

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 SETISTM 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.

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 & Dausend, 1976), shoot tips (Hammatt & Grant, 1998; Németh, 1979; Sedlák & Paprštein, 2008), embryos (Ivanička & 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 & 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 & Berthouly, 2002; Teisson & 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 & 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 minutes, followed by 70% (v/v) ethanol washing for 30 seconds. 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 & 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 were optimized according to the experiments carried out by Sharma et al. (2017). MS basal media was supplemented with 0.44 µM of N⁶-benzyladenine (BA), 0.29 µM gibberellic acid (GA₃), 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 (9,87 GE / day). 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 minutes every 8 hours. 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. After 28 days, shoot length and number of shoots were recorded. Relative Growth Rate (RGR) was calculated as $(\ln W_2 - \ln W_1) \times 100 / (t_2 - t_0)$, 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 & Garnier, 1996). All plants were maintained under standard conditions (light photoperiod and temperature).

Rooting and acclimatization

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 minute and put on a commercial SIRO® Turfa 45-0 mixed with perlite (80:20), and planted in plastic trays with 80 cm³ per cell. The induced explants were maintained in the climatic chamber at 25 °C under a 16h light photoperiod. 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 pigments such as 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 seconds each, ultrasonic bath for 10 minutes and centrifuged at 10,000 g for 10 minutes 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/mL and calculated using the following equations:

$$\text{Chlorophyll a (Chl a)} (\mu\text{g/mL}) = 13.36 A_{664} - 5.19 A_{649}$$

$$\text{Chlorophyll b (Chl b)} (\mu\text{g/mL}) = 27.43 A_{649} - 8.12 A_{664}$$

$$\text{Carotenoids} (\mu\text{g/mL}) = (1000 A_{470} - 2.13 \text{ Chl a} - 97.63 \text{ Chl b})/209$$

Flavonoids

Total flavonoids were quantified 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 hour and centrifuged for 15 min, 10000 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 (0-125 µg, $y = 201.89x - 15.633$, $R^2 = 0.9951$).

Malondialdehyde (MDA) quantification

The same supernatant used for the photosynthetic pigments analysis was then used for the MDA assay. The MDA quantification protocol was based on (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 $(A - B)/157.000 \times 106$, in which

$A = [(A_{532}+TBA) - (A_{600}+TBA) - (A_{532}-TBA - A_{600}-TBA)]$ and $B = [(A_{440}+TBA - A_{600}+TBA) \times 0.0571]$. Each absorbance reading was repeated three times.

Proline quantification

Total proline in the extracts was measured 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 minutes 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 hour 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 success rate was evaluated after 28 days for two genotypes. The establishment percentage was 56% for *Prunus avium* and 62% for Gisela 6. Overall, the results were consistent with previous studies for both genotypes. The establishment was performed in the spring with a low percentage of humidity, allowing for better results. Consequently, the lower rate of contamination was related to the season, particularly due to the unusually dry conditions during spring. 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 & Jones, 1991).

In this work, 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 & Burgos, 2007).

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 presents several 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 1.0 cm for both treatments, the difference was statistically significant ($p = 0.0054$), indicating a variation in distribution 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 ($p < 0.0001$) (Table 1). 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 cm. However, shoot length was significantly higher in TIB 2.1 cm compared to semi-solid 2.0 cm, with $p = 0.0004$ (Table 1). 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 & 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 hyperhydricity 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). Hyperhydricity 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 & De Carlo, 2018; Etienne & Berthouly, 2002; Gatica-Arias et al., 2008; Musa & Lyam, 2012; Sota et al., 2021).

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

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.

Significant ($p < 0.05$)					
Parameter	Group	<i>n</i>	Median	<i>p</i> -value	
N° 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		
N° 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		

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; 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.

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

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 54%. 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 3).

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.

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 & Vandercook, 1985).

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 height (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) ^b	2.5 (n=10) ^b	2.0 (n=11) ^b	2.93 (n=10) ^b

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 ($p > 0.05$) (Table 4). 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 ($p < 0.0001$) (Table 4). 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 differences were found when comparing semi-solid and TIB. Similar results were previously obtained (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.

