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ORIGINAL ARTICLE

Sweet cherry rootstock micropropagation using SETIS™ bioreactor: evaluation, challenges, and biochemical characterization of regenerated shoots

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Abstract

In vitro propagation of *Prunus* sp. using semi-solid media allows clonal multiplication but requires frequent subcultures and higher labor and material costs. In contrast, temporary immersion bioreactors (TIBs) promoted faster shoot elongation, higher biomass accumulation, and improved overall shoot quality, while reducing handling time and the cost per regenerated plant. This work aimed to establish in vitro selected rootstocks of *Prunus avium* and Gisela 6 (*Prunus cerasus* x *Prunus canescens*) and compare the multiplication and rooting processes of shoots obtained in different culture conditions, in semi-solid media and SETIS™ bioreactors a system that alternates brief liquid immersion with aeration to enhance growth and shoot quality. Process efficiency and shoot growth parameters were evaluated, including in vitro establishment percentages, shoot length, number of shoots, and Relative Growth Rate (RGR). Physiological and biochemical analysis were also conducted, such as photosynthetic pigments content, total flavonoids, malondialdehyde (MDA), and proline levels. The in vitro percentage establishment was 56% for *P. avium* and 62% for Gisela 6, consistent with seasonal conditions favoring lower contamination. While no statistical differences were observed in the number of shoots between systems or genotypes, shoot elongation was significantly higher in TIB, particularly for Gisela 6. The RGR index confirmed the superior performance of TIB in promoting shoot proliferation, and rooting capacity was also improved, with 57% rooting in Gisela 6 and 44% in *P. avium* from TIB compared to 16% and 10% in semi-solid medium, respectively. Biochemical analyses revealed that *P. avium* propagated in TIB showed reduced pigment levels and signs of stress likely related to hyperhydricity, whereas Gisela 6 exhibited greater stability across both systems. Overall, the results demonstrate that TIB is a more efficient alternative to semi-solid media for the micropropagation of sweet cherry rootstocks, enhancing shoot quality, growth, and *ex vitro* rooting, although genotype-specific responses highlight the need for further optimization.

Key message

Temporary immersion bioreactors enhance shoot growth and relative growth rate in sweet cherry rootstocks, offering a cost-effective alternative to semi-solid media for large-scale micropropagation.

Keywords Large-scale production · *Prunus* spp. · Rootstocks · Semi-solid media · Temporary immersion bioreactors (TIB)

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Introduction

Sweet cherry (*Prunus avium* L.) is a well-known Rosaceae tree, cultured mainly for its edible fruits. Although cultivated in both Hemispheres, Prunus species are naturally distributed throughout the Northern Hemisphere, being highly represented in Europe (Baránek et al. 2006). Almonds, apricots, and peaches are among the most representative Prunus crops, and prefer warmer temperate regions influenced by a Mediterranean climate, while cherries and plums are better adapted to cooler temperate areas of the world. However, regardless of their climatic preferences, all Prunus species require adequate winter chilling to ensure effective fruit set and optimal production (Guajardo et al. 2020).

In Portugal, the sweet cherry production is mostly located in the interior north and centre of the country. According to Portuguese statistics (INE 2021), the 2021 harvest was considered the most productive of the last 50 years, with a total of 23.9 thousand tons. These outcomes are driven by the growing number of specialized cherry farms adopting improved cultivars and advanced technologies, highlighting the increasing demand for large-scale production of high-quality rootstocks and scion varieties to support the continuous improvement of this production sector.

Grafting is the most common method used for sweet cherry propagation, resulting in orchard trees composed of two genetically distinct components: the scion (aerial part - cultivar) and the rootstock. This grafted union creates a strong cultivar-rootstock relationship, which significantly influences performance and productivity, including parameters such as blossom, fruit set, fruit size, sugar content (Albacete et al. 2015), as well as tolerance to biotic and abiotic stresses (Guajardo et al. 2020; Opazo et al. 2020).

To maintain high-quality growing standards required by the worldwide industry, it is imperative to implement clonal propagation systems for elite scion and rootstock materials (Mauro et al. 2020). Tissue culture techniques facilitate the rapid production of genetically identical plants, requiring minimal space, supplies and time. However, the application of these achievements in fruit trees, particularly in Prunus, is often complex and must overcome the intrinsic biological characteristics of the different genotypes, including their recalcitrance to the current propagation methods (Martínez-Gómez et al. 2005).

Several methods have been used to establish *P. avium* cultivars and rootstocks with efficient micropropagation protocols. Described methodologies for the in vitro propagation of cherries include the use of stem sections between nodes from one-year-old shoots (Feucht and Dausend 1976), shoot tips (Hammatt and Grant 1998; Németh 1979; Sedlák and Paprštein 2008), embryos (Ivaniĉka & Pretová, 1980), and in vitro micrografting (Amiri 2006). Overall, these studies have shown a significant dependence on genotype for both propagation and regeneration abilities (Feeney et al. 2007; Godoy et al. 2017; Zilkah et al. 1992).

The use of liquid media-based methods is often regarded as the optimal solution for micropropagation, due to superior nutrient availability, improved contact with explants, and the ability to support faster growth and higher multiplication rates compared to semi-solid media (Preil 2005). However, technical challenges such as anoxia and hyperhydricity can counterbalance these advantages (Etienne and Berthouly 2002). Temporary immersion systems (TIS) have become one of the most popular methods to overcome such problems (Hwang et al. 2022; Vidal et al. 2015), by enabling temporary contact between the plants and the liquid medium, thus avoiding continuous immersion and providing adequate oxygen transfer to the cultures (Alvard et al. 1993; Etienne and Berthouly 2002; Teisson and Alvard 1995). The increased absorption of nutrients via the liquid medium together with the renewal of the air inside the bioreactors may improve the physiological state of the explant (Vidal and Sánchez 2019).

The purpose of using bioreactors is to control chemical or physical parameters to create optimal growth conditions. The main objective is to achieve a high yield and quality of explants while minimizing production costs through the integration of low-cost devices and automated facilities (Preil 2005). These systems operate by periodically immersing plantlets in liquid medium using timed cycles of immersion and drainage, regulated by solenoid valves (Hwang et al. 2022; Kim et al. 2020). An advanced example is the SETIS™ bioreactor, which improves upon previous TIS designs with a compact, user-friendly structure that maximizes surface utilization. It features a multi-stage process involving nutrient transfer through compressed air, timed immersion, gravity-based drainage, and active ventilation to optimize growth conditions (Vervit, 2024).

This work aimed to establish the traditional rootstock *P. avium* and the commercial rootstock Gisela 6 (*Prunus cerasus* x *P. canescens*) in vitro and compare the multiplication and rooting processes of shoots in different culture conditions: semi-solid media and temporary immersion bioreactors (TIB) using SETIS™ system. The efficiency of the newly developed protocol was assessed by the evaluation of shoot multiplication and growth parameters, including in vitro establishment percentages, shoot length, number of shoots, and Relative Growth Rate (RGR), as well as by the measurement of physiological and biochemical parameters, such as photosynthetic pigments content, total flavonoids, malondialdehyde (MDA), and proline levels.

Materials and methods

Plant material

P. avium (Pa), commonly known as wild cherry and used as the standard seedling rootstock for sweet cherry 'Franco de Cerejeira' and Gisela 6 (G6) rootstocks were kindly provided by Viveiros Miguel Vaz Miranda do Corvo, Portugal (40.101997° N, 8.311087° W). The plant material was collected directly from a mother plant field during spring sprouting and stored on ice until it was brought to the laboratory and established in vitro as described in the following sections.

In vitro establishment

Young branches with 20 cm were cut and stored on ice. In the laboratory, the nodal segments were cut and immediately immersed in a solution (1.g L^{-1}) of the fungicide Mancozebe (Bayer®) for 15 min, followed by 70% (v/v) ethanol washing for 30 s. The plant material surface was then sterilized with 5% (w/v) sodium hypochlorite (Sigma-Aldrich®) containing (2–3) drops of Tween 20 (Sigma-Aldrich®) for 10 min. After the surface disinfection, the plant material was washed 3 times with sterile deionized water. Lastly, the explants were cut and placed in MS (Murashige and Skoog 1962) (Duchefa Biochemie) medium without plant growth regulators (PGRs) in assay tubes measuring 16 mm in diameter and 150 mm in length. The initial number of nodal segments cultured in vitro, was 37 for Gisela 6 and 53 for *P. avium*. The cultures were maintained in a growth chamber at 25 °C under a 16-hour light photoperiod. The percentage of in vitro establishment was taken after 28 days of culture.

