × 5 = 2,500 plants.
2. **Weekly Media Preparation**:
   - Total media prepared per week: 15 × 5 = 7515 × 5 = 75 liters.
   - Weekly jar capacity: 75 × 12 = 90075 × 12 = 900 jars.
   - Weekly plant capacity: 900 × 10 = 9,000 plants.

**Labor Schedule:**

| Task | Person | Plants/Media per Day | Total per Week | Total per 8 Weeks |
|---|---|---|---|---|
| Plant Cutting | Cutter 1 | 500 | 2,500 | 20,000 |
| Media Preparation | Preparer | 15 liters | 75 liters | 600 liters |

**Shipping and Regeneration Schedule:**

1. **Every 8 Weeks**:
   - 10,000 plants are shipped.
   - 10,000 plants are prepared for the next cycle.

**Quality Control:**

- Continuous monitoring of plant health and growth conditions.
- Ensuring sterile conditions in media preparation and plant handling.
- Regular inspections to ensure uniform plant size and development.

This model ensures the synchronous propagation and shipment of 10,000 *in vitro* plants every 8 weeks, with an additional 10,000 plants ready for the next cycle. By maintaining a consistent daily workflow, implementing quality control measures, and optimizing labor and resources, regular and reliable plant shipments are achievable. The model involves starting with 4,000 plants, which regenerate into 20,000 plants over 8 weeks, necessitating the use of 2,000 jars and approximately 167 liters of media. Daily tasks include cutting and preparing 500 plants and managing 15 liters of media, supporting 1,800 plants. Weekly, 2,500 plants are cut, and 75 liters of media are prepared, filling 900 jars and supporting 9,000 plants. This structured approach ensures all plants are of uniform size and quality at the time of shipment. Labor efficiency is maximized by having one cutter and one media preparer, ensuring tasks are evenly distributed and production targets are met. Continuous monitoring and maintaining sterile conditions are crucial for quality control. Integrating high-quality standards in production and packaging ensures that plants survive transport and meet market expectations. This model effectively balances resource use, labor efficiency, and market demands, ensuring sustainable and profitable plant cloning operations.

The strategic plan for industrial *in vitro* plant production leverages optimized use of plant growth regulators (PGRs) and effective management of stock sizes to enhance productivity and meet market demands. High concentrations of Benzylaminopurine (BAP), up to 5.0 mg/L for bananas and 2.0 mg/L for woody crops, are employed initially to rapidly increase stock numbers. Subsequent subcultures use lower BAP concentrations (0.1 mg/L) to control regeneration rates, ensuring sustainable production and efficient shipment management. Combining BAP with Naphthaleneacetic acid (NAA) at a 1/10 ratio further enhances initial stock proliferation. A regeneration rate of 2× is maintained to support consistent stock availability. Additionally, BAP levels are adjusted according to desired stock size and customer demand, which helps manage labor effectively. During this phase, developing a robust root system is prioritized, a practice widely adopted across various crop species to ensure the health and viability of the plants. This strategic approach ensures that *in vitro* plant production remains efficient, scalable, and responsive to market needs.

In Figure 4. the top section highlights the core objectives: optimizing resource and labor management, maintaining high-quality production, and aligning with market demands. The bottom section details the critical elements of the tissue culture cloning process, including genetic optimization, strategic partnerships, efficient logistics, advanced technologies, and stringent quality control measures. Together, these elements ensure scalable, sustainable, and market-responsive plant propagation.

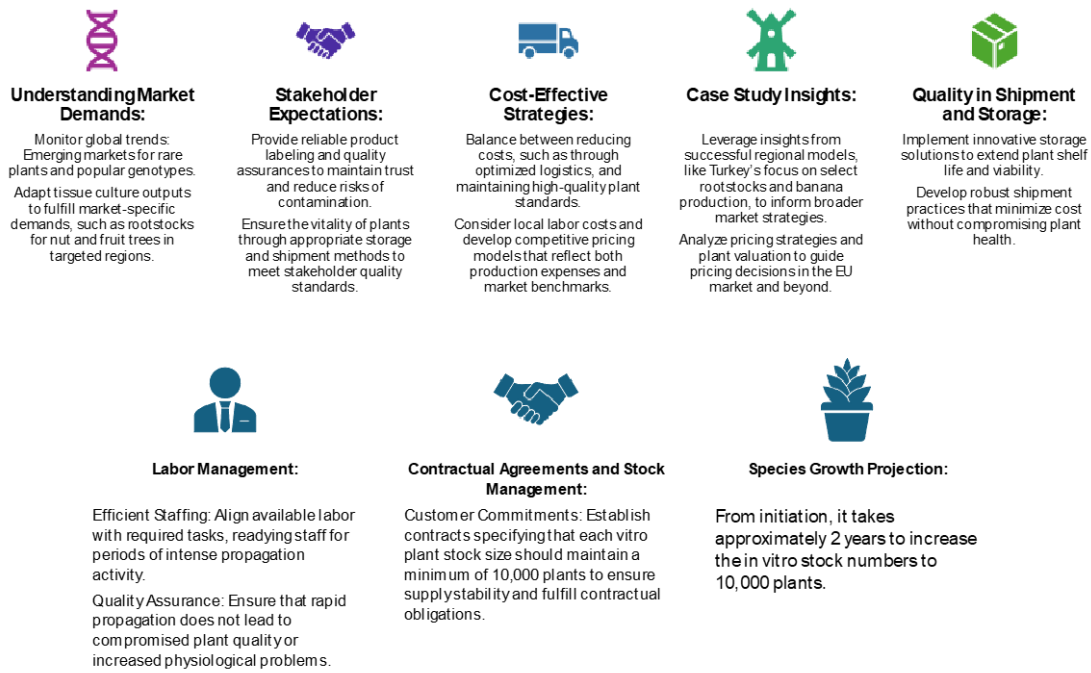


**Figure 4.** Strategic Framework for Industrial *In vitro* Plant Production

## 8. CONCLUSION

The importance of resource and labor management is underscored by the model's structure. With calculated labor force needs, daily media preparation, and media consumption, the approach ensures the consistent handling of 10,000 plants every 8 weeks, with another 10,000 ready for the next cycle. This systematic method includes optimizing labor and resources, maintaining rigorous quality control, and ensuring all plants are uniform in size and quality at shipment time. Market preferences highlight the necessity of aligning production strategies with consumer demands and economic viability. Growing trend crops, managing costs effectively in both tissue culture and greenhouse environments, and maintaining high standards in packaging and plant material quality are crucial. Countries with lower labor costs often provide cheaper *in vitro* plants, necessitating a focus on quality to remain competitive. Operational risks, such as labor availability and energy price

fluctuations, are mitigated through skilled workforce management, regular training, and optimized workflow schedules. Implementing automation technologies and maintaining a supportive work environment further enhance productivity and reduce risks. In conclusion, the strategic plan for industrial *in vitro* plant production integrates advanced tissue culture techniques, optimized resource use, and market-aligned production strategies. This approach ensures high-quality, sustainable, and profitable plant cloning operations, meeting both current market demands and future growth potential. The comprehensive management of technological, market, and operational risks solidifies this model as a robust framework for the *in vitro* plant production industry.

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# Micropropagation of *Phalaenopsis* using temporary immersion system

**Bora Onur Hallaç[1], Yeşim Yalçın Mendi[2], Soner Yağ[3]**

*[1,3] FSB Biotech Company, Adana, Türkiye*

*[2] Department of Horticulture, Faculty of Agriculture, University of Çukurova, Adana, Türkiye*

bora@fsbbiotech.com

## Abstract

Phalaenopsis orchids are the most popular among indoor potted and flowering ornamental plants. While the annual consumption amount in Turkey is 6,000,000 units, this figure reaches hundreds of millions of units in the world. Although there are serious processes at every stage of orchid production, the healthy supply of seedling material is of great importance. Phalaenopsis seeds, although quite small, require artificial medium for germination since they do not contain endosperm. The production of this plant, whose vegetative propagation in nature is not commercially efficient, is done using tissue culture techniques. In generative production methods, since the plants do not have clone characteristics, production is achieved vegetatively, not by seeds, but by using various growth regulators in artificial media of structures taken from various organs of the plant. Direct organogenesis and somatic embryogenesis methods are used in commercial production. In the project, somatic embryogenesis was used as the method. The flower stems of the plants were sterilized with chemicals such as sodium hypochlorite (0.5% - 1.5%) and mercuric chloride (0.05% - 0.1%) and then transferred to the in-vitro environment. After the first shoots were obtained, PLB was obtained by applying thidiazuron at concentrations between (1mg - 4mg). Media such as Vacin&Went, Hyponex, ½ MS were used in the preparation of these media. The resulting PLBs were replicated in the next step. Replicated PLB structures; They were transferred to media containing activated carbon and grown in clusters. Plants that reached a certain maturity were sorted and transferred to rooting media. Rooted plants were removed from the medium and transferred to quick-plug growing media in order to adapt to external conditions. Here, the temperature was gradually increased to 28 °C and the light intensity to 5000 lux. Humidity was gradually reduced to 85%. Protective practices have been carried out against bacteria, fungi, insects and mites. The resulting plants were defined as seedlings ready to be potted. Semi-solid culture methods are generally used throughout the world in the commercial production of Phalaenopsis orchids. Both semi-solid culture and bioreactors with temporary immersion systems were used in the project. The bioreactors used were designed by FSB Biotechnology. Comparisons made at all stages showed that bioreactors have superior qualities compared to semi-solid culture media in parameters such as tillering rate, growth rate and plant quality.

***Keywords:*** *Phalaenopsis, micropropagation, ornamental*

## 1. INTRODUCTION

Phalaenopsis (moth orchids) is one of the most popular among orchid species because of their specially beautiful and long-lasting flowers, and can cultivate quite easily in the

artificial conditions. As a monopodial plant Phalaenopsis are traditionally propagated by the cutting or division of off-shoots, however, these methods results low multiplication rate and hamper the growth of the mother plant, making them ineffective for large scale production. Therefore, tissue culture may be an efficient and alternative tool for propagation of this orchid species (Kosir et al., 2004).

The general aim of this study is to use higher quality and more efficient systems in Phalaenopsis propagation. It was aimed to overcome many key problems caused by the traditional solid system, such as high labour, phenolic problems, lower proliferation efficiency, difficult acclimatization process, low waiting times of plants in jars, and energy inefficiency by using the bioreactor system. The problems in solid culture will be explained and compare with bioreactor systems as follows:

- Plants grown in solid culture are cut one by one by workers in biosafety cabinets and are carefully planted in agar or gelrite medium. Since each plant requires separate media for induction, propagation (many times with subculture) and rooting, each media change means great labor in solid culture. Just because of this situation, European manufacturers have shifted their production to countries where labour expenses is low. The workmanship here is very important and abundant.
- In solid culture, plants use sucrose in the medium as a carbon source rather than carbon dioxide in the air. This removes the normal metabolism of plants from the process in their natural environment. After a while, plants produce chemicals called phenolics due to this situation, and this gradually poisons the nutrient environment. Although there are different ways to escape from this situation in solid culture such as using activated charcoal, all solution suggestions are temporary and far from the real solution.
- Solid cultures provide less tillering in a longer time compared to the bioreactor.
- Since plants cannot come into contact with the outdoor air, the adaptation process of plants that have completed the laboratory process to the outdoor environment is more difficult and painful.
- Placing fewer plants per unit area, less tillering, and the length of the process also mean a serious energy consumption. After all, plant rooms are places that are air-conditioned and illuminated 24 hours a day.
- In times of personnel shortage or workload, or when orders must be kept on hold, plant waiting time in solid culture is limited.

To solve these problems, it is aimed to achieve quality and cost optimization by using a temporary immersion system instead of classical semi-solid culture. For this purpose, the bioreactor system we developed within the company was used. The designed bioreactor system is similar to RITA bioreactors. With the effect of the clean air passing through the filter in the lower chamber, the ambient liquid rises up and comes into contact with the plants, and after 5 minutes of immersion, it returns to its place with the effect of gravity. While this process is taking place, clean air is provided through the filter on the top cover.

## 2. MATERIAL AND METHODS

### 2.1. Material

Phalaenopsis amabilis, V3 and purple hase varieties were used as plant materials in this study. The experiments carried out were determined as sterilization and shoot acquisition, somatic embryo extraction, tillering of the obtained somatic embryos, growth stage, rooting stage and acclimatization.

### 2.2. Method

#### 2.2.1. Sterilization and induction stage

In the first stage of the process was carried out on sterilizing the material and placing in the culture medium. It was aimed to obtain a sterile plant material through sterilization and induction. All tissue culture studies were carried out with sterile plants and the resulting healty seedling material without viruses. In this respect, all plant tissue culture studies started with sterilization and all processes were carried out in a sterile condition (Park et al., 2002, 2004). Sterile plants were also the starting materials for other work stages, and contaminated tissues were eliminated. Different tissues such as leaf, root, stem and flower stalk were used as starting plant tissue for Phalaenopsis orchids.

In this study, flower stalks were used because it reacted faster, its sterilization was relatively easier than other plant tissues, and its bacterial load was even lower. The fastest proliferation cycle of bacteria within the plant occured through transmission bundles. Therefore, the buds on flower stalks that are smaller in diameter were used as the source of explant. One week before culture, the plants were treated with 20 mg/l dosage of streptomycin sulfate, 20mg/l gentamicin sulfate and 10 mg/l tetracycline hydrochloride in the greenhouse. In this way, it was aimed to reduce the total bacterial load systemically. The flower stems of the plants taken from the greenhouse were cut by leaving one bud at each node. After the cut materials were thoroughly washed, they kept in antifungal solutions, placed in antibiotic-containing solutions, and then sterilized with various chemicals, as stated in the attached trial groups. After the sterilization solution was poured into the cabinet, it was rinsed five times with autoclaved pure water and if there were damaged structures, stemming and planting were done. Culture conditions were 27 $^{o}$C, 2500 lux light intensity and 16/8 photoperiod.

Although bioreactors and liquid culture media were used as media throughout our study, solid culture experiments were also carried out in test tubes for the initial culture and induction of plants. In this way, it was possible to work with a larger number of materials. The sterilization ratio in this study was 10%. At least 100 sterile explants were required to continue other stages. For this reason, 900 entries were made in solid culture and 100 in liquid culture (as many as the number of bioreactors). In liquid culture, 200 ml/piece and solid culture will be 50 ml/piece, one tube/bioreactor for each plant.