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	8.695 µg/g DW (n = 12)	0.5899	No
		Semi-solid	9.309 µg/g DW (n = 12)		
	Chlorophyll b	TIB	2.921 µg/g DW (n = 12)	0.4776	No
		Semi-solid	3.058 µg/g DW (n = 12)		
	Carotenoids	TIB	2.031 µg/g DW (n = 12)	0.1135	No
		Semi-solid	2.281 µg/g DW (n = 12)		
	Flavonoids	TIB	72.54 mg catechin eq./g DW (n = 12)	0.4428	No
		Semi-solid	63.86 mg 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	4.614 µmol/g DW (n = 3)	>0.9999	No
		Semi-solid	5.301 µmol/g DW (n = 3)		
Pa	Chlorophyll a	TIB	4.828 µg/g DW (n = 12)	<0.0001	Yes
		Semi-solid	8.284 µg/g DW (n = 12)		
	Chlorophyll b	TIB	1.925 µg/g DW (n = 12)	<0.0001	Yes
		Semi-solid	2.940 µg/g DW (n = 12)		
	Carotenoids	TIB	1.204 µg/g DW (n = 12)	<0.0001	Yes
		Semi-solid	2.181 µg/g DW (n = 12)		
	Flavonoids	TIB	63.04 mg catechin eq./g DW (n = 12)	0.2657	No
		Semi-solid	73.51 mg 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)		

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 & 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 & 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 3). 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 & 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 & Munné-Bosch, 2019; Polivanova & Bedarev, 2022). For this parameter, no significant differences were observed between propagation systems within each genotype (Table 3). 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 & 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 3), however higher values of proline content were noted 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 presents the first report of sweet cherry rootstocks propagation using the SETIS™ system. The success of *in vitro* establishment, explants were collected and established *in vitro*, with successful establishment rates of 56% for *P. avium* and 62% for Gisela 6. Assessment of shoot proliferation in the SETIS™ bioreactor showed superior results in shoot length and RGR compared to 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. 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.

Declarations

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.

Ethics declaration

Not applicable.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1. Akdemir, H., Süzerer, V., Onay, A., Tilkat, E., Ersali, Y., & Çiftçi, Y. O. (2014). Micropropagation of the pistachio and its rootstocks by temporary immersion system. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 117(1), 65–76. <https://doi.org/10.1007/s11240-013-0421-0>
2. Albacete, A., Martínez-Andújar, C., Martínez-Pérez, A., Thompson, A. J., Dodd, I. C., & Pérez-Alfocea, F. (2015). Unravelling rootstock×scion interactions to improve food security. *Journal of Experimental Botany*, 66(8), 2211–2226. <https://doi.org/10.1093/jxb/erv027>
3. 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. *Plant Cell, Tissue and Organ Culture*, 81(1), 27–36. <https://doi.org/10.1007/s11240-004-2618-8>

4. Alvard, D., Cote, F., & Teisson, C. (1993). Comparison of methods of liquid medium culture for banana micropropagation. *Plant Cell, Tissue and Organ Culture*, 32(1), 55–60.
<https://doi.org/10.1007/BF00040116>
5. Alvarenga Venutolo, S. (2015). Micropropagación masiva de *Stevia 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
6. Alves, V., Pinto, R., Debiasi, C., Santos, M. C., Gonçalves, J. C., & Domingues, J. (2021). Micropropagation of *Corema album* from adult plants in semisolid medium and temporary immersion bioreactor. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 145(3), 641–648.
<https://doi.org/10.1007/s11240-021-02034-1>
7. Amiri, M. E. (2006). *In vitro* techniques to study the shoot-tip grafting of *Prunus avium* L. (cherry) var. Seeyahe Mashad. *Journal of Food, Agriculture and Environment*, 4, 151–154.
8. Aragón, C. E., Escalona, M., Capote, I., Pina, D., Cejas, I., Rodriguez, R., Cañal, M. J., 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. *In Vitro Cellular & Developmental Biology. Plant*, 41(4), 550–554.
<https://www.jstor.org/stable/4293899>
9. Baránek, M., Raddová, J., & Pidra, M. (2006). Comparative analysis of genetic diversity in *Prunus* L. as revealed by RAPD and SSR markers. *Scientia Horticulturae*, 108(3), 253–259.
<https://doi.org/10.1016/j.scienta.2006.01.023>
10. Bates, L. S., Waldren, R. P., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil*, 39(1), 205–207. <https://doi.org/10.1007/BF00018060>
11. Benelli, C., & De Carlo, A. (2018). *In vitro* multiplication and growth improvement of *Olea europaea* L. cv Canino with temporary immersion system (Plantform™). *3 Biotech*, 8(7), 317.
<https://doi.org/10.1007/s13205-018-1346-4>
12. Casanova, E., Moysset, L., & Trillas, M. I. (2008). Effects of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots. *Biologia Plantarum*, 52(1), 1–8. <https://doi.org/10.1007/s10535-008-0001-z>
13. Chakrabarty, D., Hahn, E., Yoon, Y., & Paek, K. (2003). Micropropagation of apple rootstock M. 9 EMLA using bioreactor. *Journal of horticultural science & biotechnology*, 78, 605–609.
<https://doi.org/10.1080/14620316.2003.11511671>
14. Cristina, M.-A. M., Eucario, M.-Á., Luis, S.-C. J., & Jabín, B.-B. J. (2023). Evaluation of the effect of different culture systems on photomixotrophic capacity during *in vitro* multiplication of pitahaya (*Hylocereus undatus*). *South African Journal of Botany*, 159, 396–404.
<https://doi.org/10.1016/j.sajb.2023.06.013>
15. Druart, P. (2013). Micropropagation of *Prunus* species relevant to cherry fruit production. *Methods in Molecular Biology (Clifton, N.J.)*, 11013, 119–136. https://doi.org/10.1007/978-1-62703-074-8_9

