The influence of air dehydration time in the cryopreservation process of *Polyscias filicifolia* transformed roots on their genetic stability and phytochemical profile

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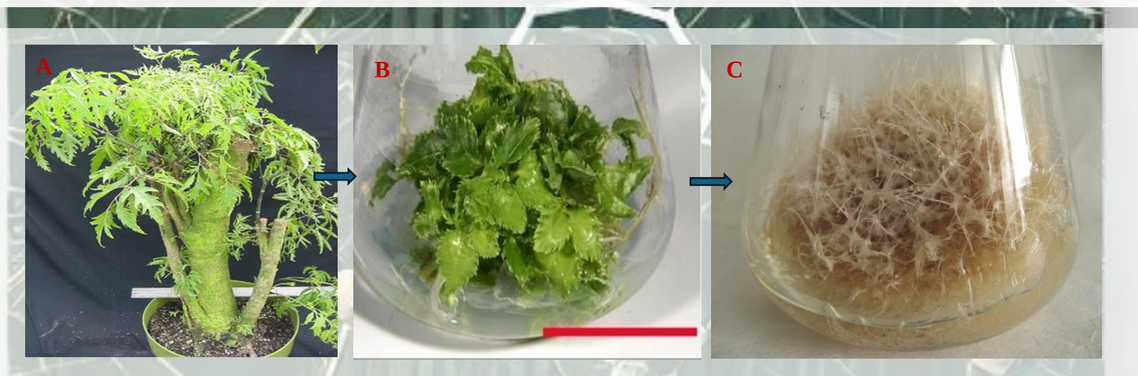

Fig. 1 Roots of *P. filicifolia* obtained in *in vitro* culture using the bacterial strain *Agrobacterium rhizogenes* ATCC 15834.

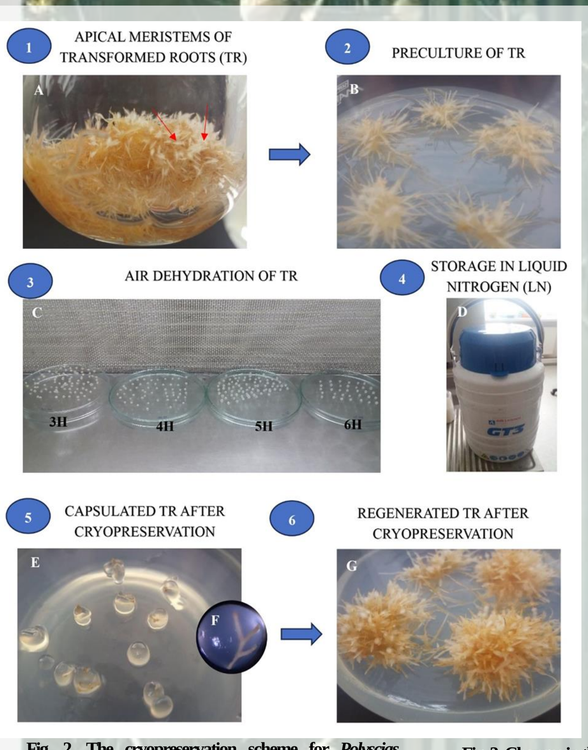

Fig. 2. The cryopreservation scheme for *Polyscias filicifolia* K-1 transformed root line.