### 2.2.2. Obtaining protocorm from various organs of plants

In commercial propagation of Phalaenopsis varieties by tissue culture, direct organogenesis or somatic embryogenesis is used as the propagation method. In somatic embryogenesis, embryos are obtained vegetatively from various parts of plants. In special media, the plant is encouraged to form embryos under the influence of various hormones. This method, which is widely used in tissue culture applications, is a common method used especially in plants such as orchids, which take a long time to tiller and have relatively less tillering compared to other plants (Kosir et al., 2004). Another advantage of this method is that it does not require leaf cutting for grading purposes before placing it in the rooting medium. In this way, it eliminates the need to cut for grading the Phalenopsis plant, which has a longer rooting process than many other plants. However, its disadvantage is that the rooting process is longer and it requires changing the medium one more time during the rooting period. When the studies in the literature are examined, it is seen that the bioreactor increases the proliferation frequency of embryos (Barough et al., 2024). On the other hand, It greatly simplifies the necessary media change by adding a third discharge valve and filter to the bioreactor, for the rooting stage and does not require taking it back into the cabinet and changing the environment. When these advantages are considered, the propagation method that will work most compatible with the bioreactor system will be somatic embryogenesis. Somatic embryogenesis method was used in our studies. The sterile shoot formed in the buds on the flower stem as a result of induction was divided into three parts as stem, leaf and root in the cabin. Leaves and roots were cut into thin sections between 0.5-1mm. There was some waiting for the cut surfaces of the cut structures to dry. In the next step, they were kept in a liquid condition containing ½ MS medium for about 2 hours, and then the trial groups were taken to the previously created bioreactors. The plants first left in the dark for about a week, then incubated at 25 $^{o}$C, 2500 lux light intensity, with a 16/8 photoperiod. The culture condition was refreshed every 15 days. Somatic embryos were formed within 2-3 months and the outputs created here were the input products of the next work package. The trial groups created at this stage were available in this study.

### 2.2.3. Reproduction of PLB structures

The protocorms created from roots, stems and leaves in the previous work stage were separated from the main structures that form them in the bioreactors system. Root and leaf extensions were cleaned and the leaves of the created protocorms exceeding one centimeter were cut off, leaving only the stems. Protocorms were propagated by tillering according to the principle of forming protocorms from the stem; accordingly, the leaves and stems that cut during this process were not used and thrown away. In this process, since the possibility of vitrification in the liquid culture medium was higher than in other processes, it was necessary to increase the amount of sucrose and, if necessary, add heavy metal ions due to their growth regulatory effects (Julkifle et al., 2010).

### 2.2.4. Growth of PLB structures

In orchid propagation method with somatic embryos, generally, after the desired protocorms were propagated in sufficient quantities, the desired amount was cut and separated in the cabin and taken for rooting. After three months, it was necessary to renew the nutrient content in the rooting medium (Huaiyiu, 2011). Our experiences with solid culture showed that there was a waste in the production made in this way and the desired rooting quantity could not be achieved. Since the protocorms were very small, their stems could be damaged during the cutting process and rooting medium in clusters without cutting. When the materials were transferred to the new rooting medium, the plants were classified that have reached 2-3 cm in length and the roots. More homogeneous structures emerged in terms of the number and length of leaves. This had become an innovation that is more satisfied by ornamental buyers from whom we supply seedlings.

At this point, similar application was carried out in bioreactor experiment. Because the mixtures in the grading becomes difficult, the protocorms were transferred to the bioreactors without being separated in clusters.

### 2.2.5. Rooting of plants in *in-vitro* environment

The plants were cut in the cabin and completely separated from their siblings. Plants that were less than 2.5 centimeters tall and had no more than three leaves were grouped and separated. These plants were transferred back to the growth medium so that they could develop further.

Plants with over 2.5 cm were transferred to the rooting medium in order to ensure their rooting at the same time. A bioreactor setup containing 200 ml of rooting nutrient medium for 15 plants were used for this process.

### 2.2.6. Acclimatization and obtaining seedlings for potting

The amount of light in the area where the plants were located was adjusted to 2000 lux for the first 5 days, and then to 3000 lux. The greenhouse temperature was 28 $^{o}$C which is the key information for the vegetative development of orchid plants. Humidity was adjusted to 90% for the first 3 days, then 75%. It was essential that the greenhouse had an external aluminum curtain. Because the only material that reflects the heating radiation effect of the sun was aluminum. Thus, the invisible damage caused by sunlight to plant cells was prevented. If these conditions were not met, orchids lost strength in the short term and then became extremely vulnerable to bacteria such as *Erwinia* and fungi such as fusarium, and often the plants were lost.

The air was constantly sterilized in the area where the plants were located with the help of a device containing H14 HEPA filters and ensured that the air in the plant area was constantly clean. After root development was completed, the plants were ready for planting in pots.

## 3. RESULTS AND DISCUSSION

### 3.1. Sterilization and induction phase

Regarding sterilization protocols with 0.05% mercury (II) chloride application for half an hour; it gave 42% sterilization result. When the inductions created were evaluated, the most successful induction medium was the medium, which was the medium in which 10 mg/l TDZ, 0.5 mg/l NAA and 20 g sucrose were solidified with 2 g/l gelrite in addition to ½ MS medium, with an induction rate of 92.31%. It gave the most successful and healthy result. It was observed that the induction rate increased with the increase in the amount of TDZ, but decreased with the increase in the sucrose concentration. In addition to the trial groups, another group with the same characteristics as the I16 medium but with a TDZ concentration of 15 mg/l was created. Induction was observed in all plants in this condition. Images of the results obtained are as follows.

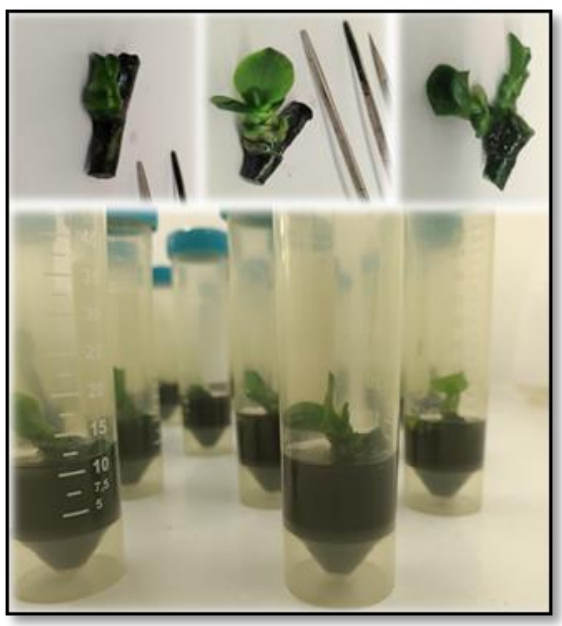

**Figure 1.** Initition stages of flower stalks

### 3.2. Obtaining protocorm from various organs of plants

The shoots obtained from the flower stem were transferred to sterile paper. Leaves and roots between 0.5 and 1 mm were cut into thin strips. Root and leaf pieces were immersed in MS solution, which added 2200 mg per liter and were left in this solution for 2 hours. By this way, nutritional uptake was ensured. All plants were left in the dark condition for a week. The formation of excessive amounts of phenolic exudates was prevented. After one week, incubation was provided at 25 $^{o}$C and 2500 lux light intensity. The medium was changed every 15 days. No contamination was observed in micro- leaves obtained by meristem culture. In other explant sources, 55% endogenous

bacterial contamination occurred. Fungal contamination occurred at a rate of 3%. Considering that the fungal contaminations was eliminated in the previous stage; it was concluded that it was caused by workers. Bacterial contaminations was observed to be of plant origin. Confirming the output of the previous work stage, the superiority of meristem culture in eliminating bacterial pathogens came to the fore. It was decided to use meristem culture in all phalaenopsis production stages from now on. In the relevant period, protocorm-like structures (PLB) began to be observed at the end of the 8th week. The sizes of PLBs formed in different plant genotypes in the same protocols were observed to be in the range of 1-8 mm. This showed that genotype was one of the most important factors affecting PLB size. On the other hand, it had been observed that the formation of phenolic substances was much more intense, especially in varieties with purple flowers. Among the experimental groups, the most successful medium showed 100% protocorm formation in all roots. In some medium, 70% protocorm formation in leaf explants were observed. The average number of protocorms formed from leaves was 3.1 per explant, and the number of protocorms formed from roots was found to be 1.1 per explant. Although the protocorms formed in the leaf were more in number, the protocorms obtained from the root were found to be more suitable for propagation in terms of shape, hardness and quality. Images of the results obtained are as follows.

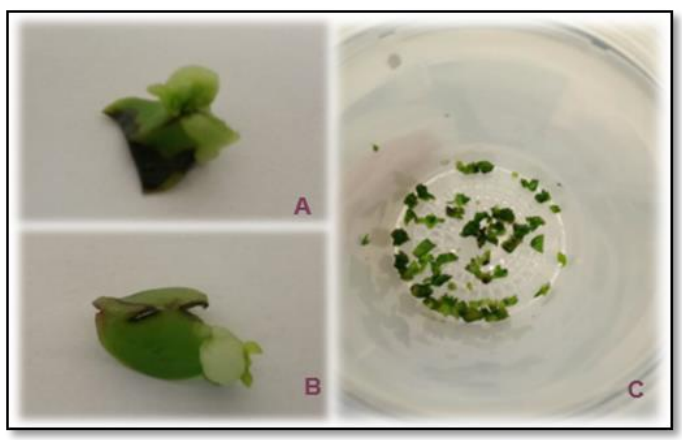


**Figure 2.** Protocorm formed at the leaf tip (A), protocorm formed at the leaf cut side (B), protocorm formed in the bioreactor (C)

### 3.3. Reproduction of PLB structure

In this stage, it was aimed to reproduce the tissue pieces included in the previous work stage using tissue culture methods. For bioreactors; the media was prepared by mixing the relevant components without the need for heating. After similar procedures were carried out in solid culture media, agar was added and the nutrient mixture was boiled at 100 $^{o}$C for 2 minutes to dissolve the agar material. It had been observed that the nutrients poured into glass jars solidify within an hour. The created bioreactors and glass jars were

sterilized in an autoclave at 121 ºC and under 15 PSI pressure for 15 minutes. Planting media were transferred to laminar flow sterile cabinets.

The surroundings of all jars transferred into the cabin were wiped with a mixture containing 70% ethanol and their outer walls were cleaned. The medium in which the plantings will be made consists of papers sterilized by autoclaving in aluminum foil. The scalpel and forceps were sterilized by heat treatment for 5 seconds using a glass bead sterilizer at each plant material change. The plants obtained in the previous work stage were taken into the cabin, again by applying ethanol to the outer surfaces and the covers were carefully opened.

Plants were obtained from the organs where protocorms were formed by cutting them with a scalpel. Somatic embryos were transferred to the media containing the relevant experimental groups and cultured. The use of 20 mg/l Zeatine increased the tillering rate and high sucrose rates were observed to have negative effects on tillering. The best result was found with the use of 20 mg/l Zeatin and 20 g/l sucrose, obtaining 3.10 plants per explant. The images below show photos of the results obtained.

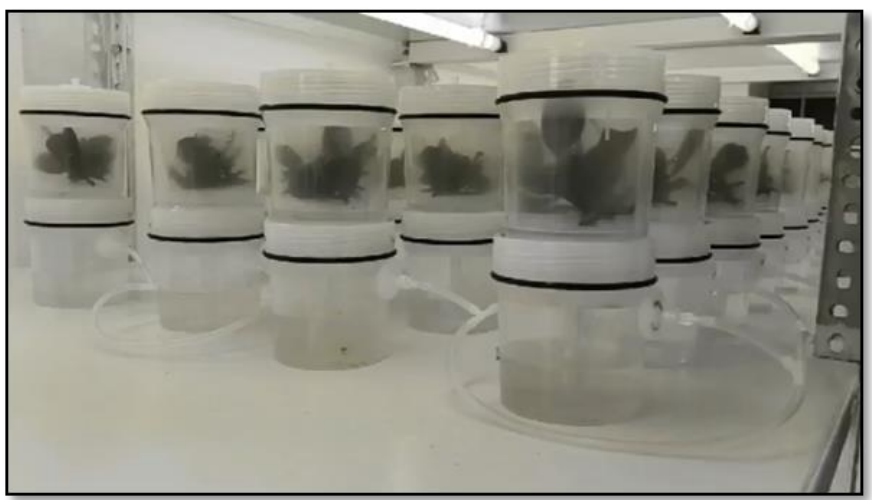

**Figure 3.** Plants in bioreactor system

**Figure 4.** Phalaenopsis plant clumps in bioreactors

### 3.4. Enlargement of PLB structures

The media containing agar were heated to 100 degrees Celsius and boiled, and the medium was poured into jars. For bioreactors, no heating process was performed, and the relevant chemicals were dissolved by mixing. All media were sterilized at 121 $^{o}$C Celsius and 15 PSI pressure for 15 minutes and cooled. The external surfaces of the jars and bioreactors containing the nutrients transferred to the vertical flow cabinets and the plants obtained in the previous work package were cleaned by applying a mixture containing 70% ethanol before being taken into the cabinet. All tools used were sterilized by autoclaving, and the scalpels and forceps with which the planting was carried out were sterilized for each plant material with a quartz sterilizer.

The protocorms taken on sterile papers were cleaned with a scalpel from the necrotic tissues around them, without separating them from their siblings. Protocorms in clusters, which were thoroughly cleaned were transferred to the bioreactor and solid culture media. Although the formation of phenolic substances is widely observed in orchids, the formation of phenolic substances increases especially in cutting areas. And again, especially small-sized plants are more intensively affected by phenolic substances. In order to eliminate this negativity caused by the process, protocorm structures were not completely divided into their siblings, but large plants were divided again in a sisterly manner. Again, during this process, care was taken to make as much grading as possible. In tissue culture applications, even though the plants are clones, equal height formation is not observed due to reasons such as the percentages of utilization of the medium, pH changes and the times when they start tillering are not the same. This situation was overcome by using techniques such as grouping plants with similar sizes and cutting the long leaves of plants with very tall leaves. In this study, grading operations were carried out in this stage and they were divided at the first planting, leaving plants with an average of 1-3 tillers. In order to carry out the slaughtering and separation processes more efficiently in the following stages, the division process was left to the next stage. The longest plant height was obtained as 4 cm in the medium containing 0.75 g/l activated charcoal and 40 g/l sucrose.

### 3.5. Rooting of plants in *in-vitro* environment

In the growth medium, protocorm clusters were brought to a length of at least 1 cm. Their connections with the trunk structure were cut and their shoots were cut. After the cut shoots were thoroughly cleaned from phenolic residues, they were transferred to bioreactors and solid culture media. In order to prevent contamination that may occur during the process, the edges of the container were exposed to the burner flame. Plants cultured in this way have created the final rooting condition. The plants that emerge from here will be ready to be transferred directly to the greenhouse. The best results were obtained with a 100% rooting rate in media containing 20 g/l sucrose and 0.25 g/l activated charcoal. A similar ratio was obtained in the medium containing 25 g/l sucrose

and 0.25 g/l activated charcoal. It was observed that the rooting rate decreased as the amount of activated charcoal increased. Images of the results obtained are shown below.