16. Elazab, D., Capuana, M., Ozudogru, E. A., 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), Artigo 6. <https://doi.org/10.3390/horticulturae9060680>
17. Escalona, M., Lorenzo, J. C., González, B., Daquinta, M., González, J. L., Desjardins, Y., & Borroto, C. G. (1999). Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. *Plant Cell Reports*, 18(9), 743–748. <https://doi.org/10.1007/s002990050653>
18. Etienne, H., & Berthouly, M. (2002). Temporary immersion systems in plant micropropagation. *Plant Cell, Tissue and Organ Culture*, 69(3), 215–231. <https://doi.org/10.1023/A:1015668610465>
19. Falqueto, A. R., da Silva Júnior, R. A., Gomes, M. T. G., Martins, J. P. R., Silva, D. M., & Partelli, F. L. (2017). Effects of drought stress on chlorophyll *a* fluorescence in two rubber tree clones. *Scientia Horticulturae*, 224, 238–243. <https://doi.org/10.1016/j.scienta.2017.06.019>
20. Feeney, M., Bhagwat, B., Mitchell, J. S., & Lane, W. D. (2007). Shoot regeneration from organogenic callus of sweet cherry (*Prunus avium* L.). *Plant Cell, Tissue and Organ Culture*, 90(2), 201–214. <https://doi.org/10.1007/s11240-007-9252-1>
21. Fernandez-García, N., Piqueras, A., & Olmos, E. (2008). Sub-cellular location of H₂O₂, peroxidases and pectin epitopes in control and hyperhydric shoots of carnation. *Environmental and Experimental Botany*, 62(2), 168–175. <https://doi.org/10.1016/j.envexpbot.2007.08.004>
22. Feucht, W., & Dausend, B. (1976). Root induction *in vitro* of easy-to-root *Prunus pseudocerasus* and difficult-to-root *Prunus avium*. *Scientia Horticulturae*, 4(1), 49–54. [https://doi.org/10.1016/0304-4238\(76\)90064-9](https://doi.org/10.1016/0304-4238(76)90064-9)
23. Gatica-Arias, A. M., Arrieta-Espinoza, G., & Espinoza Esquivel, A. M. (2008). Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuaí. *Electronic Journal of Biotechnology*, 11(1), 101–112. <https://doi.org/10.4067/S0717-34582008000100010>
24. Godoy, S., Tapia, E., Seit, P., Andrade, D., Sánchez, E., Andrade, P., Almeida, A. M., & 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. *In Vitro Cellular & Developmental Biology - Plant*, 53(5), 494–504. <https://doi.org/10.1007/s11627-017-9856-z>
25. Guajardo, V., Solís, S., Almada, R., Saski, C., Gasic, K., & Moreno, M. Á. (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. *Scientific Reports*, 10(1), 1467. <https://doi.org/10.1038/s41598-020-58271-5>
26. 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. *The ISME Journal*, 16(1), 122–137. <https://doi.org/10.1038/s41396-021-01060-3>