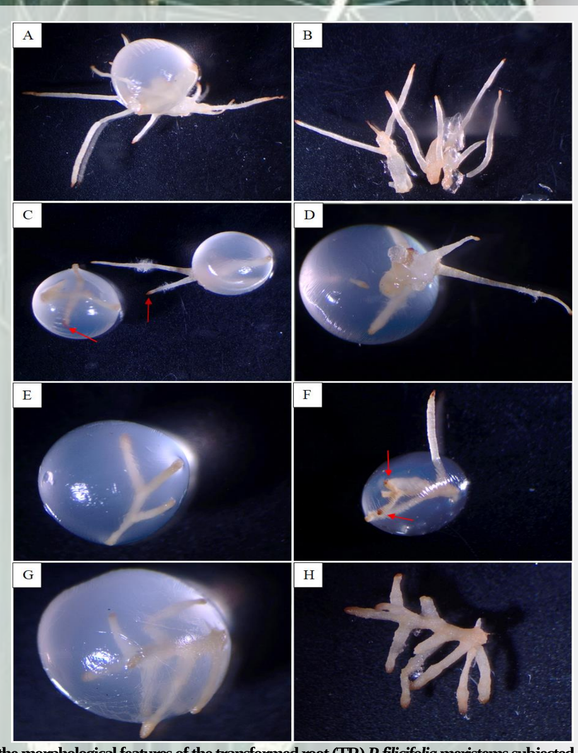

Fig. 3. Changes in the morphological features of the transformed root (TR) *P. filicifolia* meristems subjected to various forms of cryopreservatoin after two weeks of culture on solid DCR-M medium: (A) non-cyopreserved TR; (B) after capsule removal, demonstrating a number of visible lateral roots without any meristem damage; (C) after cryopreservation and osmotic dehydration (OD), demonstrating visible brown ends to the apical meristems; (D) after cryopreservation and OD combined with three-hour air dehydration (AD), demonstrating visible callus tissue with thin lateral roots, (E) after cryopreservation and OD combined with four-hour AD carried, demonstrating short lateral roots; (F) after cryopreservation and OD combined with five-hour AD, demonstrating a few long thin lateral roots with brown apical meristems; (G and H) after cryopreservation and OD combined with six-hour AD, demonstrating very numerous, thick lateral roots without any signs of meristems browning.

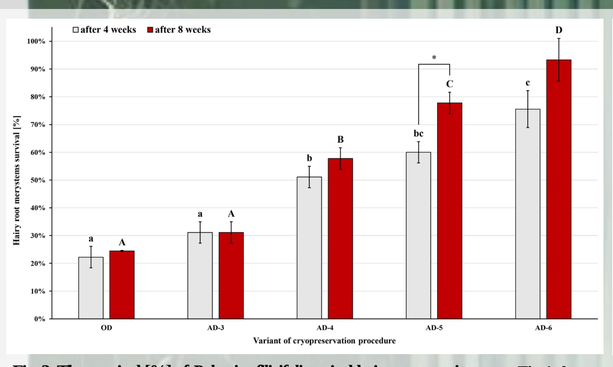

Fig. 3. The survival [%] of *Polyscias filicifolia* apical hairy root meristems subjected to dehydration procedures: OD – osmotic dehydration without air dehydration (AD); AD-3: OD combined with three-hour AD; AD-4: OD combined with four-hour AD; AD-5: OD combined with five-hour AD; AD-6: OD combined with six-hour AD. Asterisks (*) indicate statisically significant differences between data obtained from the same variant cultivated for four or eight weeks according to Tukey's HSD post-hoc test ($p<0.05$). Various lower/upper-case letter above each bar indicate statistically significant differences between culture variants according to Tukey's HSD post-hoc test ($p<0.05$).

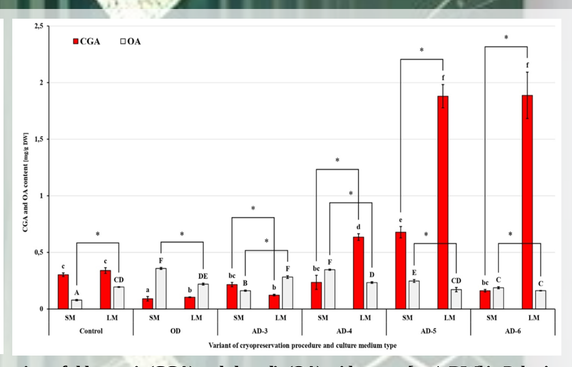

Fig 4. A comparison of chlorogenic (CGA) and oleanolic (OA) acid content [mg/g DW] in *Polyscias filicifolia* transformed roots (TR) cultivated on solid medium (SM) or in liquid (LM) DCR-M medium after six week– after OD and three-hour air dehydration (AD); AD-4 – after OD and four-hour AD; AD-5 – after OD and five-hour AD; AD-6 – after OD and six-hour AD. Asterisks (*) indicate statistically significant differences between data obtained during cultivation on solid or in liquid medium within the same culture variant according to Tukey's HSD post-hoc test ($p < 0.05$).