**Figure 5.** Rooting stages of plants

**Figure 6.** Seperating of clumps to the single plants and rooting under *invitro* condition (TIS)

### 3.6. Acclimatization

Plants were monitored daily and checked for light intensities, temperature, wetness of the plug environment, humidity, diseases and pests. Acclimatization phase; it was of critical importance for tissue culture applications. In the *in-vitro* condition, stable access to all micro and macro elements and carbon resources needed by the plant was provided and the temperature and humidity requirements were met uninterruptedly. On the other hand, because plants and their nutrient media were sterile; they continued their lives protected from pests such as bacteria, fungi, insects and mites. When plants were transferred from such a sheltered condition to the condition where climate parameters were less stable and where they were in contact with disease agents, serious problems occured. On the other hand, plants that used sucrose and other chemical and organic resources as carbon sources throughout their development could not adapt quickly enough to reuse carbon dioxide in the

external environment. Especially, failure to adjust the humidity parameter correctly caused fungal diseases to occur and spread in high humidity conditions; In low humidity conditions, it could cause the plant to lose water and die. When we think of these aspects, acclimatization was considered one of the most difficult stages of tissue culture applications. This situation creates greater problems in Phalaenopsis orchids than in other plants. In Phalaenopsis plants, plants with broad leaves and large stems must be created *in vitro*. The disadvantage of this is that the leaf sections are large. Although it is a factor that increases the rate of photosynthesis, in plants with large leaf sections, plants lose moisture very quickly and die. On the other hand, phalaenopsis orchids are extremely sensitive to solar radiation. Therefore, acclimatization can only be carried out in high-tech greenhouses with aluminum curtains and climate automation.

The acclimatization period of plants takes an average of 120 days. In this process, certain practices must be carried out to keep the plants alive, to ensure that they develop the necessary root structure, to prevent them from succumbing to diseases and pests, and to acclimate them to natural climatic conditions. These applications are summarized in the table 1.

**Table 1**. The cultural conditions in greenhouse in 4 months period

| **Implementation** | **I.Month** | **II.Month** | **III.Month** | **IV.Month** |
|---|---|---|---|---|
| Light | 1000 lux | 1000 lux | 2000 lux | 3000 lux |
| Humidity | %95-%85 | %85-%75 | %75-%70 | %75-%70 |
| Temperature | 25 °C | 27 °C | 28 °C | 28 °C |
| EC | - | 0.5 mS/cm | 0.5 mS/cm | 0.8 mS/cm |
| pH | 5.6 | 5.6 | 5.6 | 5.6 |
| Fosetyl- Al | 10 mg/l-every week | 10 mg/l-every week | - | - |
| Deltamethirin | - | - | 20 mg/l | 50 mg/l |
| Diflubenzuron | - | - | 25 mg/l | 70 mg/l |
| Bifenazate | - | - | 15 mg/l | 15 mg/l |

A total of 400 plants were planted and the numbers and factors related to plant losses of these planted plants were classified morphologically and stated in the table 2.

**Table 2.** The reasons of plant loss in 4 months

| **The reason of plant loss** | **I.Month** | **II.Month** | **III.Month** | **IV.Month** |
|---|---|---|---|---|
| Low humidity | 11 | 3 | - | - |
| Bacteria | 2 | 1 | - | - |
| Fungus | 5 | 1 | - | - |

In total, 14 plants lost water due to low humidity conditions, 3 plants were lost due to bacterial disease (*Erwinia amylovora*) and 6 plants were lost due to fusarium. As a result of acclimatization, 377 plants were successfully turned into seedlings. 94.25% chance of success was achieved. Losses of up to 10% are tolerable in commercial seedling production. The total wastage rate at the end of this study was only 5.75%.

**Figure 7.** Acclimatization of rooted plantlets

## 4. CONCLUSION

*In vitro* propagation of Phalaenopsis orchids offers a powerful and efficient method for the large-scale production of these popular and commercially valuable plants. The technique's ability to produce disease-free, genetically uniform plants year-round makes it an indispensable tool in both commercial horticulture and conservation efforts. Additionally, it supports the rapid multiplication of plant stocks, contributing to the sustainability and availability of Phalaenopsis orchids. However, the process is not without its challenges. The need for stringent aseptic conditions, the cost of setting up and maintaining the necessary facilities, and the requirement for technical expertise can pose significant hurdles. Moreover, the transition of plants from *in vitro* to ex vitro conditions demands careful management to ensure survival and healthy growth. Balancing the benefits and challenges, *in vitro* propagation remains a highly advantageous technique for the cultivation of Phalaenopsis orchids. Its successful application can lead to substantial gains in orchid production and conservation, making it a valuable practice for orchid enthusiasts, commercial growers, and conservationists alike.

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# *WORKING GROUP 5:*

# Communication, dissemination, and technology transfer

# PInK-NET: Resuming the network idea of *in vitro* tree labs in Germany

**Andrea Rupps[1], Franka Thiesen[3], Juliane Raschke[1], Madlen Walther[1], Emma Ehmke[1], Lena Safranek[2], Julia Eckard[2], Antje Schmidt[2], Winston Beck[2], Ben Bubner[3], Anne-Mareen E. Eisold[3], Virginia Zahn[4], Alexander Fendel[4], Tobias Bruegmann[4]**

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

*[2]Dept. of Urban Plant Ecophysiology, Thaer-Institute of Agricultural and Horticultural Sciences, Humboldt- Universität zu Berlin, Lentzeallee 55/57, 14195 Berlin, Germany*

*[3]Thünen Institute of Forest Genetics, Groups of Provenance & Breeding Research and Pathogen Resistance Research, Eberswalder Chaussee 3a, 15377 Waldsieversdorf, Germany*

*[4]Thünen Institute of Forest Genetics, Group Genetic Technologies, Sieker Landstrasse 2, 22927 Grosshansdorf, Germany*

*andrea.rupps@hu-berlin.de*

## Abstract

Research groups with focus on *in vitro* culture and clonal tree propagation are scarce in Germany. The ADIVK (Association for German *in vitro* cultures) acted as the only existing German *in vitro* research association from 2005-2023. Following the end of ADIVK, there was an urgent need for a collaboration of research groups in Germany focussed on *in vitro* culture of woody plants. In summer 2023 three institutes from Berlin/Brandenburg (Institute of Biology and Thaer Institute, Humboldt- Universität zu Berlin; Thünen Institute of Forest Genetics, Waldsieversdorf and Grosshansdorf) founded a new national cooperation combining knowledge, expertise, and researchers focused on *in vitro* culture of woody plants: the PInK-net (plant *in vitro* culture network). Beyond continuous material and methodological exchange, the aims are mutual support in scientific and administrative procedures, submission of common project proposals, and troubleshooting at specific cultivation steps. These comprise different multiplication techniques, like somatic embryogenesis as well as micro cuttings for various conifers and deciduous trees, the establishment of *in vitro* cultures using different explants, handling of subsequent culture and storage, solving contamination problems and finally stabilisation of further development up to acclimatisation of young trees. We are committed to sharing details about the application of stress tests, analysis of pathogen infestation and molecular processes. Another common objective is the involvement and information of public and private stakeholder groups at an early entry point. By this, we hope that clonal reproduction will become socially accepted and economically affordable for nurseries and forestry in our country in the foreseeable future.

***Keywords:*** *PInK-net, national scientific association, vegetative propagation of conifers and deciduous trees, in vitro techniques*

## 1. FOUNDING OF THE PINK NETWORK

From 2005 to 2023, the Association of German *In vitro* Cultures (ADIVK) as sub-group of the central association for horticulture in Germany served as a platform for connecting researchers and industry professionals involved in plant tissue culture and *in vitro* propagation. Unfortunately, the ADIVK had to break off due to personal and formal reasons.

To continue scientific cooperation and exchange in Germany on multiplication and modification of trees in *in vitro* culture, scientists out of four research groups from two German institutions (Humboldt-Universität zu Berlin: Department of Plant Evolution and Biodiversity and Department of Urban Plant Ecophysiology; Johann Heinrich von Thünen Institute: Groups of Provenance & Breeding Research and Pathogen Resistance Research and Group Genetic Technologies) agreed to join their expertise and facilities. By bringing together experts from different backgrounds and disciplines working with various tree species, the association also facilitates interdisciplinary research and development initiatives that address key challenges in plant propagation, conservation, biotechnology, and genetic improvement.

It was already at the first meeting, when the demand and advantages of working in a cooperation became obvious and the network PInK-net ("Netzwerk für pflanzliche In-vitro-Kultur") was founded as a German association of research institutions for plant *in vitro* cultures with focus on woody plants. Fig. 1 was taken at the founding day (30.08.2023) at the Späth Arboretum, Berlin.


**Figure 1.** Founding member assembly at the initial PiNK-net meeting in June 2023 at Späth-Arboretum, Berlin. From left to right: Antje Schüttig, Franziska Past, Anne-Mareen Eisold, Julia Eckardt, Lena Safranek, Titus Hinze, Juliane Raschke, Kurt Zoglauer, Franka Thiesen, Andrea Rupps, Emma Ehmke

As primary tasks, the network operates as collegial support group at different levels: the transfer of material, methods, specific details thereupon as well as researchers themselves, troubleshooting assistance at specific steps of the *in vitro* culture process, sample preparation as well as analytical steps. Administrative questions may be tackled in common and the process of project acquisition could be eased by collaborative approaches. Accordingly, the network may provide significant contributions to the field of plant *in vitro* culture, including the development of new tissue culture protocols, improved propagation techniques, and the introduction of novel genetic technologies fortree breeding and conservation.

Additionally, the network aims to engage in public relations activities to promote the importance of *in vitro* culture research, upcoming challenges in forestry, and the advantages of clonal reproduction for science, forestry, and today's and future demands.

## 2. PINK-NET MEMBERS

### 2.1. Humboldt-Universität zu Berlin (two institutes: Inst. of Biology and Thaer Inst.)

#### 2.1.1. Dept. of Plant Evolution and Biodiversity, Späth-Arboretum, Inst. of Biology

The focus of the team "Plant Development" of the Group Plant Evolution and Biodiversity lies on the initiation, analysis, and utilization of somatic embryogenesis in various conifer species. The group was originally established in the 1980s and led by Prof. Kurt Zoglauer until his retirement. Since 2020, the expertise of the research team has been continued with a new focus. Meanwhile, the group built up high experience with the system of somatic embryogenesis in conifers (Zoglauer et al., 2002; Rupps et al., 2015; Walther et al., 2021; Hassani et al., 2022). A number of conifer species have been intensively studied in various projects including European larch (*Larix decidua*), hybrid larch (*L.* × *eurolepis*), Douglas fir (*Pseudotsuga menziesii*), maritime pine (*Pinus pinaster*) and various fir species and hybrids: *Abies alba, A. nordmanniana, A. bornmuelleriana, A. procera, A. lasiocarpa, A. alba* × *A. nordmanniana, A. concolor* × *A. grandis*. A number of genotypes of most of these species are in cryopreservation - more than 600 genotypes of Nordmann fir, solely. For *L. decidua* and *L.* × *eurolepis*, the system of somatic embryogenesis has been highly advanced and is about to be transferred into practise (Zoglauer et al., 2002; Kraft & Kadolsky, 2018; Benneckenstein & Tietje, 2018) in tight cooperation with plant nurseries (see article on the "Joint Project OPAL" in this issue).

The research questions are currently centred about (i) how and why already differentiated cells can return to the embryogenic state, if somatic embryos differ from their zygotic counterparts in any regard, (ii) how genotypes differ in terms of their developmental behaviour and (iii) whether genetic or epigenetic markers can be identified thereof. In parallel, different aspects with practical importance are addressed, in particular, which culture steps influence or maintain the embryogenic potential and how the process chain can effectively be transferred to nurseries.

**Figure 2.** Team ”Plant Development“ (from left to right):

Juliane Raschke, Idris and Jana Seifert, Kurt Zoglauer, Emma Ehmke, Johannes Gürth, Madlen Walther, Tim Kortekamp, Anika Dreilich, Andrea Rupps.

### 2.1.2. Dept. Urban Plant Ecophysiology, Thaer-Institute of Agricultural and Horticultural Sciences

The research group has 15 years of experience in *in vitro* propagation, set up by a team of four main scientists with different research foci (see projects below). Our common topic lies in refining micro cutting methods, which are crucial for plant cloning and propagation. The team established advanced *in vitro* techniques to address various environmental stresses, including drought stress, salt stress, and late frosts. These methods are essential for improving plant resilience and ensuring sustainable growth under adverse conditions. Furthermore, the group aims to apply biomarker analyses to better understand plant stress responses and adaptation mechanisms. Recently, the team has successfully established somatic embryogenesis for common ash (*Fraxinus excelsior*), marking a significant milestone in regenerative plant research.

Currently, the team cultures a diverse range of genera, including *Acer, Betula, Fraxinus, Hippophae, Juglans, Tilia, Quercus, Yucca, Rosa,* and *Cannabis*.

To address this, three nurseries, two research institutions, and municipalities collaborated to produce climate-adapted tree assortments for Berlin-Brandenburg, focusing on own-root and stress-tolerant street trees. They developed screening and certification methods for these plants. The project demonstrated that stress-tolerant

woody plants can be selected and grown *in vitro* more quickly. From 14 genera sampled, 40 clones were established *in vitro* and protocols were adapted for 21 clones. Modified media tailored to genotypes were key. Stress tests for drought, excess nitrogen, heat, frost, and de-icing salts were conducted *in vitro* on 9 clones. *In vivo* drought stress tests on young potted plants were more revealing, showing clear distinctions in drought tolerance among clones of *Tilia*, *Acer*, and *Platanus* based on biomarkers, shoot-root development, and budding behavior. For instance, a Dutch lime clone showed high drought tolerance. Sufficient plants were produced *in vitro* and provided to nurseries for young plant production. These potentially drought-tolerant, own-root plants will be planted in demonstration areas for evaluation and marketing. The new rapid *in vitro* test procedure can shorten the selection and production period by at least two years, reducing costs.

**Figure 3.** Main researchers at the Dept. Urban Plant Ecophysiology and their current projects: Winston Beck (ESPRITNUSS), Lena Safranek (FraxForFuture), Julia Eckardt and Antje Schüttig (Trees4Streets).

Project FraxForFuture: As part of a nationwide project, strategies for conserving the common ash (*Fraxinus excelsior*) against ash dieback (caused by the fungal pathogen *Hymenoscyphus fraxineus*) are being developed. This disease deteriorates wood quality, kills trees, and increases safety costs, causing financial losses for forestry businesses. *In vitro* culture is key, creating a clone bank of resistant, tolerant, and susceptible genotypes using seed germination, meristem multiplication, and somatic embryogenesis. Seed germination *in vitro* involves sterilizing seeds, dissecting embryos, and germinating them on a phytohormone medium. Meristem propagation uses meristems from buds as explants for further micro-cutting propagation. Somatic embryogenesis prepares zygotic embryos with growth regulators to form identical embryos, which are then germinated. The resulting plant material is used for further research and can be planted after clone testing.

Project ESPRITNUSS: The project aims to further develop and apply propagation methods from the previous SUPERHYBRID project for hybrid trees. Hybrid *J.* ×

*intermedia* trees are being created for the German market with seed orchards for controlled hybridization and uniform *Juglans* hybrid seed production. The project also aims to produce triploid multiple hybrids, including "super hybrids", and to propagate selected genotypes *in vitro* and through other vegetative methods like stooling and layering. Trial plots with 33 different genotypes were planted in Mecklenburg-Western Pomerania andRhineland-Palatinate, both areas in Germany, to test their suitability. The project records morphological, phenological, and vitality characteristics and evaluates site suitability in north-eastern and south-western Germany. Selected genotypes will be introduced in "SilvaSELECT" based on these results. So far, selected plus trees grafted on *J.* × *intermedia* rootstocks have shown strong vigor and vitality, but ongoing compatibility tests are needed for conclusive silvicultural recommendations.