Shoot proliferation in semi-solid and SETIS™ bioreactor

The culture media for shoot propagation was chosen according to Sharma et al. (2017). MS basal media was supplemented with 0.44 µM of N^6-benzyladenine (BA), 0.29 µM gibberellic acid (GA_3), 3% (w/v) sucrose, 0.7% (w/v) agar and the pH was adjusted to 5.8. After twelve subcultures, propagation assays were conducted in semi-solid media in plastic Microbox containers (Sac O2) with a cover diameter of 118 mm, base diameter of 90 mm, height of 120 mm, and fill volume of 870 mL, complemented with white filters providing a gas exchange (GE) rate of 9.87 day^{-1}. For each Microbox containing 150 mL of medium, $N=10$ explants were used, totaling $N=60$ per treatment. Both nodal and apical shoots (1 cm long) were used as explants in all treatments.

In parallel, shoots were cultured in SETIS™ bioreactors. The culture vessel had a volume of 6 L and the media vessel 4 L. Stage 1 corresponded to the stationary phase, with the medium remaining in the lower container. In stage 2, the medium was circulated to contact the explants for 5 min every 8 h. For each bioreactor containing 2 L of medium, $N=60$ explants were used. Both nodal and apical shoots were also employed in the SETIS™ assays. No forced ventilation was applied between immersion cycles because the available equipment did not allow independent control of gas exchange. After 28 days, shoot length and number of shoots were recorded. Relative Growth Rate (RGR) was calculated as (Ln W2 – Ln W1) × 100 / (t2 – t0), where W1 and W2 are, respectively, the initial and final fresh weights (g), and t0 and t2 represent the starting and ending times of the 4-week subculture (Poorter and Garnier 1996). All plants were maintained under standard conditions (light photoperiod and temperature).

Rooting and aclimatization

The explants coming from the two types of propagation were rooted *ex vitro* by basal dipping with 1 g L^{-1} of Indole-3-butyric acid (IBA), for 1 min and transferred to a commercial SIRO® Turfa 45 − 0 mixed with perlite (80:20), and planted in plastic trays with 80 cm^3 per cell. Shoots approximately 1.5–2.0 cm in length and containing at least two internodes were used as explants; smaller or poorly developed shoots were discarded prior to rooting. The induced explants were maintained in the climatic chamber at 25 °C under a 16 h light photoperiod. The number of explants evaluated for rooting differed among genotypes and propagation systems due to differences in plant availability and survival (G6 semi-solid $n=25$; G6 TIB $n=20$; *P. avium* semi-solid $n=40$; *P. avium* TIB $n=23$). Rooting percentage was calculated based on these totals. Root number and root length were measured only in rooted plantlets presenting sufficiently developed and intact root systems suitable for evaluation. After 28 days, the rooting rate, the root length, and the number of roots per explant were registered.

Biochemical analysis

After 28 days of culture, explants from both the semi-solid medium and temporary immersion bioreactor treatments consisting of shoots including both stems and leaves, were immediately flash-frozen in liquid nitrogen, ground to a fine powder using a mortar and pestle under liquid nitrogen to prevent degradation, then lyophilized and stored at room temperature until being analyzed. Explants showing a high level of hyperhydricity were discarded prior to analysis.

Photosynthetic pigments quantification

The analysis of photosynthetic pigments was carried out to evaluate the physiological performance of plants grown under two different culture systems: semi-solid medium and temporary immersion bioreactor (TIB).

Photosynthetic pigment analysis was performed using 12 biological replicates per treatment, and chlorophyll a, chlorophyll b and carotenoids were determined according to Lichtenthaler (1987). Approximately 10 mg of dry lyophilized tissue powder was extracted using 1 mL of 80% cold ethanol, followed by 3 times vortexing for 10 s each, ultrasonic bath for 10 min and centrifuged at 10,000 g for 10 min at 4 °C. A total of 750 µl of supernatant was obtained per sample. From this, 300 µl were transferred to a microcentrifuge tube and diluted 1:1 with 80% cold ethanol. Then, 150 µl per sample were placed in a microplate well, and the absorbances were read at 470, 649, and 664 nm using a SpectraMax PLUS 384 (Molecular Devices, San Jose, CA, USA). The results were expressed in µg/g DW and calculated using the following equations:

$$\text{Chlorophyll a (Chl a)} = 13.36\,A664 - 5.19\,A649$$

$$\text{Chlorophyll b (Chl b)} = 27.43\,A649 - 8.12\,A664$$

$$\text{Carotenoids} = (1000\,A470 - 2.13\,\text{Chl a} - 97.63\,\text{Chl b})/209$$

Flavonoids

Total flavonoids were quantified using 12 biological replicates per treatment according to Zhishen et al. (1999) with some adaptations by Martins et al. (2022). Briefly, 1 mL of methanol (70%, v/v) was added to 10 mg of dry tissue powder. Samples were kept on an orbital shaker at 700 rpm, 25 °C for 1 h and centrifuged for 15 min, 10,000 g, 4 °C. The supernatant was collected, and the extraction was repeated twice. The final volume was adjusted to 2.5 mL with methanol (70%, v/v).

For quantification, a 60 µL sample was mixed with 28 µL sodium nitrite (5%, w/v). The samples were incubated in the dark for 6 min, followed by the addition of 28 µL aluminium chloride (10%, w/v). Finally, 120 µL sodium hydroxide (4%, w/v) was added. The absorbance was read at 510 nm in a microplate reader. The concentration of flavonoids was determined as catechin equivalents from a standard curve (0-125ug, $y = 201.89x - 15.633$, $R^2 = 0.9951$).

Malondialdehyde (MDA) quantification

The same supernatant used for the photosynthetic pigments analysis was then used for the MDA assay. The MDA quantification was performed using 6 biological replicates per treatment based on the protocol described by Hodges et al. (1999) and Landi, (2017). An equal volume (250 µl) of the supernatant was mixed with the TBA-containing reaction solution (+TBA; 0.5% TBA in 20% (w/v) trichloroacetic acid) in a 1.5 mL screw-capped microcentrifuge tube. In parallel, the same procedure was carried out using the TBA-free reaction solution (−TBA; 20% (w/v) trichloroacetic acid). Blank tubes consisted of 250 µL of 80% ethanol with 250 µL of positive reaction solution or negative reaction solution. The tubes were incubated at 95 °C for 30 min in a heat block (BOECO Thermo-shaker for Microtubes TS-100). The reaction tubes were cooled at room temperature (RT) and centrifuged at 3,000 g for 10 min at 4 °C, and the supernatant was recovered. 150 µL of each sample or blank were transferred to a clear 96-well microplate and the absorbance of each well was measured at 440, 532 and 600 nm. MDA equivalents were calculated in (nmol/mL) as (A – B)/157.000) × 106, in which A = [(A532+TBA) − (A600+TBA) − (A532−TBA−A600−TBA)] and B = [(A440+TBA−A600+TBA) × 0.0571]. Each absorbance reading was repeated three times.

Proline quantification

Total proline in the extracts was measured using 3 biological replicates per treatment, following the method described by Bates et al. (1973). Briefly, 1.5 mL of 3% (v/v) sulfosalicylic acid was added to 50 mg of lyophilized plant material, which was then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant (500 µL) was collected and mixed with 500 µL of a solution containing 1.25 g of ninhydrin, 30 mL of glacial acetic acid and 20 mL phosphoric acid. The samples were incubated at 100 °C for 1 h and subsequently cooled on ice. The reaction mixture was extracted with toluene, and the absorbance of the chromophore-containing toluene was measured at 520 nm using quartz cuvettes in a Thermo Scientific™ GENESYS™ 140 Visible Spectrophotometer. Proline concentration was determined using a standard curve prepared with L-Proline (0–15 mg, $y = 0.0096x + 0.2471$, $R^2 = 0.9668$).

Experimental design and statistical methods

To compare genotypes under different proliferation system conditions, physiological and biochemical data were analyzed using non-parametric statistical methods, as the data did not follow a normal distribution. Pairwise comparisons were performed using the Mann–Whitney U test ($P < 0.05$). All statistical analyses were conducted using GraphPad Prism (v. 8.4.3 for Windows, San Diego, CA, USA). Results are presented as medians.

Results and discussion

In vitro establishment

In the present study, the in vitro establishment rate was evaluated after 28 days for two genotypes. Successful establishment percentages were obtained (56% for Prunus avium and 62% for Gisela 6). Nodal buds were collected and established in vitro from the year branches (Fig. 1). The choice of explant is a critical factor influencing the success of in vitro establishment. In sweet cherry, both nodal buds and shoot tips can be used as explants due to their genetic stability (Druart 2013). In woody fruit trees, the success of the initial stage of micropropagation (in vitro establishment) is influenced by several factors, including the type of explant, the sterilization procedure, the physical and chemical conditions of the culture medium, as well as the meteorological conditions at the time of explant collection (Leal et al. 2007; Pérez-Tornero and Burgos 2007). In our case, the establishment was performed during spring, in a period with low percentage of humidity due to unusually dry conditions, which may have influenced the obtained results. Several studies have proven that explants collected in spring or summer were found to be less contaminated than those collected in autumn or winter (Hutchison 1984; Modgil et al. 1999; Webster & and Jones, 1991).