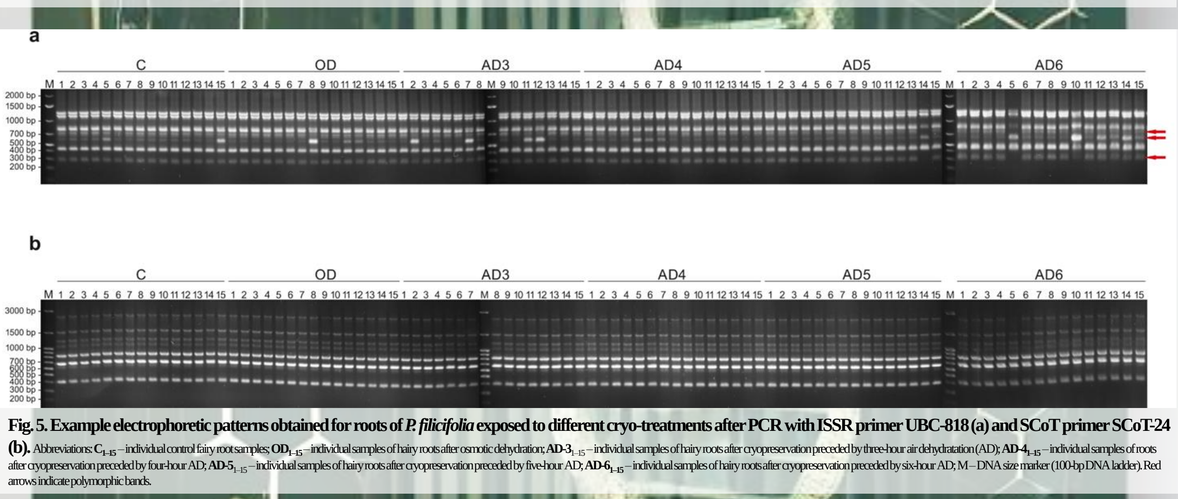

Fig. 5. Example electrophoretic patterns obtained for roots of *P. filicifolia* exposed to different cryo-treatments after PCR with ISSR primer UBC-818 (a) and SCoT primer SCoT-24 (b). Abbreviations: C_{1-15} – individual control fairy root samples; OD_{1-15} – individual samples of hairy roots after osmotic dehydration; $AD\text{-}3_{1-15}$ – individual samples of hairy roots after cryopreservation preceded by three-hour air dehydration (AD); $AD\text{-}4_{1-15}$ – individual samples of roots after cryopreservation preceded by four-hour AD; $AD\text{-}5_{1-15}$ – individual samples of hairy roots after cryopreservation preceded by five-hour AD; $AD\text{-}6_{1-15}$ – individual samples of hairy roots after cryopreservation preceded by six-hour AD; M – DNA size marker (100-bp DNA ladder). Red arrows indicate polymorphic bands.

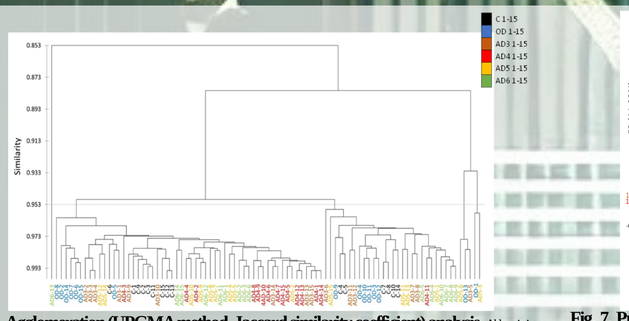

Fig. 6. Agglomeration (UPGMA method, Jaccard similarity coefficient) analysis. Abbreviations: C_{1-15} – individual control hairy root samples; OD_{1-15} – individual samples of hairy roots after osmotic dehydration; $AD\text{-}3_{1-15}$ – individual samples of hairy roots after cryopreservation preceded by three-hour air dehydration (AD); – individual samples of roots after cryopreservation preceded by four-hour AD; $AD\text{-}5_{1-15}$ – individual samples of hairy roots after cryopr,eservation preceded by five-hour AD; $AD\text{-}6_{1-15}$ – individual samples of hairy roots after cryopreservation preceded by six-hour AD Abbreviations: control (C_{1-15}, osmotically dehydrated (), cryopreserved with three-hour air desiccation ($AD\text{-}3_{1-15}$), cryopreserved with four-hour air desiccation ($AD\text{-}4_{1-15}$), cryopreserved $OD_{1-15}$with five-hour air desiccation (AD51–15), and cryopreserved with six-hour air desiccation ($AD\text{-}6_{1-15}$).