## 2.2. Thünen Institute of Forest Genetics (two locations: Waldsieversdorf & Grosshansdorf)

The Johann Heinrich von Thünen Institute – Thünen Institute in brief – is a federal research institute at the interface of science, politics and society. At the Institute of Forest Genetics, three research groups work on various topics related to forest genetic and have been using tissue culture as a fundamental approach since decades. Currently, the tissue culture collection includes 14 tree genera (Table 1), which are used for several purposes.

**Table 1.** List of species available in the *in vitro* collection and their purpose

| # | Genera | | Purpose |
|---|---|---|---|
| **1** | *Ailanthus* | Tree of heaven | Genetic transformation and genome editing |
| **2** | *Acer* | Maple | Conservation of high value genotypes |
| **3** | *Alnus* | Alder | Infection studies |
| **4** | *Betula* | Birch | Conservation of high value genotypes |
| **5** | *Fagus* | Beech | Methodological improvement of recalcitrant species, genetic transformation, genome editing |
| **6** | *Fraxinus* | Ash | Infection studies, genetic transformation, genome editing |
| **7** | *Larix* | Larch | Conservation of genotypes |
| **8** | *Picea* | Spruce | Conservation of genotypes |
| **9** | *Populus* | Poplar | High mass production, stress experiments, genetic transformation, genome editing |
| **10** | *Quercus* | Oak | Methodological improvement, interaction studies |
| **11** | *Robinia* | Robinia | Conservation of genotypes |
| **12** | *Salix* | Willow | Conservation of high value genotypes |
| **13** | *Taxus* | Yew | Conservation of genotypes |
| **14** | *Ulmus* | Elm | Conservation of rare species |

Tissue culture techniques play a crucial role as tools for breeding and conservation purposes. Since trees grow slowly and have long generation cycles, biotechnological methods on woody plants are particularly helpful and accelerate breeding processes.


**Figure 4.** Members of the Groups Provenance & Breeding Research and Pathogen Resistance Research (A) – Anne-Mareen E. Eisold, Ben Bubner, Franka Thiesen; members of the group Genetic Technologies (B) – Alexander Fendel, Virginia Zahn, Susanne Jelkmann, Alice-Jeannine Sievers, Tobias Bruegmann

### 2.2.1. Groups Provenance & Breeding Research and Pathogen Resistance Research

Tree breeding research concerns the optimization of biomass and valuable/high-end wood production in several tree species by conducting provenance trials with a variety of tree species with high economic impact. To improve the resilience and resistance of this tree species against biotic and abiotic detrimental factors, pathogen resistance research investigates the impact of pathogens as well as the stability and genetic structure of forest tree populations.

Therefore, diverse methodological experiences are required, including (i) establishment of *in vitro* cultures from buds, seeds, shoot tips (Fig. 5), (ii) mass propagation, (iii) long-term conservation, (iv) *in vitro* resistance tests, and (v) genotyping through microsatellite analysis.


**Figure 5.** *In vitro* clones of the four species *Acer pseudoplatanus, Fagus sylvatica, Quercus petraea*, and *Ulmus laevis,* established from buds and shoot tips.

### 2.2.2. Group Genetic Technologies

The Thünen working group "Genome Research" (led by Matthias Fladung) has been working with genetic engineering in poplars since the 1990s (e.g., Müller et al., 2020; Kumar & Fladung, 2001; Fladung et al., 1997; Walter et al., 2010). Building on this experience, the junior research group "Genetic Technologies" was established to advance the biotechnological processing of forest trees using *in vitro* methods, genetic engineering, and new breeding technologies (NBT, also new genomic technologies – NGT).

To enable advanced biotechnological research, the group is working on the optimization of tissue culture, including the *in vitro* establishment, propagation and sustainable cultivation of various tree species. The European beech (*Fagus sylvatica*) and the Asian tree of heaven (*Ailanthus altissima*) were newly transferred to *in vitro* culture. Protocols have been developed to isolate protoplasts from various tree species, enabling microscopic detection of genetic changes. Research primarily involves poplars (e.g., *Populus* × *canescens*) as fast-growing model trees, though other species like common ash (*Fraxinus excelsior*) and pedunculate oak (*Quercus robur*) are also studied. Key projects include the genetical modification of candidate genes for genetic studies, with a current focus on the genetic basis of drought stress tolerance. Notably, one of Europe's first CRISPR/Cas9-mediated gene knockouts in trees targeted flower induction genes in *P.* × *canescens* (Bruegmann et al., 2019).

Despite challenges like *in vitro* recalcitrance, slow growth, and the hesitantly increasing number of genome-sequenced species, the use of NGT in tree research is increasing (Bruegmann et al., 2023). Recognized as powerful research tool, the group is optimizing CRISPR/Cas delivery and editing efficiency, while investigating off-target effects to ensure biosafety. This biotechnological work contributes to the climate change adaptation of trees, ecosystem preservation, and the optimization of trees as a renewable resource.

## 3. NEXT PLANNED JOINT ACTIVITIES

Members of the PInK-net will participate at the public “Long Night of Science” in Berlin on June 22nd, 2024. We will set up demonstrations about *in vitro* cultures of coniferous and deciduous trees, offer join-in games for (not only) children, and present our recent studies in talks between the famous trees and bushes of the Späth Arboretum in order to inform the interested public about our network and further scientific progress.

Additionally, we will commonly setting up cryo selections to further our research and collaboration efforts. This will allow us to work together more efficiently and effectively in our research projects.

We will also be focusing on the inclusion of further members into PInk-net, welcoming new researchers and scientists who share our passion for advancing knowledge and research in our field. We are happy to welcome colleagues from the Northwest German Forest Research Institute, Department Forest Genetic Ressources, Göttingen & Hann. Münden, and Julius Kühn-Institut (JKI), Institute for Breeding Research on Fruit Crops, Dresden/Pillnitz, who applied for membership during the CopyTree Conference in Riga, 2024.

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# The wonderful forest world inside a glass jar: Micropropagation as an educational tool

**Puri Covelo, Conchi Sánchez, Mª José Cernadas, Anxela Aldrey, Ricardo Castro, Carmen Fuentes, Nieves Vidal**

*Dept. of Plant Production, Misión Biológica de Galicia (MBG), CSIC, Avda de Vigo s/n, 15705, Santiago de Compostela, Spain*

nieves@mbg.csic.es

## Abstract

In our research group, Woody Plant Biotechnology, we are committed to scientific communication and outreach activities for people of all ages, all educational levels and with different functional and cognitive abilities. One of our educational and outreach activities is called "the wonderful forest world inside a glass jar", and consists of taking our *in vitro* cultures out of the laboratory, in glass jars. For more than fifteen years we have been traveling with them to classrooms, theatres, streets, hospitals, gardens and fairs. These "portable" or "traveling" forests allow us to present scientific concepts such as photosynthesis, genetic diversity, gene expression, ecosystems, geochemical cycles, as well as current challenges as climate change and germplasm conservation. We also use everyday objects and design hand-made tools and materials to dramatize the laboratory work and the life of trees in the forest. Our goal is that children and adults experiment and play, make questions and share ideas enjoying the beauty of *in vitro* trees. We want to encourage interest in science at all ages, encourage scientific vocations in the youngest and promote interest in nature conservation at all levels. All of us contribute to the organization of talks and experiments, as well as subdividing the plants, preparing the jars and other materials, transporting them and organizing the stand, as well as interacting with the public and developing new ideas with their feedback.

***Keywords:*** *children, general public, hands on, outreach, scientific concepts*

## 1. INTRODUCTION

Besides carrying out experiments and producing results, researchers have to effectively communicate scientific knowledge, scientific methodology, research findings, and discoveries to a range of audiences in a clear, accessible, and meaningful way, using strategies known as Dissemination and Communication. Following the European Commission guidelines, Dissemination means making results available to the scientific community, industry, commercial players and policymakers, by publishing in scientific magazines, going to scientific and/or targeted conferences and uploading results in databases (European Commission. 2023). It helps to explain the wider relevance of

science to society, build support for future research and innovation funding, ensure the uptake of results within the scientific community, and open up potential business opportunities for novel products or services.

Communication refers to articles in mainstream newspapers and magazines, websites, TV and radio channels (European Commission. 2024). Successful communication requires a clear language and attractive scientific subject with outstanding results that can catch the media's attention (European Commission. 2024; Sota, 2023).

Outreach activities are related to communication but imply an interaction between the sender and the receiver of the message: the researcher and the public (European Commission. 2015; Ayala, 2018). Outreach activities can take several forms, such as school presentations, workshops, public talks and lab visits, science festivals, and educational programs, with the goal of making science accessible and engaging (Ayala, 2018). In our research group we are committed to scientific communication by performing outreach activities for people of all ages, all educational levels and with different functional and cognitive abilities.

Here we present the activity "the wonderful forest world inside a glass jar" that consists of using our *in vitro* cultures as an educational tool to present scientific concepts such as photosynthesis, genetic diversity, gene expression, ecosystems, geochemical cycles, as well as current challenges as climate change and germplasm conservation. We take our "portable forests" to classrooms, theatres, streets, hospitals, gardens and fairs (Figure 1a), and also participate in open science days in our institute with other research groups. We use jars that are kept tightly closed to prevent microbial contamination, so they can be admired or given as special gift to schools or associations, whereas others will be available for opening and challenging the senses by looking, touching, smelling and starting the questions of how life is possible in a glass jar. We use everyday objects and design hand-made tools and materials to dramatize the laboratory work and the life of trees in the forest. We believe that when children and adults can have fun while they experiment and play, they feel more engaged with science and nature. Over the last sixteen years we have carried out more than 300 activities with more than 6500 participants.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Plant material consists in *in vitro* cultures of the main trees of Northwest Spanish native forests: oaks, chestnuts, hazelnuts, alders, wild cherries, hawthorns, birches, willows and cork oaks that we cultivate in our laboratory as already described (Vieitez et al., 1994; Sánchez et al., 1997; San José et al., 2020; Covelo et al., 2019; Sánchez et al., 2024; Dasilva,et al., 2019; Gago et al., 2021; Sánchez et al., 2013; Gago et al., 2022). For preparing attractive cultures we choose shoots of different species and sizes and inoculate them together in jars that frequently came from canned vegetables. We adapt

media formulation and plant growth regulator combinations so they can be suitable for growing several species together. Pedunculate and cork oak, chestnut and willow can easily share the same jar, whereas hazelnuts, hawthorns, plums and alders could grow together in a different medium as they normally require more cytokinin. We balance big and small shoots, include some with roots so they will live longer. In big jars we insert little animal toys -previously bleach-treated- to simulate a real forest (Figure 1). Some of the jars will be tightly closed to prevent microbial contamination, so they can be admired or given as special gift to schools or associations, whereas others will be available for opening, touching, smelling and practice plant transfer or subculturing.

**Other materials include:**

- jars with agar but without plants (so people can touch the agar or practice plant inoculation),
- small twigs with dormant buds,
- a kitchen sieve that acts as a laminar flow cabinet,
- colourful playdough shaped as microbes,
- forceps plus scalpels,
- a sprayer, a little pot with peat, a plastic soft drink or water bottle with the top cut off and used to make a small greenhouse,
- a sun shaped cushion pillow and a laser sword,
- small gifts for the public (flowers, bookmarkers, pencils, seeds…)
- A brochure with a protocol of the event where the phases of *in vitro* culture are explained in simple language and with attractive pictures.


**Figure 1. a)** Stand prepared for an outreach event in a garden. **b)** Detail of jars with shoots of different woody plants and little animals ready to be taken to an outreach event.

The quantities and sizes of all these things need to be matched to the size of the audience. They have to be safely packed in portable boxes that can be transported in a car and moved to the exact location of the event.

## 2.2. Methods

Materials can be used in many ways depending on the age and interest of the audience. Besides, the time for preparation the explants for the jars and the public manipulation will depend on the number of people and the type of interaction, ranging from 3 weeks to 6 weeks if more than one subculture is needed. For this reason it is better to plan the activities in advance and propagate a constant number of plants specifically for this purpose.

When we go to a school or an association we first contact the teacher or responsible person to ask what the room we are going to use is like (dimensions, arrangement of tables, chairs, screen, computer...), the number and age of the participants, if they have any special need, allergies, or a special interest in some approach, etc. We ask the teachers to be with us in the class so we (1 to 3 researchers) can safely manage a group of 15-25 children. Depending on the age and duration of the event we can make a power point presentation with aspects of the plants that are difficult to see with the naked eye, or bring an extra number of plants in plastic containers, since small children want to touch everything and we want to minimize the risk of accidents and broken glass. We also try to make the experience last over time and leave them with a jar or two to take care of for a few weeks. For children under 12 years of age we also organize drawing or writing contests about the scientific experience they have had. These stories and illustrations are a valuable feedback about what they learned and what they felt during the activity.

When we go to a fair we have to ask in advance for one or two tables to setup our stand, as well as chairs for sitting for a while if we are going to stay there for about 3-4 h. In fairs the public are normally families and we have to be prepared to all sort of situations and be extremely careful with objects that can be accidentally misused (scalpels) or broken (jars).

In all situations we seek interaction. First of all, we introduce ourselves and immediately ask the public their names and listen to their stories about their favourite trees. Usually as soon as we show one of our jars the public start making questions. If not, we try to encourage them to look, touch and talk, always respecting their personal preferences and interests. After all, the goal is to provide an experience in which "science" and "fun" go together. We use the sun cushion and the laser sword for talking about the amount of energy plants need to synthesize big molecules like carbohydrates and subsequently all the components of living matter. We talk a bit about all the elements that plants take from the soil, use the sieve and the playdough microbes for talking about asepsis. The asepsis chat is very attractive to children who are in hospital, as they know quite a lot about it and can empathize with the trees living in the jars. When our audience is able to use forceps and scalpels we let them to micropropagate oak or chestnut shoots. They cut one shoot into about 4 sections and inoculate them in a new jar. Then we ask to calculate how many plants they will have next year if they started with a single shoot big enough to be cut in 4 pieces and every month they can repeat the multiplication process with the shoots obtained the previous one. A typical question we receive is if they can take the trees home and plant them in a pot or in the

garden. Here we can discuss root formation, sometimes we can talk about cell types, plant growth regulators, totipotence, differentiation, de-differentiation. Usually we talk about transpiration, humidity, stomata, climate adaptations, how to build a mini-greenhouse for acclimation of *in vitro* plants. There is no limit to their questions, if they keep asking, we are prepared to make comparisons and give explanations in a simple language. When it is possible they go home with a rooted willow in a small pot made from a recycled milk container and covered with a plastic bottle.