27. 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>
28. Hammatt, N., & Grant, N. J. (1998). Shoot regeneration from leaves of *Prunus serotina* Ehrh. (Black cherry) and *P. avium* L. (wild cherry). *Plant Cell Reports*, 17(6–7), 526–530. <https://doi.org/10.1007/s002990050436>
29. Hartmann, H. T., Brooks, R. M., Hartmann, H. T., & Brooks, R. M. (1958). Propagation of Stockton Morello cherry rootstock by softwood cuttings under mist sprays. *Proceedings of the American Society for Horticultural Science*, 71, 127–134. <https://eurekamag.com/research/014/156/014156205.php>
30. Hodges, D. M., DeLong, J. M., Forney, C. F., & Prange, R. K. (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>
31. Hutchison, J. (1984). Factors affecting shoot proliferation and root initiation in organ cultures of the apple 'Northern Spy'. *Scientia Horticulturae*, 22(4), 347–358. [https://doi.org/10.1016/S0304-4238\(84\)80006-0](https://doi.org/10.1016/S0304-4238(84)80006-0)
32. Hwang, H.-D., Kwon, S.-H., Murthy, H. N., Yun, S.-W., Pyo, S.-S., & Park, S.-Y. (2022). Temporary Immersion Bioreactor System as an Efficient Method for Mass Production of In Vitro Plants in Horticulture and Medicinal Plants. *Agronomy*, 12(2), Artigo 2. <https://doi.org/10.3390/agronomy12020346>
33. INE. (2021). *Estatísticas Agrícolas 2021*. Statistics Portugal. https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_publicacoes&PUBLICACOESpub_boui=31589846&PUBLICACOESmodo=2
34. Iv Aní & Pre. (1980). Embryo culture and micropropagation of cherries *in vitro*. *Scientia Horticulturae*, 12(1), 77–82. [https://doi.org/10.1016/0304-4238\(80\)90040-0](https://doi.org/10.1016/0304-4238(80)90040-0)
35. Ivanova, M., & Van Staden, J. (2011). Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 104(1), 13–21. <https://doi.org/10.1007/s11240-010-9794-5>
36. Jiroutova, P., Kovalikova, Z., Toman, J., Dobrovolna, D., & Andrys, R. (2021). Complex Analysis of Antioxidant Activity, Abscissic Acid Level, and Accumulation of Osmotica in Apple and Cherry *In Vitro* Cultures under Osmotic Stress. *International Journal of Molecular Sciences*, 22(15), 7922. <https://doi.org/10.3390/ijms22157922>
37. Kevers, C., Franck, T., Strasser, R. J., Dommès, J., & Gaspar, T. (2004). Hyperhydricity of Micropropagated Shoots: A Typically Stress-induced Change of Physiological State. *Plant Cell, Tissue and Organ Culture*, 77(2), 181–191. <https://doi.org/10.1023/B:TICU.0000016825.18930.e4>
38. 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. *Horticulture, Environment, and Biotechnology*, 61(4), 779–785. <https://doi.org/10.1007/s13580-020-00257-3>

39. 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>
40. Leal, D. R., Sánchez-Olate, M., Avilés, F., Materan, M. E., 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* (pp. 381–390). Springer Netherlands. https://doi.org/10.1007/978-1-4020-6352-7_35
41. Lichtenthaler, H. K. (1987). Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Em *Methods in Enzymology* (Vol. 148, pp. 350–382). Academic Press. [https://doi.org/10.1016/0076-6879\(87\)48036-1](https://doi.org/10.1016/0076-6879(87)48036-1)
42. Lopes, T., Correia, M., Pedrosa, A., Baltazar, E., Canhoto, J., & Correia, S. (2023). Improved micropropagation of *Prunus* spp. Rootstocks using temporary immersion systems. *Acta Horticulturae*, 1359, 249–254. <https://doi.org/10.17660/ActaHortic.2023.1359.32>
43. Mancilla-Álvarez, E., Pérez-Sato, J. A., Núñez-Pastrana, R., Spinoso-Castillo, J. L., & Bello-Bello, J. J. (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>
44. Martínez-Estrada, E., Islas-Luna, B., Pérez-Sato, J. A., & Bello-Bello, J. J. (2019). Temporary immersion improves *in vitro* multiplication and acclimatization of *Anthurium andreaeanum* Lind. *Scientia Horticulturae*, 249, 185–191. <https://doi.org/10.1016/j.scienta.2019.01.053>
45. Martínez-Gómez, P., Sánchez-Pérez, R., Rubio, M., Dicenta, F., Campus Universitario de Espinardo, Gradziel, T. M., University of California, Sozzi, G. O., & Universidad de Buenos Aires. (2005). Application of Recent Biotechnologies to *Prunus* Tree Crop Genetic Improvement. *Ciencia e Investigación Agraria*, 32(2), 73–96. <https://doi.org/10.7764/rcia.v32i2.308>
46. 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. *Frontiers in Plant Science*, 13. <https://doi.org/10.3389/fpls.2022.1011542>
47. Mauro, R. P., 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>
48. Modgil, M., Sharma, D. R., & Bhardwaj, S. V. (1999). Micropropagation of apple cv. Tydemann's Early Worcester. *Scientia Horticulturae*, 81(2), 179–188. [https://doi.org/10.1016/S0304-4238\(98\)00259-3](https://doi.org/10.1016/S0304-4238(98)00259-3)
49. Monteiro, T. R., Freitas, E. O., Nogueira, G. F., & Scherwinski-Pereira, J. E. (2018). Assessing the influence of subcultures and liquid medium during somatic embryogenesis and plant regeneration in oil palm (*Elaeis guineensis* Jacq.). *The Journal of Horticultural Science and Biotechnology*, 93(2), 196–203. <https://doi.org/10.1080/14620316.2017.1360156>