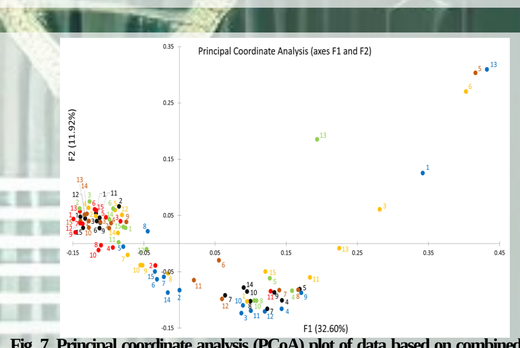

Fig. 7. Principal coordinate analysis (PCoA) plot of data based on combined ISSR and SCoT markers. The F1 and F2 axes are indicated by the first and second coordinates, respectively, and the values in parentheses show the percentages explaining the community variation. Black, blue, brown, red, orange, and green symbols represent control hairy roots (not cryopreserved) (C_{1-15}), individual control hairy root samples non-cryopreserved; (OD_{1-15}) individual samples of hairy roots after osmotic dehydration (OD), ($AD\text{-}3_{1-15}$) individual samples after three-hour air dehydration (AD) and cryopreservation; ($AD\text{-}4_{1-15}$), individual samples after four-hour AD and cryopreservation; $AD\text{-}5_{1-15}$)(individual samples after five-hour AD and cryopreservation; ($AD\text{-}6_{1-15}$) individual samples after six-hour AD and cryopreservation.

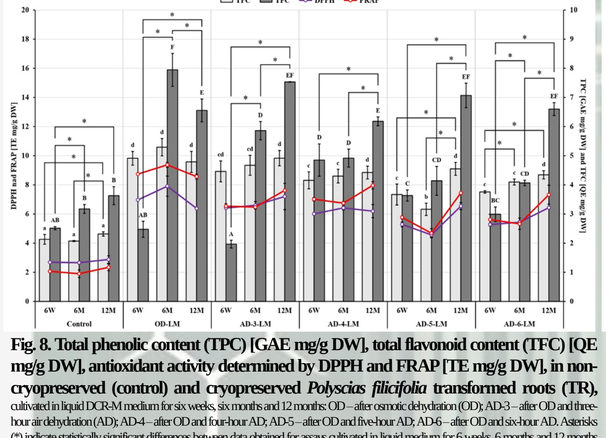

Fig. 8. Total phenolic content (TPC) [GAE mg/g DW], total flavonoid content (TFC) [QE mg/g DW], antioxidant activity determined by DPPH and FRAP [TE mg/g DW], in non-cryopreserved (control) and cryopreserved *Polyscias filicifolia* transformed roots (TR), cultivated in liquid DCR-M medium for six weeks, six months and 12 months: OD – after osmotic dehydration (OD); AD-3 – after OD and three-hour air dehydration (AD); AD-4 – after OD and four-hour AD; AD-5 – after OD and five-hour AD; AD-6 – after OD and six-hour AD. Asterisks (*) indicate statistically significant differences between data obtained for assays cultivated in liquid medium for 6 weeks, 6 months and 12 months within the same culture variant according to Tukey's HSD post-hoc test ($p < 0.05$).

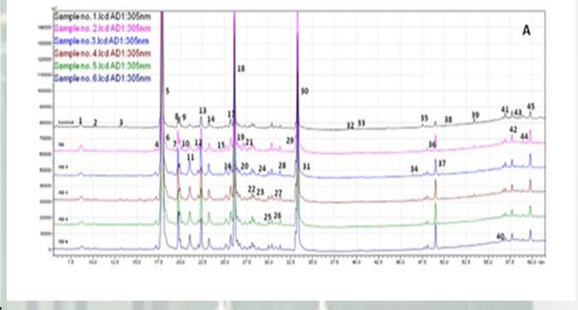

Fig. 9. HPLC-PDA-ESI-MS chromatograms of methanolic extract derived from *Polyscias filicifolia* transformed roots cultivated in liquid DCR-M under various culture conditions.

1 Introduction

Cryopreservation is a critical tool in the conservation of valuable plant genetic resources, particularly for medicinal species like *Polyscias filicifolia*, a member of the Araliaceae family (Fig.1).This plant is known for its rich production of secondary metabolites such as triterpenoid saponins and phenolic compounds, which have shown antioxidant, antibacterial, and antimutagenic properties. However, one of the main challenges in plant cryopreservation is maintaining genetic, physiological, and biochemical integrity over long-term storage.

Hairy root cultures, obtained through infection with *Agrobacterium rhizogenes*: ATCC 15834, are highly suitable for this purpose because of their rapid growth and enhanced biosynthesis of secondary metabolites compared to undifferentiated tissues. In this study, we aimed to optimize the cryopreservation of *Polyscias filicifolia* hairy roots using encapsulation-dehydration, a technique designed to protect root meristems during freezing, while ensuring the recovery of genetically stable and biochemically active material post-regeneration.