## 3. RESULTS

Our group started educational activities about twenty years ago going to secondary schools to give lectures about our research to the students that were close to go to University. The teachers asked us to give talks to large groups of students, to cover as many topics as possible. The talks took place in the centers' assembly hall, and we spoke from the platform somewhat away from the students. Even if we prepared the talks carefully, choosing what we considered stimulating experiments, we would invariably notice a sharp decline on students' attention five minutes after starting a presentation. So, as a matter of necessity this conventional approach was substituted by dialogs and "hands on" experiments involving smaller groups in smaller classrooms with more time for listening to them than for talking ourselves. The change in the students' attitude encouraged us to extend their age to a range between 3 and "ninety something" and to develop many of the activities not just for inside a classroom but out in the street or fairs. This was the germ of the idea for a "portable" or "traveling" forest and the jars decoration. Our approach was well received by the educative community and repeat invitations to go to schools and fairs started to grow. Figure 2 shows the evolution of our activities and the number of participants in the last sixteen years.

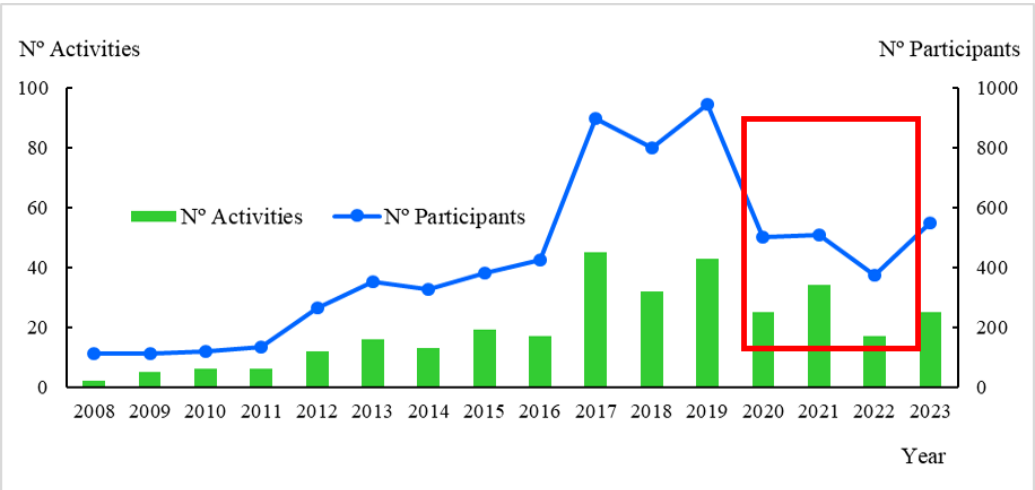


**Figure 2.** Evolution of the number of activities and participants in "The wonderful forest world inside a glass jar" outreach event over a 16-year period. The red rectangle corresponds to restrictions to the celebration of public events during lockdown periods due to Covid-19.

We carried out 317 activities, averaging 19.8 per year, these were developed in classrooms, theatres, mental health associations, streets, hospitals, gardens and fairs. We estimate about 6800 participants (averaging 425 per year and 24 per activity) were engaged with our activities. The participation figures are approximate because we can count the number of students attending an indoor educational activity, whereas it is quite difficult to be accurate in counting the number of children, teenagers, parents and grandparents who participate in outdoor activities or in indoor fairs.

Figures 3 and 4 illustrate activities in which we do not know *a priori* the number, age or interests of the attendees, and Figure 4 activities planned with a school or with the members of an association.

**Figure 3.** Indoor and outdoor outreach activities in fairs.

**Figure 4.** Outreach activities inside a classroom for students (left) and for users of a mental health centre (right).

To improve our performances we used several types of feedback: the opinion of teachers and parents, the contents of the stories and drawings that children made for the contests and our own feelings during and after the sessions.

## 4. DISCUSSION

Conducting fun, scientifically sound activities with a diverse audience requires planning, resources, and time. But they also produce benefits for researchers and society. Outreach activities allow scientific knowledge to be brought closer to people it would not normally reach. For scientists it is a way to get closer to society, to discover what normal people think about scientific activity, to establish contact with the youngest members of the world that have the potential to become the scientists that will solve the world problems in the future. As highlighted by Silvana Ovaitt (formerly S. Ayala) in her essay "7 Reasons why you should do outreach" (Ayala, 2018), teaching others is one of the best ways to learn". She enumerates other benefits, like that in addition to helping publicize our research, outreach activities allow us to meet our obligations with funding agencies, make it possible to connect with peers, neighbours, teachers, parents, adults and kids with unique stories and even "out of the box" points of view.

We have to keep in mind that some of these children, teenagers or adults will vote about funding for education and science programs; they will become leaders of schools, universities, companies and even government. Maybe they will not remember how photosynthesis works, but hopefully they will keep a warm feeling and empathy for science and scientists.

We agree completely with Silvana's conclusions regarding outreach. In our case we have found collaboration opportunities, established contacts with people of other fields. Sometimes when a member of the public discovers something new and shares this amazement with us we experience a golden moment in which we remember similar moments in our own lives, why we love what we do and come back to the laboratory reconciled with the world of the investigation.

We had to learn to be humble, and recognized that sometimes a child or a grandparent question makes us realize we don't know all the answers or even as much as we might think about our own work. The trick is to lose fear and don't hide the truth but turn those moments into an opportunity to interact honestly with the public. Later we can revisit the fundamental aspects of our work with another vision, which will surely bring new professional ideas.

We are grateful to the members of the staff of our institute, as well as our predoc and post doc students who have participated in the activities during these years with genuine enthusiasm. We also appreciate the contribution of the teachers, nurses, social workers who collaborated with us and gave us feedback and support. During these years we have

collaborated with other organizations as EPSO (European Plant Science Society) to celebrate the Fascination of Plants Day (18 May), with UNESCO and UN-Women to celebrate the International Day of Women and Girls in Science (11 February). Over the years we have received funding from the following organizations: Asociación Galega de Comunicación de Cultura Científica e Tecnolóxica (Divulgaccion), City of Culture of Galicia, Council of Pontevedra, Council of Santiago de Compostela, CULTURGAL, Educa Barrié and Fundación Barrié, Faro Educa and Faro da Escola (journal Faro de Vigo), FECYT, Parque Naútico de Castrelo do Miño, Pint of Science, Salón del Libro Infantil y Juvenil de Pontevedra, Unidade de Cultura Científica da Delegación do CSIC en Galicia, Xacobeo 2021, Xunta de Galicia, and invaluable help from family and friends.

## 5. CONCLUSIONS

The outreach activities that we have carried out during these years have had positive effects on all of us both emotionally and scientifically. "The wonderful forest world inside a glass jar" has allowed us to meet with a diverse range of people sharing ideas and feelings about science and nature. In our opinion, the main points required to carry out effective communication and be able to enjoy it are to keep the groups small, use simple language, let the audience touch, play, ask and give their opinion, always be respectful of their ideas, value their contributions, show joy and appreciation for their successes and smile as much as we can.

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# Joint project OPAL in Germany: Development and evaluation of methods for the efficient production of hybrid larch and fir clones

**Juliane Raschke, Jana Seifert, Madlen Walther, Emma Ehmke, Andrea Rupps**

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

andrea.rupps@hu-berlin.de

## Abstract

As part of the national project OPAL, somatic embryogenesis (SE) is being investigated, optimized, and applied to mass propagate genotypes of *Larix x eurolepis*, two *Abies* species (A. nordmanniana and A. bornmuelleriana) and three Abies-hybrids (*A. grandis* x *concolor*, *A. concolor* x *grandis*, *A. alba* x *nordmanniana*) relevant to forestry. The objective is to transfer this knowledge to small and medium-sized enterprises for large-scale, ready-to-market production. To achieve this, the individual steps of the SE *in vitro* process are being refined, in close collaboration with tree nurseries and a seed trader, with the shared goal of ensuring the availability of resilient plant material for robust and economically viable forestry.

***Keywords:*** *German joint project OPAL, somatic embryogenesis, Larix sp., Abies sp., hybrids, transfer into practice*

## 1. INTRODUCTION

### 1.1. Background

European forests are threatened by progressing climate change. Drought during the summer months, storm disasters, and the massive spread of pests are leading to the loss of forested areas (Hanewinkel et al., 2013). The conversion to climate-adapted forests and the long-term preservation of forest genetic resources is therefore one of the most pressing tasks facing not only the German forestry sector. While in recent decades the focus of conserving forest genetic resources has been on *in situ* measures, the loss of *in situ* resources and valuable seed stands will necessitate a strengthening of suitable *ex situ* techniques (Park et al., 2016). At the same time, there is a need for reforestation with adapted trees (Hylander et al., 2022). Larch and fir species and hybrids are characterized by high drought tolerance and stability (Paul et al., 2019).

The aim of OPAL is to advance specific technologies and bring them to marketability to efficiently produce robust genotypes of both genera in large numbers regardless of seed availability. Based on somatic embryogenesis (SE) as the preferred technique for clonal propagation of conifers, specifically suitable clone variety mixtures can be compiled

and produced in a scalable manner (Park et al., 2016). The basis for our work is a genetically diverse clone bank of existing, cryopreserved genotypes. OPAL aims to optimize critical points of the biotechnological process chain, and to anchor all steps in the participating nurseries and horticultural companies, thereby completing the transition from research to applied mass production (Fig. 1).

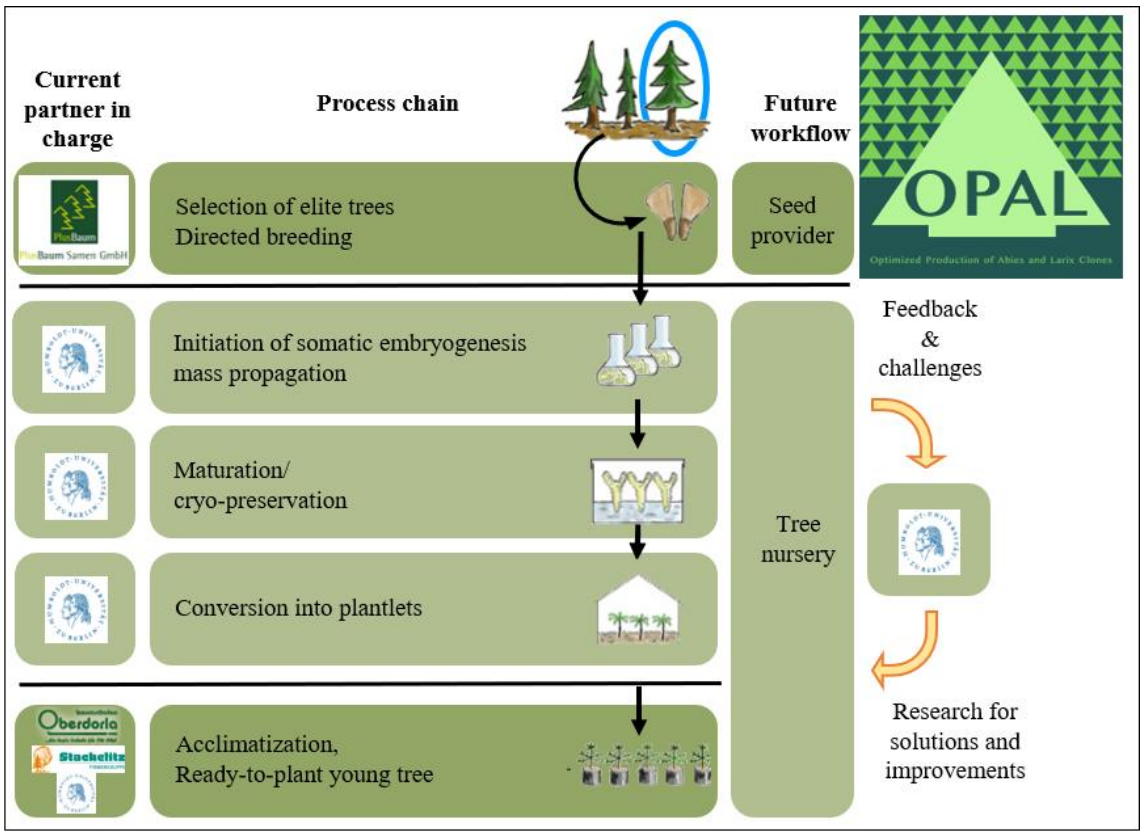


**Figure 1**. Overview of our current somatic embryogenesis process chain with the aim to be handed over completely to users in near future: our project partners PlusBaum Samen GmbH, Baumschulen Oberdorla GmbH and Baumschule Stackelitz GmbH & Co. KG are represented by their logos (*see picture above*)

## 1.2. SE as means of clonal propagation

SE is induced by application of plant growth regulator-induced stress to zygotic embryos. A sub-cultivation interval of 3-4 weeks allows for rapid proliferation of the embryogenic cultures and easy scale-up. During the maturation phase early embryos develop into cotyledonary embryos, which are then prepared for conversion and germination into seedlings on a plant growth regulator (PGR)-free culture medium. Several test plots have already been successfully planted, although acclimatization of *in vitro* derived trees remains challenging especially for *Abies* plantlets.

Early cryopreservation of embryogenic genotypes retains their juvenile state and allows long-term storage for later use (Cyr & Klimaszewska, 2002). In previous projects, a clone bank containing over 800 characterized *Abies spp.* genotypes and 260 genotypes of hybrid larch was established.

## 2. OPAL'S MAIN OBJECTIVES: Focus on the optimization of single SE process steps

### 2.1. Maintenance and propagation of somatic embryo cultures

The SE cultures (Fig. 2 A and B) are sub-cultivated on a monthly basis – a balancing act to retain vitality while limiting the accumulation of undesirable traits, such as a reduction in the quantity and quality of somatic embryos. This process can be accelerated by shorter multiplication schedules. In OPAL, the aim is to specify the morphological features of embryogenic cultures that characterize their developmental potential and determine their suitability for proper maturation and further growth. Additionally, efforts are made to ensure that the young embryos selected for maturation are in the same developmental phase during the multiplication cycle, as synchronicity is crucial for maturation success.

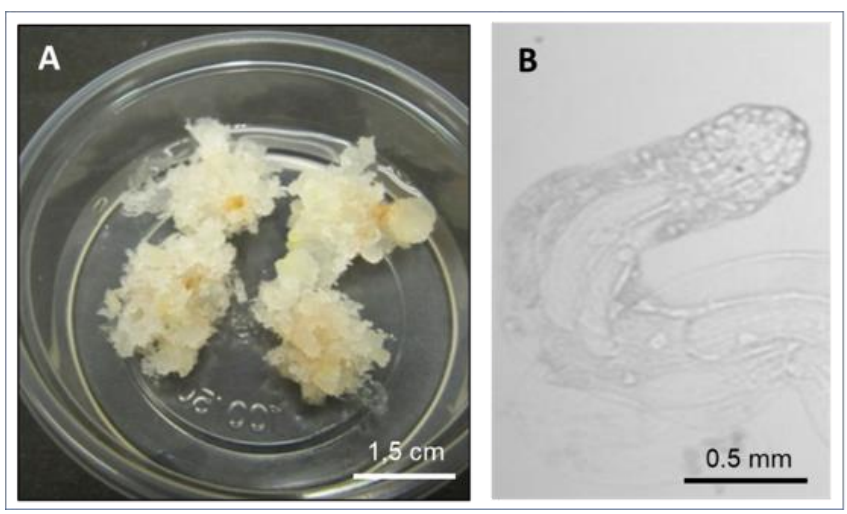


**Figure 2.** Maintenance stage of tissue culture: **A)** proliferation of embryogenic cultures of *A. nordmanniana* on SH culture medium in plastic vessels; **B)** microscopic picture of a single early somatic embryo of *Larix decidua*

### 2.2. Maturation of somatic embryos

During the maturation process, the somatic embryos develop into cotyledonary embryos (Fig. 3 A and B). This step is essential for defining the value of the genotype in the lab production chain. The productivity of cotyledonary, morphologically ideal embryos varies greatly among genotypes, often leading to the exclusion of up to one-third of successfully initiated and propagated clones due to no or very low maturation capacity.