50. Morales, M., & Munné-Bosch, S. (2019). Malondialdehyde: Facts and Artifacts. *Plant Physiology*, 180(3), 1246–1250. <https://doi.org/10.1104/pp.19.00405>
51. Moreno, R. J. L., Morales, A. V., & Gradaille, M. D. (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.
52. Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, 15(3), 473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
53. Musa, M., & Lyam, P. (2012). The Potential of Temporary Immersion Bioreactors (TIBs) in Meeting Crop Production Demand in Nigeria. *Journal of Biology*. <https://doi.org/10.5296/JBLS.V3I1.1156>
54. Németh, G. (1979). Benzyladenine-Stimulated Rooting in Fruit-Tree Rootstocks Cultured *in vitro*. *Zeitschrift für Pflanzenphysiologie*, 95(5), 389–396. [https://doi.org/10.1016/S0044-328X\(79\)80209-3](https://doi.org/10.1016/S0044-328X(79)80209-3)
55. 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. *Agricultural Water Management*, 228, 105897. <https://doi.org/10.1016/j.agwat.2019.105897>
56. 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 Journal of Botany*, 42, 280–288. <https://doi.org/10.3906/bot-1709-14>
57. Pérez-Tornero, O., & Burgos, L. (2007). Apricot micropropagation. Em S. M. Jain & H. Häggman (Eds.), *Protocols for Micropropagation of Woody Trees and Fruits* (pp. 267–278). Springer Netherlands. https://doi.org/10.1007/978-1-4020-6352-7_25
58. Picoli, E. A. T., Otoni, W. C., Figueira, M. L., Carolino, S. M. B., Almeida, R. S., Silva, E. A. M., Carvalho, C. R., & Fontes, E. P. B. (2001). Hyperhydricity in *in vitro* eggplant regenerated plants: Structural characteristics and involvement of BiP (Binding Protein). *Plant Science*, 160(5), 857–868. [https://doi.org/10.1016/S0168-9452\(00\)00463-5](https://doi.org/10.1016/S0168-9452(00)00463-5)
59. Polivanova, O. B., & Bedarev, V. A. (2022). Hyperhydricity in Plant Tissue Culture. *Plants*, 11(23), 3313. <https://doi.org/10.3390/plants11233313>
60. Poorter, H., & Garnier, E. (1996). Plant growth analysis: An evaluation of experimental design and computational methods. *Journal of Experimental Botany*, 47(9), 1343–1351. <https://doi.org/10.1093/jxb/47.9.1343>
61. Preil, W. (2005). General introduction: A personal reflection on the use of liquid media for *in vitro* culture. Em A. K. Hvostlef-Eide & W. Preil (Eds.), *Liquid Culture Systems for in vitro Plant Propagation* (pp. 1–18). Springer Netherlands. https://doi.org/10.1007/1-4020-3200-5_1
62. Quiala, E., Cañal, M.-J., Meijón, M., Rodríguez, R., Chávez, M., Valledor, L., de Fera, M., & Barbón, R. (2012). Morphological and physiological responses of proliferating shoots of teak to temporary