In recent years, significant advances have been made in the development of in vitro propagation protocols for woody species, particularly those of the genus *Prunus*, due to their high economic value for fruit and rootstock production. However, conventional micropropagation using semi-solid media can present limitations, such as low multiplication rates, high labor costs, and physiological disorders, including hyperhydricity or callus formation. To address these challenges, alternative propagation systems, particularly TIS have been increasingly adopted. TIS allow for periodic contact between the explants, improving nutrient uptake and gas exchange while reducing the risk of hyperhydricity. These systems have demonstrated promising results in several *Prunus* species, showing higher shoot proliferation rates, improved plant quality, and enhanced rooting efficiency when compared to traditional methods. Lopes et al. (2023) reported successful establishment and multiplication of *Prunus* rootstocks 'GF677' and 'GXN15' using SETIS® bioreactors, achieving higher multiplication rates compared to conventional methods. Similarly, Godoy et al. (2017) developed a micropropagation procedure for sweet cherry cultivars and rootstocks using TIS, highlighting the system's effectiveness in enhancing plant quality and proliferation rates.

Shoot proliferation in SETIS™

To evaluate the shoot proliferation of *P. avium* and Gisela 6 in the two propagation systems, explants previously maintained for 12 subcultures were used, with the same media composition applied to both. The comparison between TIB and semi-solid culture systems showed significant differences in shoot development parameters for both genotypes. In *G6*, although the median number of shoots was the same (Table 1) for both treatments, the difference was statistically significant ($p = 0.0054$), indicating a variation in distribution



Fig. 1 Gisela 6 branches collected during spring for in vitro establishment. (**A**) Gisela 6-spring shoots; (**B**) Gisela 6 shoots after 28 days of in vitro culture

Table 1 Comparison of shoot development between Temporary Immersion Bioreactor (TIB) and semi-solid culture systems for G6 and Pa. Median values are shown for the number of shoots and the height of the tallest shoot. Differences between treatments were assessed using the Mann-Whitney test. Statistically significant results ($p<0.05$) are highlighted

Parameter	Group	*n*	Median	*p*-value	Significant ($p<0.05$)
Nº of shoots (G6)	TIB	63	1.000	0.0054	Yes
	Semi-solid	60	1.000		
Shoot Lenght (G6)	TIB	63	2.500	<0.0001	Yes
	Semi-solid	60	2.000		
Nº of shoots (Pa)	TIB	56	1.000	0.1203	No
	Semi-solid	61	1.000		
Shoot Lenght (Pa)	TIB	56	2.100	0.0004	Yes
	Semi-solid	61	2.000		

favoring the TIB system. Additionally, TIB promoted significantly greater shoot elongation in *G6*, with a median of 2.5 cm compared to 2.0 cm in the semi-solid system ($p<0.0001$). For *Pa*, no significant difference was found in the number of shoots between treatments ($p=0.1203$), as both had a median of 1.0. However, shoot length was significantly higher in TIB 2.1 cm compared to semi-solid 2.0 cm, with $p=0.0004$. These results indicate that TIB can enhance shoot elongation in both genotypes and may also improve shoot proliferation in *G6*. Similar results were reported by several authors for *Corema album*, *Colocasia esculenta* L. schott and *Vanilla panifolia* Jacks (Alves et al. 2021; Mancilla-Álvarez et al. 2021; Ramírez-Mosqueda and Bello-Bello 2021) in the SETIS™ system, in other TIB systems, similar results were also reported by Cristina et al. (2023) in RITA with *Hylocereus undatus*, by Elazab et al. (2023) using the ElecTIS bioreactor with *Rubus fruticosus* L., and by Sota et al. (2021) with *Malus sylvestris* (L.) Mill.The explanation for these results lies in the use of a liquid consistency medium, which enhances the physical contact between explants and the culture medium, thereby promoting a higher assimilation rate of nutrients and water compared to a semisolid culture medium (Monteiro et al. 2018). Differences between genotypes were found in TIB, where shoot length was greater in Gisela 6 compared to *P. avium*, which may be related to the hyperhydidricity seen in *P. avium*. (Fig. 2). Hyperhydricity is a physiological abnormality that directly affects propagation and is characterised by excessive hydration, yellowing, swelling, glassiness and leaf curling (Kevers et al. 2004). Hyperhydridicity has also been observed in other woody species propagated in temporary immersion systems, such as chestnut (Vidal et al. 2015), apple (Chakrabarty et al. 2003; Zhu et al. 2005), walnut (Moreno et al. 2012), and pistachio (Akdemir et al. 2014).

Several other reports have shown that immersion time is a fundamental factor in the effectiveness of liquid culture in TIS, as it affects nutrient uptake, control of hyperhydricity, RGR index, and the quality of the shoot subcultures (Albarrán et al. 2005; Alvarenga Venutolo 2015; Benelli and De Carlo 2018; Etienne and Berthouly 2002; Gatica-Arias et al. 2008; Musa and Lyam 2012; Sota et al. 2021).

In *P. avium*, hyperhydricity has been reported to be influenced by immersion frequency and the concentration

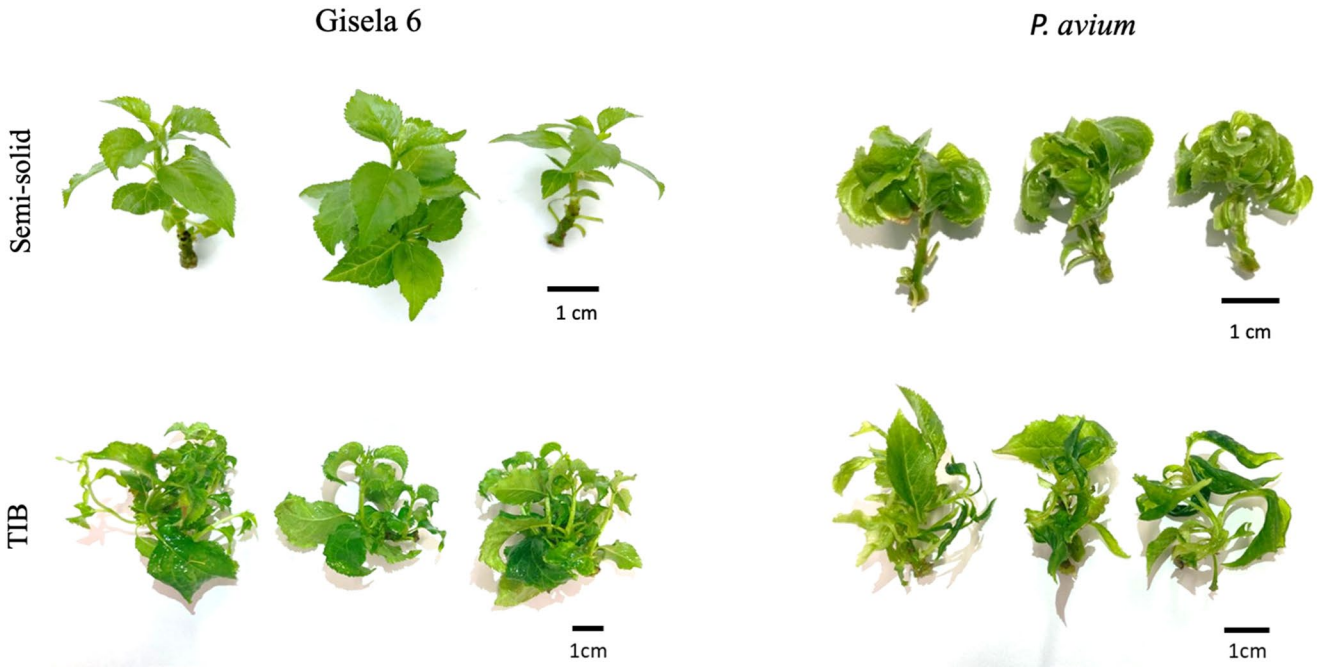


Fig. 2 Aspect of Gisela 6 and *P. avium* explants cultured on semi-solid media and TIB after 28 days of cultivation

of growth regulators, as demonstrated in the studies cited above. While these factors were not evaluated in the present work, our results suggest that similar mechanisms could underlie the occurrence of hyperhydricity in TIB cultures. The occurrence of this disorder may also stem from adjustments in growth regulator concentrations in TIB. The interaction among growth regulators in the media could increase hyperhydricity. Hence, precise modulation of cytokinin levels, such as BA, in the media is crucial for alleviating hyperhydricity problems in temporary immersion systems. Several studies found that in many species, higher cytokinin levels are associated with higher hyperhydricity (Ivanova and Van Staden 2011; Quiala et al. 2012). This relationship is believed to arise from cytokinin-induced alterations in water uptake and cell wall structure, which can disrupt normal morphogenesis (Gupta et al. 2022). While cytokinins are indispensable for shoot proliferation, their excessive levels may compromise shoot quality. In the context of liquid culture systems such as TIB, this balance becomes particularly critical, as immersion can exacerbate physiological disorders. Future experiments specifically addressing immersion schedules and hormone balance will be necessary to confirm their role in this system.