Therefore, constant optimization is required for this step, down to developing clone-specific protocols. Within the OPAL project, the effects of the sugar source, phytohormones during maintenance, and osmotic pressure during maturation on the productivity of a clone were studied. These results still need to be verified and analyzed conclusively.

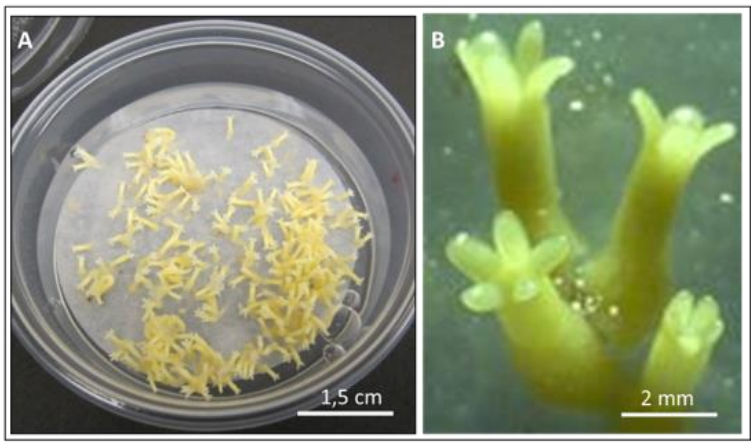


**Figure 3**. Mature embryos of *A. nordmanniana:* **A)** in a culture dish containing BLG culture medium after 12 weeks at 22°C in darkness; **B)** microscopic picture of four fully developed ("mature") somatic embryos.

## 2.3. Conversion and Acclimatization of Somatic Plants

Conversion, or "germination," refers to the development of embryos into plantlets, which are morphologically similar to seedlings. At this stage, selecting morphologically "ideal" embryos is crucial to minimize manual labor associated with plant rejects. Acclimatization involves transferring the plantlets from *in vitro* conditions to soil (Fig. 4). This transition to unsterile soil remains challenging due to several factors such as bacteria and fungi, fungus gnat, changes in humidity and ventilation, and increased UV light exposure.

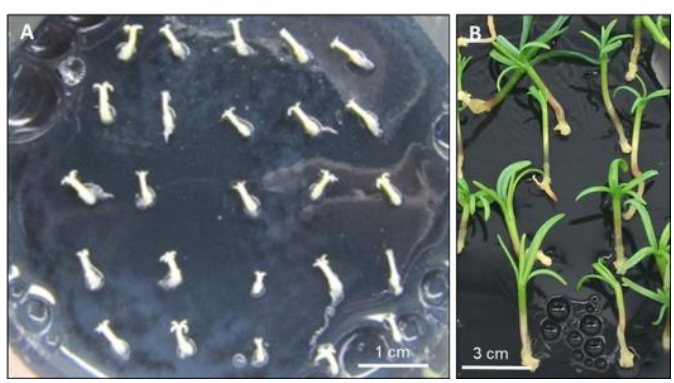


**Figure 4**. Conversion stages of somatic embryos / plants of *A. nordmanniana*: **A)** cotyledonary embryos right after transfer onto conversion medium; **B)** same plants after three weeks of growth.

Currently, all *in vitro* steps, including the initial acclimatization of *Abies* genotypes, are conducted at our facility and are still in the optimization stage, whereas *Larix* plantlets

are already send to one of our nursery partners for acclimatization. However, the goal is to streamline the entire process into an easy-to-handle method that consistently delivers stable results.

### 2.4. Bioreactor – potential for scaling up

The basic suitability of the Plantform immersion bioreactor for the propagation of embryogenic cultures of *Larix* and *Abies* cultures has already been confirmed in a previous project. In OPAL, the applicability to further process steps (maturation, conversion) is currently being tested, including the implementation of the entire *in vitro* chain in the bioreactor. Special focus is placed on the duration of the immersion intervals, additional aeration and the use of osmotically active substances.

## 3. SUMMARY AND OUTLOOK

Somatic embryogenesis (SE) is a well-established method for multiplying genotypes derived from seeds of various conifer species. When combined with an extensive clone bank, this powerful tool can accelerate adaptation to new challenges while preserving genetic diversity. The more genotypes are stored, the greater the potential for genetic diversity and adaptation, as different genotypes can be mixed.

However, due to the slow growth of conifer plantlets and their long response times to various treatments, particularly during maturation, it takes a comparatively long time to see experimental results. Follow-up experiments are required to fine-tune individual process steps. Efficient protocols are essential for making this plant production approach feasible for small enterprises, which often prioritize different aspects than academic researchers when optimizing production chains.

One vision for addressing labor-intensive process steps is the use of bioreactors to upscale output while reducing manual labor. Projects such as OPAL are crucial for transferring academic knowledge and expertise into practice and the market. This transfer is necessary to free up resources in academia, enabling further research with the experimental tool of SE in conifers for both academic purposes and future applications.

## Funding

The project OPAL (KMUi-BÖ01: OPAL – Development and evaluation of methods for the efficient production of clones of hybrid larches and fir species) is funded by the German Federal Ministry of Education and Research (BMBF) in the frame of the programme SME-innovative and runs from September 2021 to April 2025.

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# Using micropropagated trees in a community-owned revegetation project on neglected land

**Carlos Sobrino[1], Lucía Saborido[2], David García[3], Conchi Sánchez[1], Anxela Aldrey[1], Puri Covelo[1], Mª José Cernadas[1], Bruce Christie[4], Nieves Vidal[1]**

*[1]Dept. Plant Production, Misión Biológica de Galicia (MBG) sede Santiago de Compostela, CSIC, Avda. de Vigo, s/n, 15705 Santiago de Compostela, Spain.*

*[2]Community forest owners of Araño, Rianxo, A Coruña, Spain.*

*[3]Dept. Pedagogy and Didactics, Facultade de Ciencias da Educación de la Universidad de Santiago de Compostela, Spain.*

*[4]The Greenplant Company, Palmerston North 4410, New Zealand.*

*brucechristie101@gmail.com*

## Abstract

*In vitro* culture is a tool for *ex situ* biodiversity conservation enabling restoration of degraded land. This study used native trees cultured *in vitro* for replanting fire damaged forest in Galicia, where invasive species like *Acacia melanoxylon* and *Eucalyptus globulus* were establishing on neglected land. Plant material was grown from the *in vitro* germplasm collection at the MBG or from seeds collected from the proposed revegetation area. All activities were carried out with the support of local community, local government agencies, NGOs and researchers collaborating on the project. Results indicate that with adequate instruction and supervision previously inexperienced school children and adults could successfully plant micropropagated material in a prepared site that after planting did not receive the post planting care normally expected in experimental or commercial plant production plots. Technical lessons learnt included the need for acclimated plants to be tall enough to outgrow weeds, use of durable plant labels, plus the requirement for weeding and irrigation in the first summer after planting. This study is part of a broader reforestation project carried out by the Community of forest owners of Araño. LS conceived the project and organized the network of collaborations with institutions and associations. DG, leader of the Service Learning Project "Arredor do Rural", contacted the MBG to offer the possibility that a Biology student (C Sobrino) could carry out his degree project and at the same time do a service to the community. The aforementioned people, together with NV and BC, designed the main objectives and the work plan and participated in its execution, as well as in the discussions and writing of the reports. The MBG researchers propagated and provided the micropropagated plants and those from seeds.

***Keywords:*** *biodiversity, community building, forest restoration, networking, plant survival*

## 1. INTRODUCTION

The critical voices challenging climate change research are losing their impact as the effects of climate change become more indisputable. The public while still largely in denial is becoming a little more aware of nebulous concepts like the point of no return with polar ice

cap melting, the Greenland ice cover is melting 7 times faster now in 2024 than in 1990, this alone has contributed to a 1cm sea level rise. Each cm rise in sea level means another 6 million people will experience flooding every year (King, 2024). Many people have more of a casual concern perhaps than appreciation of the consequences of how short term local weather changes impact and inconvenience normal life as we experience it in the here and now rather than longer term effects.

International interest in $CO_2$ sequestration to mitigate the effects of climate change and related global disasters has focused on forest restoration and afforestation. Ambitious goals of restoring and planting 350 million ha of forests globally have seen most of the effort going into monocultures with significant loss of biodiversity and forest resilience (King, 2024; Depauw et al., 2023).

There has been more effort in afforestation rather than reforestation, so land that has been degraded by tree removal, mining, conversion to agriculture and fire in the main is not being replanted with the genetic diversity that existed in the natural forest despite diversification of plantations being a key policy recommendation (Warner et al., 2023; Lewis et al, 2019). There is increasing evidence that planting mixed (4-6) species forests (especially young forests) might actually fix 15 to 20% more carbon than single species plantations. This is a relatively new area of investigation and while the full explanation for the enhanced carbon is complicated and remains a bit obscure, it is likely that in at least some situations multiple species plantings can intercept more light, and trees may be more efficient at nutrient and water use as a community. Every effort to enhance efficiency of carbon sequestration especially at low extra cost would appear worthy of further investigation. The most difficult part going forward might be softening the recalcitrant mindset of the groups coordinating the tree planters (Warner et al., 2023; Xanthopoulus et al., 2023). Although decades of research suggest that higher species richness improves ecosystem functioning and stability, planted forests are predominantly monocultures. This highlights the need for investigative work as in this study using multiple tree species in revegetation and reforestation work collaborating with a community who is leading the project since its members are the owners and neighbors of the forest.

The Community of forest owners of Araño (650 ha) is formed by about 260 people. For this project they created a network including families, institutions and associations. The participation of the MBG and Greenplant Co. researchers took place through a Service Learning project promoted by the University. This project "Arrededor do Rural" means "Around rural life" in Galician, aims to bring together academics and rural citizens to learn and help each other. The initial objective of this study was to use native trees cultured *in vitro* for the regeneration of a riparian forest located in Araño, Rianxo, A Coruña, named "Rego do Muiño do Conde" (literally "The stream of the Mill of the Count") that had been affected by a fire fifteen years before and as a consequence was colonized by invasive introduced species, mainly *Acacia melanoxylon* and *Eucalyptus globulus* (Figure 1). Besides micropropagated plants of the *in vitro* germplasm bank of the MBG, we collected

seeds from native oak trees from a nearby natural forest (Figure 2), with the idea of germinating them and using for planting and for *in vitro* establishment. This way we would have a back-up in case the first planting seasons weren't successful.

**Figure 1.** Riparian forest "Rego do Muiño do Conde" degraded after suffering a fire and being colonized by *Eucalyptusglobulus* and *Acacia melanoxylon*

**Figure 2.** Natural forest close to the degraded area. Detail of *Quercus robur* acorn collected for germplasm preservation

To preserve a larger range of biodiversity it was planned to request the relevant permits to collect branches of woody species that did not have seeds at the time, as well as moss, ferns and herbaceous plants. Some of these materials would be established *in vitro* and others propagated conventionally. However, due to the steepness of the terrain and the fear that the machinery to remove the invasive trees would further damage the already fragile aquatic ecosystem, the permission of public administrations to act in the area was delayed without setting a date or deadline. As a consequence, the project was moved to an area located about 1.5 km away from the stream that also had been invaded by the same foreign species and named "A Hermida". After invasive trees removal and two community planting events in two consecutive years, not all of the initial objectives have been met, but progress has been made in the recovery of the area and we have learned technical lessons on how to proceed in this type of projects.

## 2. MATERIALS AND METHODS

### 2.1. Materials

The land for restoration is called A Hermida. It is located in Galicia (North Western Spain). Specifically, it belongs to Araño, Rianxo, province of A Coruña (GPS location, 42° 42′ 20.72″ N, 8° 46′ 11.31″ W). The land was unfenced and adjacent to farmland and a playground area, and had been cleared of all exotic woody plants where practicable. Figure 3 shows the location and the aspect of the place after removal of invasive plants. The area for restoration on year 2022 (Zone 1) comprised 1.8 ha and the area for the second year (Zone 2) was a bit smaller, with 1.3 ha.

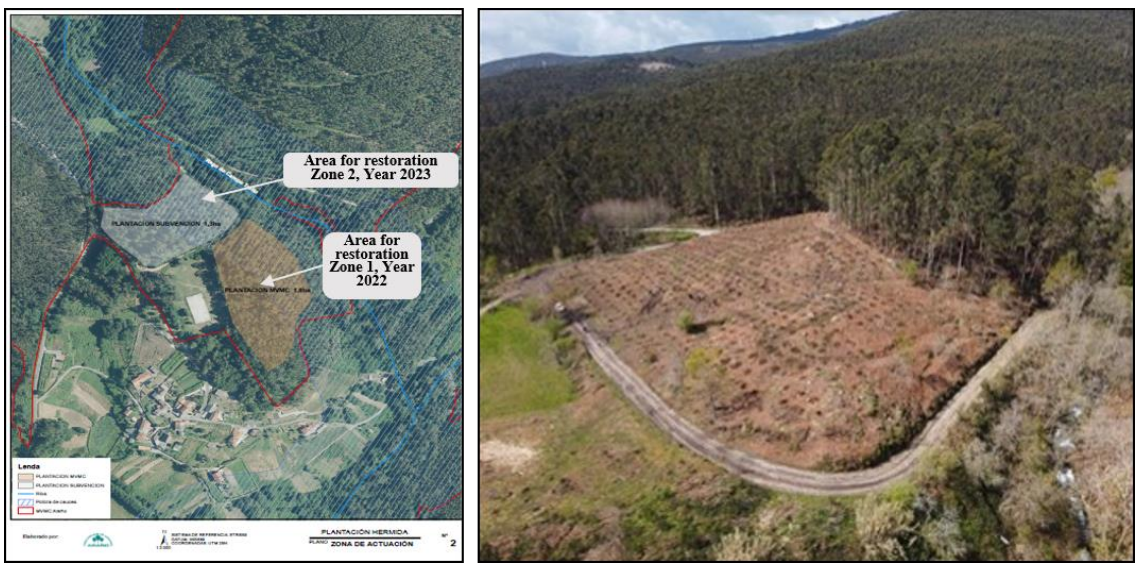


**Figure 3.** Area for restoration in "A Hermida", Araño, Rianxo.
On the left, the plan of the land, and on the right the aerial photograph of area 2 as prepared for the planting. A streamsurrounded by native vegetation can be seen in the lower right corner.

The plant material used in the project belonged to 3 categories: A) Seedlings grown in nurseries for 2-3 years and provided by associations and institutions, B) Micropropagated plants from the germplasm bank of the MBG, C) Seedlings from seeds collected in the area of the project and germinated in the MBG.