Objectives

- Develop an efficient cryobanking method for *Polyscias filicifolia* hairy roots.
- Assess the genetic stability of regenerated roots using molecular markers and flow cytometry.
- Evaluate the physiological and phytochemical stability of stored roots, focusing on the production of chlorogenic acid (CGA) and oleanolic acid (OA).
- Investigate the antioxidant potential of root extracts post-cryopreservation.

2 Materials and Methods

Plant Material:

The study was conducted on the *Polyscias filicifolia* K-1 hairy root line, established through transformation with *Agrobacterium rhizogenes*. The cultures were grown in modified DCR-M liquid medium, and apical root meristems were used for cryopreservation (Fig. 1).

Cryopreservation Protocol:

The encapsulation-dehydration method involved encapsulating apical meristems in sodium alginate capsules, followed by osmotic dehydration (OD) using sucrose solutions of increasing concentration (0.3 M to 1 M). After OD, the samples were subjected to four different air dehydration (AD) periods: 3, 4, 5, or 6 hours, before freezing in liquid nitrogen (Fig. 2, 3).

Genetic Stability:

After regeneration, the genetic stability of the roots was assessed using Inter Simple Sequence Repeats (ISSRs) and Start Codon Targeted (SCoT) markers. Flow cytometry was employed to measure nuclear DNA content, ensuring no significant changes occurred during cryopreservation.

Phytochemical Analysis:

Quantitative and qualitative analysis of chlorogenic acid (CGA) and oleanolic acid (OA) in the roots was performed using HPLC-UV-Vis and HPLC-PDA-ESI-HRMS. The antioxidant capacity of the extracts was determined using DPPH and FRAP assays.

Biomass and Growth Rate:

The growth of cryopreserved roots was measured by fresh and dry biomass increase after 6 weeks, 6 months, and 12 months of culture.

3 Results

1. Survival and Biomass Growth

The cryopreservation method, particularly with a 6-hour AD, led to a high survival rate (93%) and an impressive biomass increase (up to 3-fold) compared to control roots (Fig. 3) The roots subjected to 6-hour AD showed the best regrowth, while those subjected to shorter dehydration periods (3-4 hours) displayed lower survival rates and reduced growth (Fig. 3).

Interestingly, while prolonged dehydration (5-6 hours) improved biomass growth, it had a negative impact on genetic stability, leading to a slight increase in somaclonal variation, particularly with the 6-hour AD treatment.

2. Genetic Stability

Genetic stability, measured using ISSR and SCoT markers, revealed that the cryopreservation process did not significantly affect the overall genetic profile of the roots. The highest genetic stability was observed with 4-hour AD treatment, where the roots displayed the lowest level of polymorphism compared to control samples. Flow cytometry analysis confirmed no significant changes in nuclear DNA content between cryopreserved and control roots, indicating the absence of polyploidy or large-scale chromosomal abnormalities (Fig. 5, 6, 7).

3. Phytochemical Stability and Antioxidant Activity

Chlorogenic Acid (CGA) Production: Cryopreservation enhanced CGA content, particularly in roots subjected to 6-hour AD, which reached 2.6 mg/g dry weight (DW) after six months of regeneration and remained stable (2.2 mg/g DW) after 12 months. The production of CGA was significantly higher in cryopreserved roots compared to controls (Fig. 4, 9).

Oleanolic Acid (OA) Production: OA content remained relatively stable across all treatments and did not significantly differ between cryopreserved and non-cryopreserved roots. This suggests that OA biosynthesis is not heavily influenced by the cryopreservation process (Fig. 4).

4. Antioxidant Activity:

Extracts from roots subjected to OD and AD treatments demonstrated significantly higher antioxidant activity, as measured by DPPH and FRAP assays. The highest antioxidant potential was recorded in the OD-only variant, correlating with increased total phenolic content (TPC) (Fig.8).

4 Conclusion

Encapsulation-dehydration, particularly with a 4-hour air dehydration step, represents an optimal cryopreservation method for *Polyscias filicifolia* hairy roots, ensuring high survival rates, genetic stability, and sustained production of valuable secondary metabolites like chlorogenic acid. This approach could serve as a model for the biobanking of other medicinal plant species, ensuring their long-term conservation and availability for pharmaceutical research and development.