In addition, ventilation also plays an important role for controlling hyperhydricity in TIB systems. Poor gas exchange inside closed culture vessels leads to the accumulation of ethylene and excessive humidity, both of which contribute to abnormal tissue development. Implementing forced or passive ventilation improves the exchange of gases such as oxygen and carbon dioxide while reducing ethylene buildup, thereby helping to re-establish a more natural atmosphere around the explants (Hwang et al. 2022). Ventilation not only supports physiological processes like respiration and photosynthesis but also reduces excess internal water retention that contributes to vitrification (Martínez-Estrada et al. 2019). The importance of monitoring hyperhydricity is acknowledged. Although the number or frequency of hyperhydric shoots was not recorded in this study, assessment of shoot quality including vigour, morphology, and survival provides an indirect indication of its impact. A dedicated, quantitative evaluation of hyperhydricity would be a valuable direction for future work.

The Relative Growth Rate was also calculated. Table 2 shows the index calculated after 28 days of culture for the two types of propagation per genotype. The RGR index, as a standardised growth measure, has shown significant effectiveness when comparing growth in different propagation systems. It also offers the advantage of minimizing inherent scale differences between growing plants, allowing for fair comparisons of their performance within the examined time period (Elazab et al. 2023). Based on this index, TIB confirmed the superiority in promoting shoot proliferation, similar results were obtained by Elazab et al. (2023) and Sota et al. (2021). However, this apparent advantage was accompanied by challenges related to shoot quality, particularly hyperhydricity, indicating that further studies are required to refine culture conditions and confirm the long-term benefits of TIB.

TIB outperformed semi-solid media in *ex vitro* rooting success

The ex vitro rooting of these rootstocks proved to be a difficult process, and the results obtained cannot yet be considered efficient, as rooting rates did not exceed 57%. In comparison, an efficient rooting rate for commercial application would be expected to reach approximately which is notably lower than the 80–100% range commonly reported for propagation of other *Prunus* rootstocks The explants proliferated in TIB showed more capacity for *ex vitro* rooting when compared to the ones obtained in semi-solid media, as it is possible to verify in the (Fig. 3). The rooting percentage for Gisela 6 in semi-solid was 16% compared to TIB with 54%, in the case of *P. avium* for semi-solid was 10% compared to 44% in TIB (Table 2).

Explants of the Gisela 6 genotype derived from the TIB system showed better rooting capacity, with higher median root number and length compared to semi-solid medium (Mann-Whitney test, $p<0.05$). No significant differences were observed for Pa genotype between propagation methods (Table 3). Rooting percentages were higher in TIB for both genotypes but were not statistically analyzed. These results suggest TIB enhances rooting efficiency, especially for G6 as is possible to observe in (Fig. 4) in the acclimatisation stage. This outcome highlights that, despite initial issues with shoot quality, the capacity of shoots to recover during rooting ensures that TIB cultures ultimately deliver a comparable or even superior number of viable plantlets. Such recovery highlights the practical potential of TIB, while also indicating the need for further refinement to produce high-quality outputs consistently.

Table 2 Initial and final fresh weight and RGR index of the shoots propagated in semi-solid medium and in TIB per genotype (Gisela 6 – G6 and *P. avium* – Pa), calculated after 28 days of culture

Type of propagation	Initial weight (g)		Final weight (g)		RGR Index	
	G6	Pa	G6	Pa	G6	Pa
Semi-solid	0.04	0.04	1.38	1.58	13.02	13.07
TIB	0.05	0.04	6.21	10.07	17.54	19.74

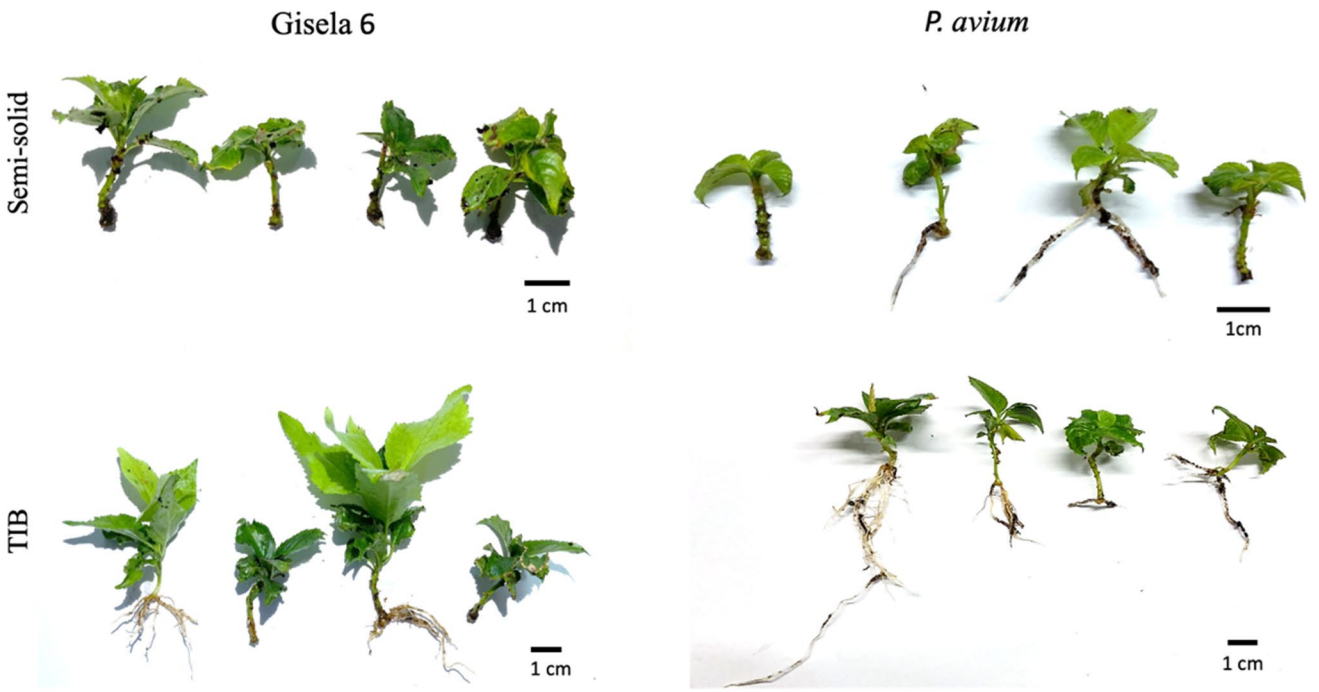


Fig. 3 Gisela 6 and *P. avium ex vitro* rooting results from semi-solid media and bioreactor, after 28 days of induction

Table 3 Rooting percentage, number of roots, and root of the longest root for G6 and Pa propagated by semi-solid medium and TIB. Values represent medians with sample sizes (n). Different letters within each column indicate statistically significant differences between propagation methods according to the Mann-Whitney test ($p<0.05$). Rooting percentages were not statistically analyzed

Type of propagation	Rooting % (G6)	Rooting % (Pa)	Number of roots (G6)	Number of roots (Pa)	Root lenght (G6)	Root lenght (Pa)
Semi-solid	16%	10%	1.0 ($n=4$) [a]	1.5 ($n=4$) [a]	0.25 ($n=4$) [a]	2.1 ($n=4$) [a]
TIB	57%	44%	2.0 ($n=11$) [a]	2.5 ($n=10$) [a]	2.0 ($n=11$) [b]	2.93 ($n=10$) [a]

Aragón et al. (2005) noted significantly improved rates of shoot survival and rooting when employing material from the TIS compared to semi-solid medium. In pineapple, liquid culture within TIS resulted in the production of shoots displaying enhanced rooting potential, thus facilitating successful ex vitro acclimatization (Escalona et al. 1999). Furthermore, shoot tips derived from *Callistephus hortensis* cultured in TIS exhibited greater size compared to those obtained from the semi-solid medium (Tisserat and Vandercook 1985).