The plants from nurseries were mainly *Quercus robur*, *Castanea sativa*, *Fagus sylvatica* and *Arbutus unedo*. The plantlets provided by the MBG corresponded to nine species -*Betula pubescens* ssp. celtiberica, *Castanea sativa*, *Corylus avellana*, *Pyrus cordata*, *Prunus domestica*, *Prunus avium*, *Quercus robur*, *Quercus suber* and *Salix viminalis*- that had been established *in vitro* from material collected in the same region of the area for restoration (Figure 4), either in natural forests or naturalized in abandoned farmland areas.

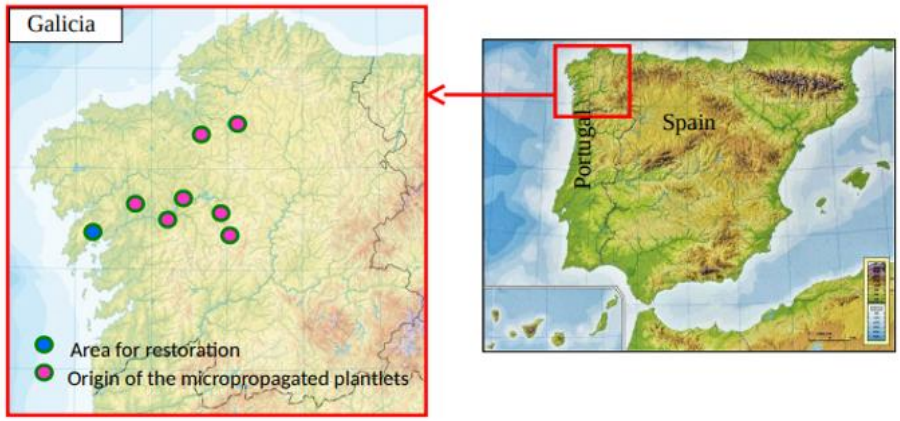


**Figure 4.** Location of restoration area and origin of the micropropagated plantlets used in the revegetation project.

## 2.2. Methods

The plants of Category B (micropropagated plantlets from MBG germplasm collection) were grown *in vitro* as described previously (Vieitez et al., 1994; Sánchez et al, 1997; Sánchez et al., 2013; Covelo et al., 2019; Dasilva et al., 2019; Gago et al.; 2021; Gago et al., 2022; Sánchez et al., 2024), acclimated in a greenhouse for 4-6 months and remained outdoors in the facilities of the MBG for at least one winter. Hazelnut plants had been established from seeds germinated *in vitro* (Sánchez et al., 2024), where the others were initiated from shoots developed on new growth from branches of mature trees.

The seedlings (Category C) were obtained by germination of *Q. robur* acorns (Figure 5a) collected in autumn with authority approval under trees close to the area for regeneration (less than 2 km). After collection acornswere washed with soapy water, air dried, and cold stratified for 4-6 months. Germination was carried out in peat:perlite (3:1) in 3 environments: phytotron, greenhouse and outdoors (Figure 5b, c). To preserve this germplasm and enable future re-introductions, some seedlings germinated in the phytotron were used for *in vitro* culture following the protocols of Vieitez et al. (1994). The basal sections of the seedlings (about 6 cm) were maintained in the pots to enable regrowth and future deployment (Figure 5d), whereas the buds of the rest of the shoot were used for *in vitro* establishment (Figure 5e-f).

The Community of forest owners of Araño organized the network of collaborations with institutions and associations that participated in the project, as primary and secondary schools -CRA de Rianxo, IES Félix Muriel-, associations -AAVV Araño, Asociación Avoar, Fundación Ria, Barbanza Ecosocial Lab - and local and regional institutions - Concello de Rianxo, Consellería Medio Rural (Xunta de Galicia). Some weeks before the planting the land was cleared of invasive trees, the holes for the new plants were prepared and filled again with soil to make planting easier in the future and children

received planting information in their schools. On the day of planting firemen, police and forest agents gave further explanations about forest importance and conservation, as well as fire prevention. During the planting the youngest children were guided by professionals and local grandparents. The firemen watered the area after the planting, as well as two weeks and one month later.

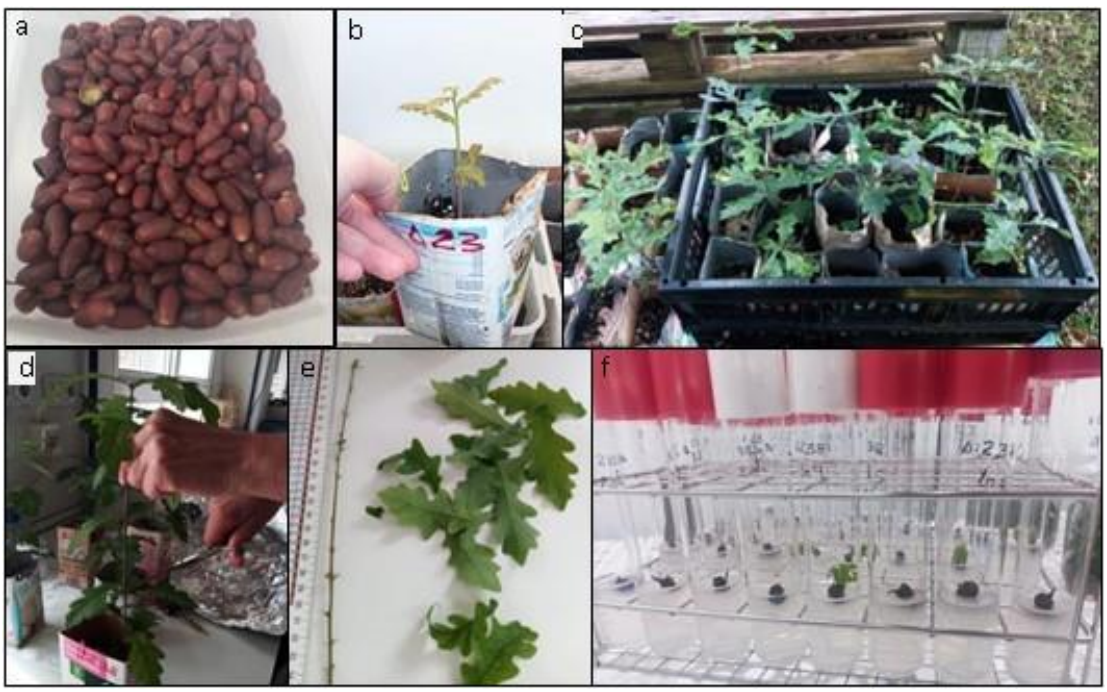


**Figure 5.** *Q. robur* plants obtained from seeds used for deployment and for *in vitro* conservation. a) acorns before cold stratification, b, c) seedlings germinated in the phytotron (b) and outdoors (c), d) seedlings selected for micropropagation, e) upper section of seedlings removed for *in vitro* establishment, f) shoots growing during the first days after *in vitro* inoculation.

## 3. RESULTS

The planting of native trees was carried out in April 2022 and March 2023 (Figure 6). In the first year, more than 350 native trees were planted with the help of 118 children aged 3-7 years, their teachers and families (mostly grandparents) who provided tools, guidance and support (El Correo Gallego 2022). In the second year, about 1000 trees were planted with the help of forest agents and 50 teenagers from the IES Felix Muriel, a secondary school that is developing an environmental project called "Monte Vivo" (*Alive forest* in English) (El Diario de Arousa, 2023). Seventy of the total number of plants came from *in vitro* culture (Figure 6a-c). Plants were labelled and the smallest were protected with plastic bottles.

Plants grew successfully during the first growing season after planting (Figure 7a). However, assessment 14 months after the second planting indicated major weed

competition especially from an indigenous fern (*Pteridium aquillinum*) that reached about 70 cm height (Figure 7b) and made it very difficult to see smaller plants even if some could still be alive (Figure 7c).


**Figure 6.** Plantation events of years 2022 (a-e) and 2023 (f-h). a, b) some of the micropropagated plants, c) micropropagated plum protected with a plastic bottle in Zone 1, d-e) children of primary school during the plantation of Zone 1, f-h) teenagers of secondary school during 2023 plantation of Zone 2.

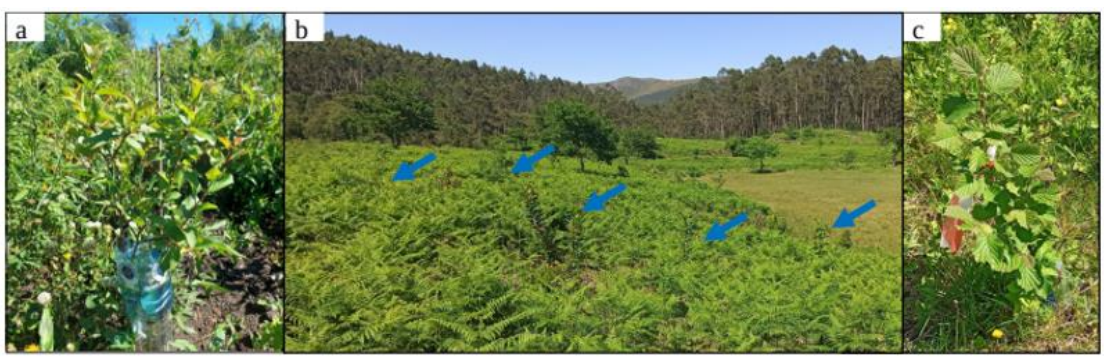


**Figure 7.** Evolution of Zone 1 during the first two years after plantation. a) micropropagated plant of Fig. 6c after the first growing season, b) Aspect of Zone 1 two years after the plantation, with blue arrows indicating the plants that grew over the fern, c) surviving micropropagated hazelnut in a small clear between the ferns in Zone 1.

With the removal of Acacia and Eucalyptus, the fern is now the dominant pioneer species at the site. This has proven to be a significant impediment to establishment of small transplants, many of which were surviving prior to the rapid growth of the fern (to 50-70 cm) in the second year after the site was initially cleared for planting. There was evidence of horses being on the site in the recent past, but it was no possible to directly connect browsing with missing plants without site monitoring. Apparently the species that were able to outgrow the fern (but without thriving) are mainly oaks, chestnuts, birches and strawberry trees that were already taller than 1 m at the moment of transplanting. Although the fern made it difficult to take accurate survival recordings, most of the smaller plants of Zone 1 were not visible, irrespective if they came from *in vitro* or conventional propagation. It seems that the same trend was follow in Zone 2, although fortunately most of those plants were larger from the beginning (about 2 m).

## 4. DISCUSSION

For the researchers from the academic world the primary goal of this study was to evaluate the potential of *in vitro* grown plants for the purpose of revegetation of degraded land, as well as develop an outreach activity to share and test scientific knowledge with a broad and heterogeneous audience (Ayala 2018). But this study is part of a community dream whose main goal was to recover their native forest and landscape while strengthening the ties that unite the community, making it possible for people of different generations, with jobs, academic training and different economic levels to work together and with enthusiasm in a common project. The collaboration of a rural community with the academic world through a Service Learning project implies recognizing the value of a way of life in contact with nature that is at risk of disappearing and that if it does, it will take with it ancient knowledge about nature and some related jobs that, as a consequence of rural depopulation and emigration, have stopped passing from parents to children and are now only in the hands of grandparents. In this sense, the project was a success. The effort of the organizers and the community, previous work in schools and guidance during the planting by teachers, families and other local statements as firemen, police and forest agents, made it possible that the young members of the region now consider the planting area belongs to them and feel committed to take care of it for the future. The main obstacle for the project was the long time needed for permits for the removal of exotic species, which was partially solved by modifying the plantation area, but that could have put at risk the whole idea if the organizers hadn't have a strong determination and commitment for carrying out the activities.

Regarding the plant material, there are still technical aspects that need to be improved. Degraded planting areas don't have the same characteristics as a farmer's field: the ground is more compacted, less fertile, unfenced and there is no irrigation. For survival, the size, stem thickness, root development and robust character of the plants have to be higher than for commercial production. Micropropagated plants need to be tall enough for standing up weed competition, and in this case we underestimated the growth capacity of

the pioneer weeds. For future interventions the use of a biodegradable weed mat "square" about 60 cm on edge could be worth trying to establish small plants (30 cm high). Alternatively growing the plants on for another 1 to 2 seasons to get them above the fern competition could facilitate field establishment. Other aspects to improve include watering during the first summer, protection against animal browsing and quality of plant labels, which weren't legible after some months in outdoor conditions. This project was a preliminary trial in which no formal experimental design was possible, as practical site constraints over-ruled consideration of blocking or replication. Randomised planting occurred, depending on the site details, but in next experiments it would be necessary to study the place with more detail and plan the situation of the plants of different species and genotypes, as well as to prepare a plan of the position of the plants to carry out adequate monitoring.

When planning the plantation, ecological criteria and public acceptance must be taken into account. The goal is not productivity but to provide ecosystemic services. The best option is to use ecologically sourced native plants, with genotypes coming from nearby locations, and make a design in which, as in this project, different species and different genotypes within species are interspersed. In this case we used about 70 micropropagated plants corresponding to 9 species and 16 genotypes, so none of the genotypes was represented by more than 5 plants in a 3 ha zone. Besides, we collected oak acorns from the area and established some of them *in vitro,* so they can provide a back-up against field plant losses. In future activities it would be interesting to cryopreserve a good number of seeds of different species to preserve the germplasm of the ecosystem (Chmielarz et al., 2011; Trusiak et al., 2023), as well as carry out outreach activities in schools and community centers to share scientific ideas and environmental concerns.

## 5. CONCLUSIONS

It is possible to use micropropagated plant material for revegetation in areas that have a lower level of technical care than experimental or plant production plots. Plantlets should be well hardened and robust enough, as soil quality can be low and they will have to endure difficult weather conditions as well as competition from weeds and browsing from wild animals. Plants maintained *in vitro* provide a back-up against field plant losses. Besides demonstrating the principle of using micropropagated plants for revegetation works, the other main outcome of the project was the involvement of a heterogeneous community of all ages who shared different expertise and that will take care of the regenerated forest.

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# Present and future perspective on knowledge-sharing and stakeholder engagement strategies on CopyTree

**Valbona Sota[1], Lucie Fischerová[2], Maurizio Lambardi[3], Stefaan P.O. Werbrouck[4]**

[1]*CopyTree Science Communication Coordinator; University of Tirana, Albania*
[2]*CopyTree Scientific Representative; Institute of Experimental Botany of CAS, Czech Republic*
[3]*CopyTree Vice-Chair; Institute of BioEconomy (IBE-CNR), Florence, Italy*
[4]*CopyTree Chair, Ghent University, Brussels, Belgium*
valbona.sota@fshn.edu.al

## Abstract

Technology transfer and stakeholder engagement within the CopyTree community are mutually beneficial and form a network outcome. The success of this process depends on the quality of the strategies used to achieve specific goals. In the context of this COST Action Network, the focus is on optimizing the interaction between Knowledge Producers (KP) and Knowledge Users (KU). Stakeholder engagement, an ongoing process of information sharing and feedback gathering, drives this ambition. The CopyTree community currently comprises 285 members from 43 countries, demonstrating strong interest and effective global distribution. Of the 173 unique affiliations, a) 52% are Universities or other educational institutions involved in micropropagation and *in vitro* technologies, b) 37% are research Institutes and centers (both public and private), some with national or governmental support, and c) 11% represents diverse stakeholders such as commercial micropropagation companies, private nurseries, scientific societies, consultants, and agribusinesses. In order to increase the involvement of KU, and to improve the bridge between research and development (R&D) for profitable knowledge acquisition, considerations should be given to (i) directly inviting companies benefiting from CopyTree results, (ii) identifying and prioritizing user interests, (iii) conducting listening sessions with targeted stakeholders to understand challenges and explore solutions, and (vi) launching awareness-raising campaigns on social media channels presenting how research and innovation on *in vitro* woody plant production can bring benefits to the society. All of the above should be managed and implemented by each Working Group independently and interdependently, while thinking and acting on the overall CopyTree mission and goals.