Impact of propagation methods on photosynthetic pigments, secondary metabolites, and stress markers

The analysis of photosynthetic pigments revealed no significant differences between the TIB and semi-solid culture systems in the G6 genotype. Chlorophyll a and b, as well as carotenoid contents, showed similar median values across both culture methods ($p>0.05$). Conversely, in the Pa genotype, significant reductions in chlorophyll a, chlorophyll b, and carotenoid levels were observed in plants grown in TIB compared to those in semi-solid culture ($p<0.0001$). These results suggest that the semi-solid system supports higher pigment accumulation in Pa, while G6 appears less affected by the culture method in terms of pigment content. However *P. avium* genotype in TIB exhibited high levels of hyperhydricity, this could be due to stress caused by the periodic exposure to the liquid environment (Cristina et al. 2023). This stress adversely affected both physiological and metabolic processes, ultimately resulting in an indirect reduction in the content of photosynthetic pigments. Physiologically, several plant processes are affected by O_2 deficiency stress. Excess H_2O coupled with O_2 deficiency can have effects on cell turgor, stomatal index, transpiration, photosynthesis, respiration, antioxidant activity and light uptake (Falqueto et al. 2017). This is caused by the presence of excess fluid in the intercellular spaces, decrease in cell adhesion and the formation of large intercellular spaces in the mesophyll, hypolignification, decrease in the formation of the epicuticular layer on leaf surfaces, changes in enzyme activity and changes in protein synthesis due to disruption of normal metabolic processes (Casanova et al. 2008; Picoli et al. 2001; van den Dries et al. 2013). In Gisela 6, no significant

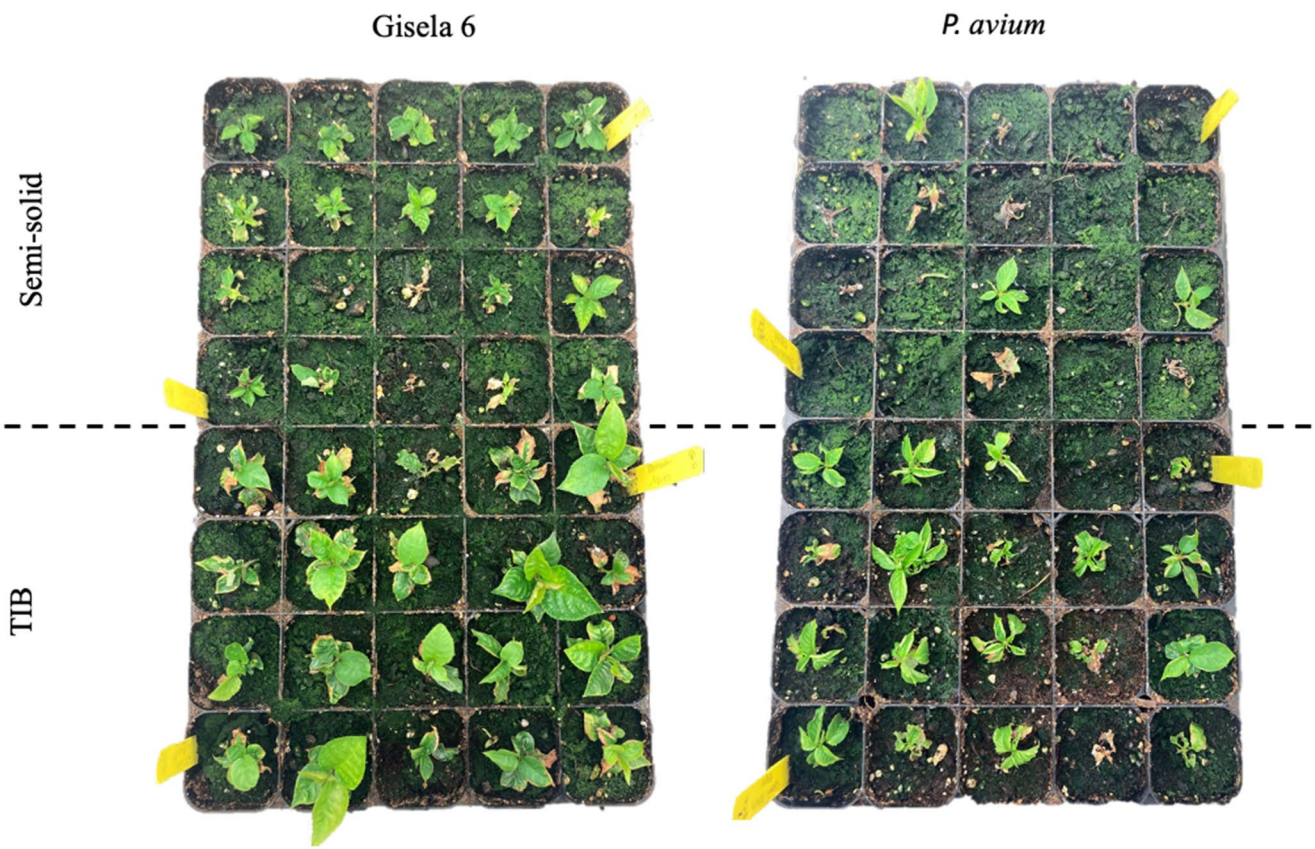


Fig. 4 Gisela 6 and *P. avium* plantlets propagated in semi-solid media and TIB in the acclimatation stage in plastic trays with 80 cm^3 per cell after 28 days of root induction

differences were found when comparing semi-solid and TIB. Similar results were previously obtained by Hwang et al. (2022) in strawberry.

Photosynthetic pigments, such as chlorophyll, carotenoids, and phycobilins are crucial for plant photosynthesis. The balance among these pigments is important for light absorption, photoprotection, antioxidant activity, regulation of light harvesting and the adaptation to environmental changes (Simkin et al. 2022). In this study, we employed dry weight as a method to assess photosynthetic pigments and other biochemical parameters. Utilizing dry weight offers the advantage of yielding more precise and consistent measurements, primarily by eliminating water interference in the samples, especially in cases of hyperhydricity.

Flavonoids represent a heterogeneous class of secondary metabolites present in plants, exhibiting multifaceted roles including UV protection, pigmentation, antioxidant properties, defence against pathogens, rhizosphere interactions and involvement in allelopathy (Weston and Mathesius 2013). These metabolites play a critical role in mitigating oxidative stress in plants by scavenging reactive oxygen species (ROS), chelating metal ions, inducing antioxidant enzymes and defending against environmental stressors. Their ability to maintain cellular redox balance is essential for plant growth, development and survival in dynamic environments (Shah and Smith 2020).

In this study, the total flavonoid content was evaluated to determine whether the type of propagation induced stress in the explants. No statistically significant differences were found between the variables Table 4. Our findings suggest that the content of flavonoids remains unaffected by both the propagation method and genotype variations. However, it is important to note that this method provides only a quantitative assessment of total flavonoids and does not account for potential qualitative differences in flavonoid composition.

Malondialdehyde (MDA) is widely recognized as a key indicator of oxidative stress because it is a primary product of lipid peroxidation, a process where free radicals, particularly ROS, attack lipids containing carbon-carbon double bonds, especially polyunsaturated fatty acids within the cell membrane (Morales and Munné-Bosch 2019). High levels of MDA are often correlated with exposure to environmental stressors, such as drought, salinity, heavy metal toxicity, high temperatures and pathogen attack. The increased MDA levels under such conditions reflect the extent of cellular damage and the organism's stress response (Morales and Munné-Bosch 2019; Polivanova and Bedarev 2022). For this parameter, no significant differences were observed between

Table 4 Biochemical parameters compared between temporary immersion bioreactor (TIB) and semi-solid culture systems in G6 and Pa genotypes. Values are presented as medians (with units and sample size). Statistical significance was assessed using the Mann–Whitney test. Significant differences ($p<0.05$) are indicated

Genotype	Parameter	Culture System	Median (unit, n)	p-value	Significant ($p<0.05$)
G6	Chlorophyll a	TIB	869.5 µg/g DW ($n=12$)	0.5899	No
		Semi-solid	930.9 µg/g DW ($n=12$)		
	Chlorophyll b	TIB	292.1 µg/g DW ($n=12$)	0.4776	No
		Semi-solid	305.8 µg/g DW ($n=12$)		
	Carotenoids	TIB	203.1 µg/g DW ($n=12$)	0.1135	No
		Semi-solid	228.1 µg/g DW ($n=12$)		
	Flavonoids	TIB	7254.0 µg catechin eq./g DW ($n=12$)	0.4428	No
		Semi-solid	6386.0 µg catechin eq./g DW ($n=12$)		
	MDA	TIB	176.0 nmol/g DW ($n=6$)	0.2403	No
		Semi-solid	151.0 nmol/g DW ($n=6$)		
	Proline	TIB	4.61 µmol/g DW ($n=3$)	>0.9999	No
		Semi-solid	5.30 µmol/g DW ($n=3$)		
Pa	Chlorophyll a	TIB	482.8 µg/g DW ($n=12$)	<0.0001	Yes
		Semi-solid	828.4 µg/g DW ($n=12$)		
	Chlorophyll b	TIB	192.5 µg/g DW ($n=12$)	<0.0001	Yes
		Semi-solid	294.0 µg/g DW ($n=12$)		
	Carotenoids	TIB	120.4 µg/g DW ($n=12$)	<0.0001	Yes
		Semi-solid	218.1 µg/g DW ($n=12$)		
	Flavonoids	TIB	6304.0 µg catechin eq./g DW ($n=12$)	0.2657	No
		Semi-solid	7351.0 µg catechin eq./g DW ($n=12$)		
	MDA	TIB	236.5 nmol/g DW ($n=6$)	>0.9999	No
		Semi-solid	238.5 nmol/g DW ($n=6$)		
	Proline	TIB	11.08 µmol/g DW ($n=3$)	0.7000	No
		Semi-solid	12.06 µmol/g DW ($n=3$)		

propagation systems within each genotype (Table 4). However, *P. avium* consistently showed higher MDA levels than G6, regardless of the culture system, indicating a possible genotype-dependent response in lipid peroxidation. The elevated MDA content in *P. avium* may be associated with the occurrence of hyperhydricity, a condition that compromises tissue structure and favors the accumulation of reactive oxygen species (ROS) (Fernandez-García et al. 2008). Increased ROS levels enhance lipid peroxidation, leading to higher MDA accumulation (Morales and Munné-Bosch 2019).

In this work, proline quantification was made to evaluate the effectiveness of the propagation systems. Proline quantification was used as an indicator to assess the stress levels in plants during the propagation stage and to evaluate the performance of the different systems. Proline content between semi-solid and TIB didn't show statistical differences in the evaluated genotypes (Table 4), however higher values of proline content were observed in *P. avium* compared to Gisela 6. Previous studies have demonstrated that proline accumulation is dependent on the species and the genotype. Different genotypes respond differently to stress conditions, affecting proline levels. Studies have shown that under adverse conditions such as drought and salinity, proline accumulation among different genotypes occurs, with some accumulating significantly more proline than others (Pazuki et al. 2018).

A study developed by Shin et al. (2020) observed that proline content increased with higher salt concentrations regardless of the genotype, indicating a genotype-independent response in tomato seedlings. However, different tomato cultivars exhibited different levels of proline accumulation in response to salt stress, highlighting the role of genotype in determining proline levels in plants. Different cultivars of apple (Rubinstep) and sweet cherry (Napoleonova and 'Kaštánka) showed higher accumulation of proline compared to other cultivars in a study made by Jiroutova et al. (2021) indicating genotype-dependent responses to osmotic stress. As an osmoprotectant, proline contributes to stress tolerance, genotypes with higher proline levels are more resistant to drought stress. This study highlights that proline accumulation can be used as a physiological indicator of plant resistance to stress tolerance, emphasizing the importance of genotype-specific responses to osmotic stress (Jiroutova et al. 2021). In another study made in Arabidopsis, the P5CS1 mutant exhibited extremely low proline

levels compared to wild-type plants, showing the role of specific genes in proline accumulation in response to stress. The capacity to accumulate proline was not consistently correlated with stress tolerance across different genotypes, indicating the complex interplay between genotype and proline accumulation in stress responses (Gurrieri et al. 2020).

Conclusion

This study provides the first report on the propagation of sweet cherry rootstocks using the SETIS™ temporary immersion system. Nodal explants showed satisfactory in vitro establishment, reaching 56% in P. avium and 62% in Gisela 6. Furthermore, shoots cultured in the SETIS™ bioreactor exhibited greater shoot length and RGR than those grown on semi-solid media. However, hyperhydricity was observed in *P. avium* in the bioreactor, affecting shoot quality. Ex vitro rooting posed challenges, with TIB shoots demonstrating better rooting capacity compared to semi-solid media, facilitating successful *ex vitro* acclimatization. Nonetheless, improvements in the rooting process are needed. Photosynthetic pigment analysis revealed a decrease in pigment content in *P. avium* due to hyperhydricity. Total flavonoids remained unaffected by the propagation method or genotype variations. MDA content was significantly higher in *P. avium* from the bioreactor, indicating oxidative stress due to hyperhydricity. Proline levels were higher in *P. avium* compared to Gisela 6, indicating genotype-dependent responses to stress conditions. These findings underscore the importance of selecting appropriate micropropagation systems and understanding their impact on plant physiology and biochemistry. While SETIS™ demonstrated advantages in shoot proliferation and rooting, challenges such as hyperhydricity-induced stress were observed, highlighting the need for optimization and improvement of micropropagation protocols. Additionally, the absence of a specific hyperhydricity index is acknowledged as a limitation of this study, and future work should include a quantitative assessment of this disorder to better understand its impact on shoot quality. The study demonstrates the feasibility of sweet cherry rootstock propagation using the SETIS™ system, offering insights into the advantages and challenges of this method compared to traditional semi-solid media. Genotype-specific responses were observed in biochemical analyses, highlighting the importance of considering genetic factors in plant propagation and stress tolerance. Further research may focus on optimizing TIB parameters to mitigate hyperhydricity and improve plant quality during propagation.

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Authors' contributions All authors contributed to the conception and design of the study. Baltazar E. conducted the micropropagation experiments and carried out the physiological and biochemical analyses. All authors contributed to data interpretation and manuscript preparation. Correia S. supervised the study and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Data availability All data supporting the findings of this study are available within the paper.

Declarations

Ethical approval Not applicable.

Conflict of interest The authors declare that they have no conflicts of interest. Sandra Correia is one of the Associate Editors of the journal at the time of submission. This had no impact on the peer-review process or the final decision.

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References

Akdemir H, Süzerer V, Onay A, Tilkat E, Ersali Y, Çiftçi YO (2014) Micropropagation of the pistachio and its rootstocks by temporary immersion system. Plant Cell Tissue Organ Cult (PCTOC) 117(1):65–76. https://doi.org/10.1007/s11240-013-0421-0

Albacete A, Martínez-Andújar C, Martínez-Pérez A, Thompson AJ, Dodd IC, Pérez-Alfocea F (2015) Unravelling rootstock×scion interactions to improve food security. J Exp Bot 66(8):2211–2226. https://doi.org/10.1093/jxb/erv027

Albarrán J, Bertrand B, Lartaud M, Etienne H (2005) Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (*Coffea arabica*) somatic embryos. Planr Cell Tissue Organ Cult 81(1):27–36. https://doi.org/10.1007/s11240-004-2618-8

Alvard D, Cote F, Teisson C (1993) Comparison of methods of liquid medium culture for banana micropropagation. Planr Cell Tissue Organ Cult 32(1):55–60. https://doi.org/10.1007/BF00040116

Alvarenga Venutolo S (2015) Micropropagación masiva de S*tevia rebaudiana* Bertoni en sistemas de inmersión temporal. Cultivos Tropicales 36(3):50–57. http://scielo.sld.cu/scielo.php?script=sci_abstract&pid=S0258 -59362015000300008&lng=es&nrm=iso&tlng=es

Alves V, Pinto R, Debiasi C, Santos MC, Gonçalves JC, Domingues J (2021) Micropropagation of *Corema album* from adult plants in semisolid medium and temporary immersion bioreactor. Plant Cell Tissue Organ Cult (PCTOC) 145(3):641–648. https://doi.org/10.1007/s11240-021-02034-1

Aragón CE, Escalona M, Capote I, Pina D, Cejas I, Rodriguez R, Cañal MJ, Sandoval J, Roels S, Debergh P, Gonzalez-Olmedo J (2005) Photosynthesis and Carbon Metabolism in Plantain (Musa AAB) Plantlets Growing in Temporary Immersion Bioreactors and during *Ex vitro* Acclimatization. Vitro Cell Dev Biology Plant 41(4):550–554. https://www.jstor.org/stable/4293899

Baránek M, Raddová J, Pidra M (2006) Comparative analysis of genetic diversity in *Prunus* L. as revealed by RAPD and SSR markers. Sci Hort 108(3):253–259. https://doi.org/10.1016/j.scienta.2006.01.023

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39(1):205–207. https://doi.org/10.1007/BF00018060

Benelli C, De Carlo A (2018) *In vitro* multiplication and growth improvement of *Olea europaea* L. cv Canino with temporary immersion system (Plantform™). Biotech 8(7);317. https://doi.org/10.1007/s13205-018-1346-4

Casanova E, Moysset L, Trillas MI (2008) Effects of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots. Biol Plant 52(1):1–8. https://doi.org/10.1007/s10535-008-0001-z

Chakrabarty D, Hahn E, Yoon Y, Paek K (2003) Micropropagation of apple rootstock M. 9 EMLA using bioreactor. J Hortic Sci Biotechnol 78:605–609. https://doi.org/10.1080/14620316.2003.11511671