***Keywords:*** *international network, micropropagation, in vitro technology transfer, awareness-raising*

## 1. BACKGROUND

CopyTree—COST Action 21157 (www.cost.eu/actions/CA21157) is a four-year action financed by the European Commission that aims to increase capacities and scientific

coordination in *in vitro* biotechnology dedicated explicitly to woody plants. This Action started in October 2022 and has been ongoing for four years. From the beginning, scientific researchers, academic staff members, agribusiness specialists, and entrepreneurs related to agriculture joined the Action.

CopyTree vision and mission aim at cooperation and coordination between all possible actors, connected directly or not, with the long-term objective of transferring knowledge on the innovations and advantages of the micropropagation of woody plants at the European level, innovation towards automation, and the newest findings that enable the mass production of plant material and the creation of genetic collections optimizing the conservation strategies of the germplasm of interest. Also, a primary goal is to simplify the methodologies and overcome the obstacles that enable the transfer of micropropagated germplasm from small-scale laboratory production to large-scale commercial micropropagation, meeting the consumers' demands and needs.

This Action members have worked through various activities to achieve the goals above, aiming to reach short-term objectives related to specific activities, but always within the CopyTree mission and vision (www.copytree.eu).

## 2. GENERAL OVERVIEW OF COPYTREE

### 2.1. Organization, bottom-up management approach, and communication processes in CopyTree

CopyTree carries out its activity in full compliance with COST rules (www.cost.eu), considering gender equality and geographical distribution and prioritizing young researchers and investigators.

The hierarchical organization of the Action (Fig. 1) is designed to facilitate effective management and the achievement of our general goal. Decision-making is not confined to the top but is carried out under a bottom-up approach. This means that every member, regardless of their position in the hierarchy, has the opportunity to be involved and influence this process and be well-informed about CopyTree activities. The Management Committee (MC), for instance, makes many decisions through the e-voting process, part of the official e-cost system. Furthermore, open calls for organizing conferences and annual meetings, training schools, short-term scientific visits, or other activities under the framework of CopyTree are launched for all community members.

On the other hand, the internal communication area serves this bottom-up management approach. This platform was created specifically to support a sense of community for sharing experiences and new ideas, participating in open discussions, and enabling further collaborations between members.

CopyTree management and organization efforts have significantly enhanced inclusiveness in decision-making and participation across various activities. This has led to the creation of equal opportunities, a key achievement of CopyTree.

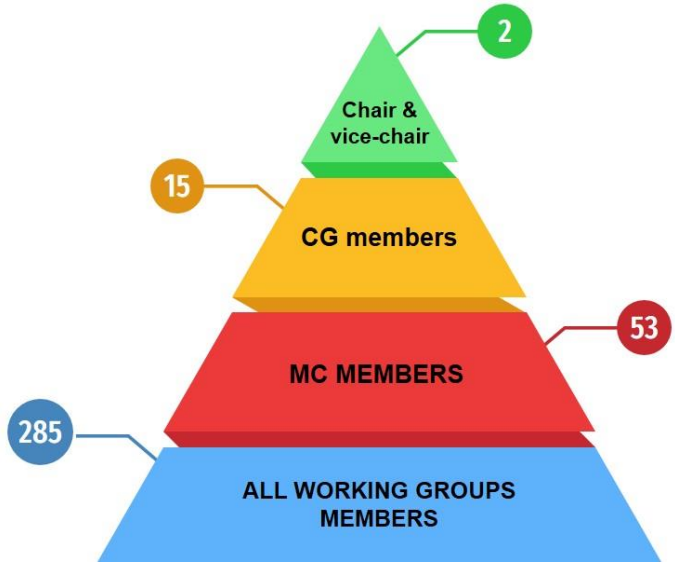


**Figure 1.** Hierarchical organization of CopyTree and membership in each level. MC – Management Committee, CG – Core Group

CopyTree two annual conferences have been resounding successes, with broad active participation from MC members and WG members, who have received full financial support for their involvement. Seven open calls for STSM (Short-term Scientific Missions) have been launched, benefiting over 20 researchers, activities that play an essential role in knowledge-sharing and technology transfer processes. The first training school focused on TIS bioreactors was a significant milestone, with 15 CopyTree members receiving training hours and contributing to knowledge sharing in this area. The launch of the second training school call, focusing on recalcitrant species and their micropropagation, promises to be another impactful event, benefiting another 15 CopyTree members. These activities so far and the high interest from the members of the CopyTree community are proof of this Action's effective management system in achieving the desired outcomes.

External and internal communication channels are necessary to facilitate communication and inclusiveness. The main communication channel is the CopyTree website (www.copytree.eu), where all the calls and detailed activity information are published. Among other things, this Action is also active on social media platforms such as:

- LinkedIn for engaging with professionals working on plant biotechnology-related areas (https://www.linkedin.com/company/copytree-costaction-21157/);
- Facebook for engaging with a broader audience that is more interested in popular science communications (https://www.facebook.com/profile.php?id=100087999720594);
- Instagram for engaging with a younger audience interested in scientific research and popular science communications (https://www.instagram.com/copytree_ca/);

- YouTube for making it easier to share videos produced by CopyTree members which fit with the mission of CopyTree and facilitating the knowledge sharing and the engagement process (https://www.youtube.com/channel/UC6lKQe0oZLI3gDoNS9ngpUA)

### 2.2. CopyTree membership categories and established working groups

CopyTree consists of 285 members from 43 countries, which is an excellent participation in the second year of Action. Of these members, 45% are young researchers and investigators, thus showing a high interest from this age group who are enthusiastic about working on *in vitro* technologies. Knowledge and expertise transfer from senior researchers to younger ones is one of the main goals of CopyTree, which has been accomplished so far, as shown by the statistics on the membership classification.

*In vitro* mass production, commercialization, and public acceptance of this biotechnological product are issues that arouse a great interest in society, enabling the involvement of many stakeholders and interested parties. Currently, of the 173 unique affiliations of CopyTree:

- 52% are Universities or other educational institutions involved in micropropagation and *in vitro* technologies,
- 7% are research Institutes and centers (both public and private), some with national or governmental support and
- 11% represents diverse stakeholders such as commercial micropropagation companies, private nurseries, scientific societies, consultants, and agribusinesses.

The data shows that most of the participants in this Action are researchers and academic staff members diligently working on scientific research and knowledge acquisition for various topics. These knowledge producers (KP) are the backbone of this community, aiding in resolving problems in small-scale plant production and propelling us towards large-scale plant production. Their scientific and practical findings are for the developing sector and the knowledge users (KU) seeking solutions and innovations in developing and commercializing their products. The KU participation, currently at 11%, is a call for further encouragement and the implementation of effective strategies for their broader inclusion.

CopyTree is structured around five working groups (WG), each with a specific focus and broad participation of members who have voluntarily joined one or more groups (Tab. 1). A positive correlation is observed between the membership percentage in each WG and affiliation distribution. As mentioned before, the scientific community part of universities or research institutes comprises the central part of membership, and this explains the high participation in the first three working groups, which are WGs directly related to scientific research.

**Table 1.** Distribution of members in CopyTree Working Groups

| **COPYTREE = 285 members / 43 countries (October 2022 - June 2024)** | | |
|---|---|---|
| **WG number** | **WG name** | **No. of members** |
| **WG1** | Recalcitrance | 176 |
| **WG2** | Diagnosis, sanitation and conservation | 159 |
| **WG3** | Scaling up and automation | 144 |
| **WG4** | Technological risk assessment, public acceptance, legislation & commercialization | 88 |
| **WG5** | Communication, dissemination and technology transfer | 89 |

These 5 WGs cover specific areas related to *in vitro* methodologies applied to woody plants. However, they are also closely related and work as a single body to achieve CopyTree overall goal (Fig. 2).

As knowledge producers, WG1, WG2, and WG3 are at the forefront of scientific research, tackling issues such as recalcitrant plant species, virus-free plant production, conservation of plant material for different time periods, and using robotics and automation to enhance plant production. Their work demonstrates CopyTree unwavering commitment to cutting-edge technologies and innovative solutions. In some cases, these WGs may interact for specific research objectives, such as virus-free production and conservation of recalcitrant species, enhancing micropropagation through automation for recalcitrant species, etc.

On the other hand, WG4 plays a essential role in large-scale plant production and commercialization, addressing concerns such as safety risk assessment, legislation, and public acceptance. This group, which includes the KU for the development sector and other third parties, is responsible for facilitating the transition from research to large-scale production. A direct connection exists between the first three groups and WG4, which ensures the transfer of biotechnological production to large-scale production using the latest scientific findings.

WG5, the final group in this chain of processes, is the general umbrella for all the CopyTree activities, particularly for knowledge sharing and stakeholder engagements. As the last link in the chain, it is directly related to all other WG activities, both on an independent and interdependent level. WG5 is a crucial group that plays a significant role in the dissemination process, engaging more third parties, raising awareness, and fostering collaboration, thereby making every stakeholder feel valued and integral to the project.

About 54% of the members of WG4 and WG5 are senior researchers, which is expected because of their professional experience in filling the gap between Research and Development and the necessity of designing effective strategies and implementing concrete measures to improve this connection.

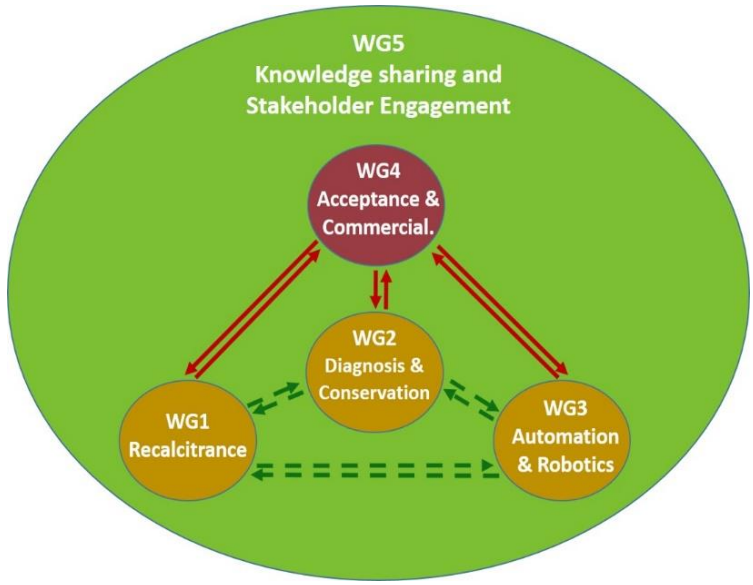


**Figure 2.** Independence and inter-dependence between CopyTree Working Groups

## 3. FUTURE PERSPECTIVES ON OPTIMIZING STAKEHOLDER ENGAGEMENT AND KNOWLEDGE-SHARING

The statistical data presented above show that the short-term objectives of CopyTree should be to find ways to optimize the interaction between Knowledge Producers (KP) and Knowledge Users (KU). Potential stakeholders important for the CopyTree community include commercial laboratories, nurseries, fruit producers, forest tree planting material, suppliers of chemicals and equipment, land owners and associated foresters, policymakers, risk assessors, biodiversity organizations, media, etc. CopyTree members collaborate and work daily for knowledge sharing and stakeholder engagement on woody plant cloning through the following activities:

- Lecturing in universities or other educational institutions to share the knowledge from senior to young researchers;
- Submitting R & D proposals on various national or international calls for projects by identifying gaps on a specific topic and justifying the need for improvement and how the society can benefit from it;
- Driving business innovation by sharing and testing new scientific findings and applications, helping the implementation of start-ups;

- Collaborating with consortiums and partnerships with various institutions and agribusinesses abroad and thus boosting agriculture biotechnological product commercialization

CopyTree has clearly defined its mission and vision of applying innovative methods to *in vitro* technology for woody plants, enhancing its commercialization and public acceptance. An effective strategy must be established to reach this objective, which can be achieved by implementing concrete actions with measurable results. This strategy must take into consideration two main questions:

- Why is this important for the society?
- Why does it matter to stakeholders and other interested third parties?

For the above, it is necessary to clearly explain the importance of this Action, focusing on its mission and vision and what is expected to benefit society. This should be done using a communication language easy to understand by different interested parties with low or medium knowledge of plant *in vitro* techniques.

### 3.1. What needs to be done to achieve the desired results for effective engagement?

Implementing concrete actions by explaining the aim and scope of this Action and avoiding scientific language about this process is crucial. This is because active participation and understanding are critical to the success of the CopyTree project.

- Directly inviting companies benefiting from CopyTree results, something that will help in a more precise explanation of CopyTree objectives and understanding of the benefits that these companies can gain from these collaborations;
- Identifying and prioritizing user interests, which is of great interest since CopyTree activities in the future can be focused on the concrete problems of interested parties directly connected to consumers;
- Conducting listening sessions with targeted stakeholders, a process that helps defining the barriers to better interaction between the research institutions and development sectors and the efforts to overcome these barriers;
- Launching awareness-raising campaigns on social media channels, which are very important to inform the general public about the advantages of *in vitro* technologies, the safety of consumption of food produced in this way, the benefits of environmental protection and biodiversity, etc. These campaigns can focus on specific topics, for example:
  - 10 reasons why ……
  - 10 advantages of ……..
  - 10 profits of ……
  - 10 perspectives on …….
  - 10 misconceptions about …….

Under this plan, all WGs are responsible for the suggested activities. Each WG will work independently within their specific topics and interact with each other to reach the consumer and provide effective public information.

## 4. CONCLUSION

The CopyTree network implements an effective management strategy for knowledge-sharing and stakeholder engagement to increase the effectiveness of innovative *in vitro* methods applied on micropropagation, sanitation, conservation and breeding of woody plant species. The community comprises 285 members from 43 countries, affiliated with universities, research institutes, and other public or private companies. The community's efforts focus on increasing cooperation between Knowledge Producers (KP) and Knowledge Users (KU), aiming to facilitate the transfer from small-scale to large-scale plant production and increase public awareness and acceptance. Future strategies for bridging the gap between research and development include concrete actions like directly involving companies that benefit from CopyTree results, conducting stakeholder listening sessions, and launching targeted social media awareness campaigns. Each working group within CopyTree is working on implementing these strategies independently and collaboratively, aligning with CopyTree mission and goals to optimize the mass production and conservation of woody plant germplasm.

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