Cristina M-AM, Eucario M-Á, Luis S-CJ, Jabín B-BJ (2023) Evaluation of the effect of different culture systems on photomixotrophic capacity during *in vitro* multiplication of pitahaya (*Hylocereus undatus*). South Afr J Bot 159:396–404. https://doi.org/10.1016/j.sajb.2023.06.013

Druart P (2013) Micropropagation of *Prunus* species relevant to cherry fruit production. Methods Mol Biology (Clifton N J) 11013:119–136. https://doi.org/10.1007/978-1-62703-074-8_9

E. Amiri M (2006) In vitro techniques to study the shoot-tip grafting of Prunus avium L. (cherry) var. Seeyahe Mashad. J Food Agric Environ 4:151–154

Elazab D, Capuana M, A Ozudogru E, Anichini M, Lambardi M (2023) Use of Liquid Culture with the ElecTIS Bioreactor for Faster Recovery of Blackberry (Rubus fruticosus L.) Shoots from Conservation at 4°C. Horticulturae 9(6):Artigo6. https://doi.org/10.3390/horticulturae9060680

Escalona M, Lorenzo JC, González B, Daquinta M, González JL, Desjardins Y, Borroto CG (1999) Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. Plant Cell Rep 18(9):743–748. https://doi.org/10.1007/s002990050653

Etienne H, Berthouly M (2002) Temporary immersion systems in plant micropropagation. Planr Cell Tissue Organ Cult 69(3):215–231. https://doi.org/10.1023/A:1015668610465

Falqueto AR, da Silva Júnior RA, Gomes MTG, Martins JPR, Silva DM, Partelli FL (2017) Effects of drought stress on chlorophyll *a* fluorescence in two rubber tree clones. Sci Hort 224:238–243. https://doi.org/10.1016/j.scienta.2017.06.019

Feeney M, Bhagwat B, Mitchell JS, Lane WD (2007) Shoot regeneration from organogenic callus of sweet cherry (*Prunus avium* L). Planr Cell Tissue Organ Cult 90(2):201–214. https://doi.org/10.1007/s11240-007-9252-1

Fernandez-García N, Piqueras A, Olmos E (2008) Sub-cellular location of H2O2, peroxidases and pectin epitopes in control and hyperhydric shoots of carnation. Environ Exp Bot 62(2):168–175. https://doi.org/10.1016/j.envexpbot.2007.08.004

Feucht W, Dausend B (1976) Root induction *in vitro* of easy-to-root *Prunus pseudocerasus* and difficult-to-root *Prunus avium*. Sci Hort 4(1):49–54. https://doi.org/10.1016/0304-4238(76)90064-9

Gatica-Arias AM, Arrieta-Espinoza G, Espinoza Esquivel AM (2008) Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuaí. Electron J Biotechnol 11(1):101–112. https://doi.org/10.4067/S0717-34582008000100010

Godoy S, Tapia E, Seit P, Andrade D, Sánchez E, Andrade P, Almeida AM, Prieto H (2017) Temporary immersion systems for the mass propagation of sweet cherry cultivars and cherry rootstocks: Development of a micropropagation procedure and effect of culture conditions on plant quality. Vitro Cell Dev Biology - Plant 53(5):494–504. https://doi.org/10.1007/s11627-017-9856-z

Guajardo V, Solís S, Almada R, Saski C, Gasic K, Moreno MÁ (2020) Genome-wide SNP identification in *Prunus* rootstocks germplasm collections using Genotyping-by-Sequencing: Phylogenetic analysis, distribution of SNPs and prediction of their effect on gene function. Sci Rep 10(1):1467. https://doi.org/10.1038/s41598-020-58271-5

Gupta R, Elkabetz D, Leibman-Markus M, Sayas T, Schneider A, Jami E, Kleiman M, Bar M (2022) Cytokinin drives assembly of the phyllosphere microbiome and promotes disease resistance through structural and chemical cues. ISME J 16(1):122–137. https://doi.org/10.1038/s41396-021-01060-3

Gurrieri L, Merico M, Trost P, Forlani G, Sparla F (2020) Impact of Drought on Soluble Sugars and Free Proline Content in Selected *Arabidopsis* Mutants. Biology 9(11):367. https://doi.org/10.3390/biology9110367

Hammatt N, Grant NJ (1998) Shoot regeneration from leaves of *Prunus serotina* Ehrh. (Black cherry) and *P. avium* L. (wild cherry). Plant Cell Rep 17(6–7):526–530. https://doi.org/10.1007/s002990050436

Hodges DM, DeLong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207(4):604–611. https://doi.org/10.1007/s004250050524

Hutchison J (1984) Factors affecting shoot proliferation and root initiation in organ cultures of the apple 'Northern Spy'. Sci Hort 22(4):347–358. https://doi.org/10.1016/S0304-4238(84)80006-0

Hwang H-D, Kwon S-H, Murthy HN, Yun S-W, Pyo S-S, Park S-Y (2022) Temporary Immersion bioreactor system as an efficient method for mass production of *In Vitro*

INE (2021) *Estatisticas Agricolas* 2021. Statistics Portugal. https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_publicacoes&PUBLICACOESpub_boui=31589846&PUBLICACOESmodo=2

Iv, Ani, Pre (1980) Embryo culture and micropropagation of cherries *in vitro*. Sci Hort 12(1):77–82. https://doi.org/10.1016/0304-4238(80)90040-0

Ivanova M, Van Staden J (2011) Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. Plant Cell Tissue Organ Cult (PCTOC) 104(1):13–21. https://doi.org/10.1007/s11240-010-9794-5

Jiroutova P, Kovalikova Z, Toman J, Dobrovolna D, Andrys R (2021) Complex Analysis of Antioxidant Activity, Abscisic Acid Level, and Accumulation of Osmotica in Apple and Cherry *In Vitro*

Cultures under Osmotic Stress. Int J Mol Sci 22(15):7922. https://doi.org/10.3390/ijms22157922

Kevers C, Franck T, Strasser RJ, Dommes J, Gaspar T (2004) Hyperhydricity of Micropropagated Shoots: A Typically Stress-induced Change of Physiological State. Planr Cell Tissue Organ Cult 77(2):181–191. https://doi.org/10.1023/B:TICU.0000016825.18930.e4

Kim N-Y, Hwang H-D, Kim J-H, Kwon B-M, Kim D, Park S-Y (2020) Efficient production of virus-free apple plantlets using the temporary immersion bioreactor system. Hortic Environ Biotechnol 61(4):779–785. https://doi.org/10.1007/s13580-020-00257-3

Landi M (2017) Commentary to: «Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds» by Hodges et al., Planta (1999) 207:604–611. Planta 245(6):1067. https://doi.org/10.1007/s00425-017-2699-3

Leal DR, Sánchez-Olate M, Avilés F, Materan ME, Uribe M, Hasbún R, Rodríguez R (2007) Micropropagation of *Juglans regia* L. Em S. M. Jain & H. Häggman (eds.), Protocols for micropropagation of woody trees and fruits. Springer Netherlands. pp. 381–390 https://doi.org/10.1007/978-1-4020-6352-7_35

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. em methods in enzymology. Academic Press. 148;350–382

Lopes T, Correia M, Pedrosa A, Baltazar E, Canhoto J, Correia S (2023) Improved micropropagation of *Prunus* spp. Rootstocks using temporary immersion systems. Acta Hort 1359:249–254. https://doi.org/10.17660/ActaHortic.2023.1359.32

Mancilla-Álvarez E, Pérez-Sato JA, Núñez-Pastrana R, Spinoso-Castillo JL, Bello-Bello JJ (2021) Comparison of Different Semi-Automated Bioreactors for *In Vitro* Propagation of Taro (*Colocasia esculenta* L. Schott). Plants (Basel Switzerland) 10(5):1010. https://doi.org/10.3390/plants10051010

Martínez-Estrada E, Islas-Luna B, Pérez-Sato JA, Bello-Bello JJ (2019) Temporary immersion improves *in vitro* multiplication and acclimatization of *Anthurium andreanum* Lind. Sci Hort 249:185–191. https://doi.org/10.1016/j.scienta.2019.01.053

Martínez-Gómez P, Sánchez-Pérez R, Rubio M, Dicenta F, Gradziel TM, Sozzi GO, Universidad de Buenos Aires (2005) Application of Recent Biotechnologies to Prunus Tree Crop Genetic Improvement. Ciencia e Investigación Agrar 32(2):73–96. https://doi.org/10.7764/rcia.v32i2.308

Martins J, Pétriacq P, Flandin A, Gómez-Cadenas A, Monteiro P, Pinto G, Canhoto J (2022) Genotype determines Arbutus unedo L. physiological and metabolomic responses to drought and recovery. Front Plant Sci 13. https://doi.org/10.3389/fpls.2022.1011542

Mauro RP, Agnello M, Onofri A, Leonardi C, Giuffrida F (2020) Scion and Rootstock Differently Influence Growth, Yield and Quality Characteristics of Cherry Tomato. Plants 9(12):1725. https://doi.org/10.3390/plants9121725

Modgil M, Sharma DR, Bhardwaj SV (1999) Micropropagtion of apple cv. Tydeman's Early Worcester. Sci Hort 81(2):179–188. https://doi.org/10.1016/S0304-4238(98)00259-3

Monteiro TR, Freitas EO, Nogueira GF,and, Scherwinski-Pereira JE (2018) Assessing the influence of subcultures and liquid medium during somatic embryogenesis and plant regeneration in oil palm (*Elaeis guineensis* Jacq). J Hortic Sci Biotechnol 93(2):196–203. https://doi.org/10.1080/14620316.2017.1360156

Morales M, Munné-Bosch S (2019) Malondialdehyde: Facts and Artifacts. Plant Physiol 180(3):1246–1250. https://doi.org/10.1104/pp.19.00405

Moreno RJL, Morales AV, Gradaille MD (2012) Towards scaling-up the micropropagation of Juglans major (Torrey) Heller var. 209 x J. regia L., a hybrid walnut of commercial interest. Proceedings of the IUFRO Working Party 2.09.02 Conference. Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management, Brno, Czech Republic

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant 15(3):473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Musa M, Lyam P (2012) The Potential of Temporary Immersion Bioreactors (TIBs) in Meeting Crop Production Demand in Nigeria. J Biol. https://doi.org/10.5296/JBLS.V3I1.1156

Németh G (1979) Benzyladenine-Stimulated Rooting in Fruit-Tree Rootstocks Cultured *in vitro*. Z für Pflanzenphysiologie 95(5):389–396. https://doi.org/10.1016/S0044-328X(79)80209-3

Opazo I, Toro G, Salvatierra A, Pastenes C, Pimentel P (2020) Rootstocks modulate the physiology and growth responses to water deficit and long-term recovery in grafted stone fruit trees. Agric Water Manage 228:105897. https://doi.org/10.1016/j.agwat.2019.105897

Pazuki A, Aflaki F, Gurel S, Ergul A, Ekrem G (2018) The effects of proline on *in vitro* proliferation and propagation of doubled haploid sugar beet (*Beta vulgaris*). Turkish J Bot 42:280–288. https://doi.org/10.3906/bot-1709-14

Pérez-Tornero O, Burgos L (2007) Apricot micropropagation. Em S. M. Jain & H. Häggman (eds.), Protocols for Micropropagation of Woody Trees and Fruits. Springer Netherlands. pp. 267–278 https://doi.org/10.1007/978-1-4020-6352-7_25

Picoli EAT, Otoni WC, Figueira ML, Carolino SMB, Almeida RS, Silva EAM, Carvalho CR, Fontes EPB (2001) Hyperhydricity in *in vitro* eggplant regenerated plants: Structural characteristics and involvement of BiP (Binding Protein). Plant Sci 160(5):857–868. https://doi.org/10.1016/S0168-9452(00)00463-5

Plants in horticulture and medicinal plants agronomy, Artigo,12(2);2. https://doi.org/10.3390/agronomy12020346

Polivanova OB, Bedarev VA (2022) Hyperhydricity in Plant Tissue Culture. Plants 11(23):3313. https://doi.org/10.3390/plants11233313

Poorter H, Garnier E (1996) Plant growth analysis: An evaluation of experimental design and computational methods. J Exp Bot 47(9):1343–1351. https://doi.org/10.1093/jxb/47.9.1343

Preil W (2005) General introduction: A personal reflection on the use of liquid media for in vitro culture. Em A. K. Hvoslef-Eide & W. Preil (eds.), Liquid culture systems for in vitro plant propagation. Springer Netherlands. pp. 1–18 https://doi.org/10.1007/1-4020-3200-5_1

Quiala E, Cañal M-J, Meijón M, Rodríguez R, Chávez M, Valledor L, de Feria M, Barbón R (2012) Morphological and physiological responses of proliferating shoots of teak to temporary immersion and BA treatments. Plant Cell Tissue Organ Cult (PCTOC) 109(2):223–234. https://doi.org/10.1007/s11240-011-0088-3

Ramírez-Mosqueda MA, Bello-Bello JJ (2021) SETIS™ bioreactor increases in vitro multiplication and shoot length in vanilla (*Vanilla planifolia* Jacks. Ex Andrews). Acta Physiol Plant 43(4):52. https://doi.org/10.1007/s11738-021-03227-z

Sedlák J, Paprštein F (2008) In vitro shoot proliferation of sweet cherry cultivars Karešova and Rivan. Hortic Sci 35(3):95–98. https://doi.org/10.17221/6/2008-HORTSCI

Shah A, Smith DL (2020) Flavonoids in Agriculture: Chemistry and Roles in, Biotic and Abiotic Stress Responses, and Microbial Associations. Agronomy 10(8):Artigo8. https://doi.org/10.3390/agronomy10081209

Sharma V, Thakur M, Kumar A (2017) An Efficient Method for *In Vitro* Propagation of Gisela 5 (*Prunus cerasus* X *Prunus canescens*)—Clonal Cherry Rootstock. Int J Curr Microbiol Appl Sci 6(8):2617–2624. https://doi.org/10.20546/ijcmas.2017.608.311

Shin YK, Bhandari SR, Cho MC, Lee JG (2020) Evaluation of chlorophyll fluorescence parameters and proline content in tomato

seedlings grown under different salt stress conditions. Hortic Environ Biotechnol 61(3):433–443. https://doi.org/10.1007/s13580-020-00231-z

Simkin AJ, Kapoor L, Doss CGP, Hofmann TA, Lawson T, Ramamoorthy S (2022) The role of photosynthesis related pigments in light harvesting, photoprotection and enhancement of photosynthetic yield in planta. Photosynth Res 152(1):23–42. https://doi.org/10.1007/s11120-021-00892-6

Sota V, Benelli C, Çuko B, Papakosta E, Depaoli C, Lambardi M, Kongjika E (2021) Evaluation of ElecTIS bioreactor for the micropropagation of *Malus sylvestris* (L.) Mill., an important autochthonous species of Albania. Hortic Sci 48(1):12–21. https://doi.org/10.17221/69/2020-HORTSCI

Teisson C, Alvard D (1995) A new concept of plant in vitro cultivation liquid medium: temporary immersion. Em M. Terzi, R. Cella, & A. Falavigna (eds.), current issues in plant molecular and cellular biology: proceedings of the VIIIth international congress on plant tissue and cell culture, Florence, Italy. Springer Netherlands. 1994;105–110 https://doi.org/10.1007/978-94-011-0307-7_12

Tisserat B, Vandercook CE (1985) Development of an automated plant culture system. Planr Cell Tissue Organ Cult 5(2):107–117. https://doi.org/10.1007/BF00040307

van den Dries N, Giannì S, Czerednik A, Krens FA, de Klerk G-JM (2013) Flooding of the apoplast is a key factor in the development of hyperhydricity. J Exp Bot 64(16):5221–5230. https://doi.org/10.1093/jxb/ert315

Vervit (2024) Setis | ABOUT SETIS™. Setis. https://setis-systems.be/about-setis

Vidal N, Sánchez C (2019) Use of bioreactor systems in the propagation of forest trees. Eng Life Sci 19(12):896–915. https://doi.org/10.1002/elsc.201900041

Vidal N, Blanco B, Cuenca B (2015) A temporary immersion system for micropropagation of axillary shoots of hybrid chestnut. Plant Cell Tissue Organ Cult (PCTOC) 123(2):229–243. https://doi.org/10.1007/s11240-015-0827-y

Webster CA,and, Jones OP (1991) Micropropagation of some cold-hardy dwarfing rootstocks for apple. J Hortic Sci 66(1):1–6. https://doi.org/10.1080/00221589.1991.11516118

Weston LA, Mathesius U (2013) Flavonoids: Their Structure, Biosynthesis and Role in the Rhizosphere, Including Allelopathy. J Chem Ecol 39(2):283–297. https://doi.org/10.1007/s10886-013-0248-5

Zhishen J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 64(4):555–559. https://doi.org/10.1016/S0308-8146(98)00102-2

Zhu L-H, Li X-Y, Welander M (2005) Optimisation of growing conditions for the apple rootstock M26 grown in RITA containers using temporary immersion principle. Planr Cell Tissue Organ Cult 81(3):313–318. https://doi.org/10.1007/s11240-004-6659-9

Zilkah S, Faingersh E, Rotbaum A (1992) *In vitro* propagation of three mxm (*Prunus avium* x *P. Mahaleb*) cherry rootstocks. Acta Hort 314:93–98. https://doi.org/10.17660/ActaHortic.1992.314.10

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