- immersion and BA treatments. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 109(2), 223–234. <https://doi.org/10.1007/s11240-011-0088-3>
63. Ramírez-Mosqueda, M. A., & Bello-Bello, J. J. (2021). SETISTM bioreactor increases in vitro multiplication and shoot length in vanilla (*Vanilla planifolia* Jacks. Ex Andrews). *Acta Physiologiae Plantarum*, 43(4), 52. <https://doi.org/10.1007/s11738-021-03227-z>
 64. Sedlák, J., & Paprštejn, F. (2008). In vitro shoot proliferation of sweet cherry cultivars Karešova and Rivan. *Horticultural Science*, 35(3), 95–98. <https://doi.org/10.17221/6/2008-HORTSCI>
 65. Shah, A., & Smith, D. L. (2020). Flavonoids in Agriculture: Chemistry and Roles in, Biotic and Abiotic Stress Responses, and Microbial Associations. *Agronomy*, 10(8), Artigo 8. <https://doi.org/10.3390/agronomy10081209>
 66. 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. *International Journal of Current Microbiology and Applied Sciences*, 6(8), 2617–2624. <https://doi.org/10.20546/ijcmas.2017.608.311>
 67. Shin, Y. K., Bhandari, S. R., Cho, M. C., & Lee, J. G. (2020). Evaluation of chlorophyll fluorescence parameters and proline content in tomato seedlings grown under different salt stress conditions. *Horticulture, Environment, and Biotechnology*, 61(3), 433–443. <https://doi.org/10.1007/s13580-020-00231-z>
 68. Simkin, A. J., Kapoor, L., Doss, C. G. P., Hofmann, T. A., Lawson, T., & Ramamoorthy, S. (2022). The role of photosynthesis related pigments in light harvesting, photoprotection and enhancement of photosynthetic yield in planta. *Photosynthesis Research*, 152(1), 23–42. <https://doi.org/10.1007/s11120-021-00892-6>
 69. 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. *Horticultural Science*, 48(1), 12–21. <https://doi.org/10.17221/69/2020-HORTSCI>
 70. 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, 12–17 June, 1994* (pp. 105–110). Springer Netherlands. https://doi.org/10.1007/978-94-011-0307-7_12
 71. Tisserat, B., & Vandercook, C. E. (1985). Development of an automated plant culture system. *Plant Cell, Tissue and Organ Culture*, 5(2), 107–117. <https://doi.org/10.1007/BF00040307>
 72. van den Dries, N., Gianni, S., Czerednik, A., Krens, F. A., & de Klerk, G.-J. M. (2013). Flooding of the apoplast is a key factor in the development of hyperhydricity. *Journal of Experimental Botany*, 64(16), 5221–5230. <https://doi.org/10.1093/jxb/ert315>
 73. Vervit. (2024). *Setis / ABOUT SETISTM*. Setis. <https://setis-systems.be/about-setis>

74. Vidal, N., Blanco, B., & Cuenca, B. (2015). A temporary immersion system for micropropagation of axillary shoots of hybrid chestnut. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 123(2), 229–243. <https://doi.org/10.1007/s11240-015-0827-y>
75. Vidal, N., & Sánchez, C. (2019). Use of bioreactor systems in the propagation of forest trees. *Engineering in Life Sciences*, 19(12), 896–915. <https://doi.org/10.1002/elsc.201900041>
76. Webster, C. A., & Jones, O. P. (1991). Micropropagation of some cold-hardy dwarfing rootstocks for apple. *Journal of Horticultural Science*, 66(1), 1–6. <https://doi.org/10.1080/00221589.1991.11516118>
77. Weston, L. A., & Mathesius, U. (2013). Flavonoids: Their Structure, Biosynthesis and Role in the Rhizosphere, Including Allelopathy. *Journal of Chemical Ecology*, 39(2), 283–297. <https://doi.org/10.1007/s10886-013-0248-5>
78. Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, 64(4), 555–559. [https://doi.org/10.1016/S0308-8146\(98\)00102-2](https://doi.org/10.1016/S0308-8146(98)00102-2)
79. 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. *Plant Cell, Tissue and Organ Culture*, 81(3), 313–318. <https://doi.org/10.1007/s11240-004-6659-9>
80. Zilkah, S., Faingersh, E., & Rotbaum, A. (1992). *In vitro* propagation of three mxm (*Prunus avium* x *P. Mahaleb*) cherry rootstocks. *Acta Horticulturae*, 314, 93–98. <https://doi.org/10.17660/ActaHortic.1992.314.10>

Figures



Figure 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



Figure 2

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



Figure 3

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

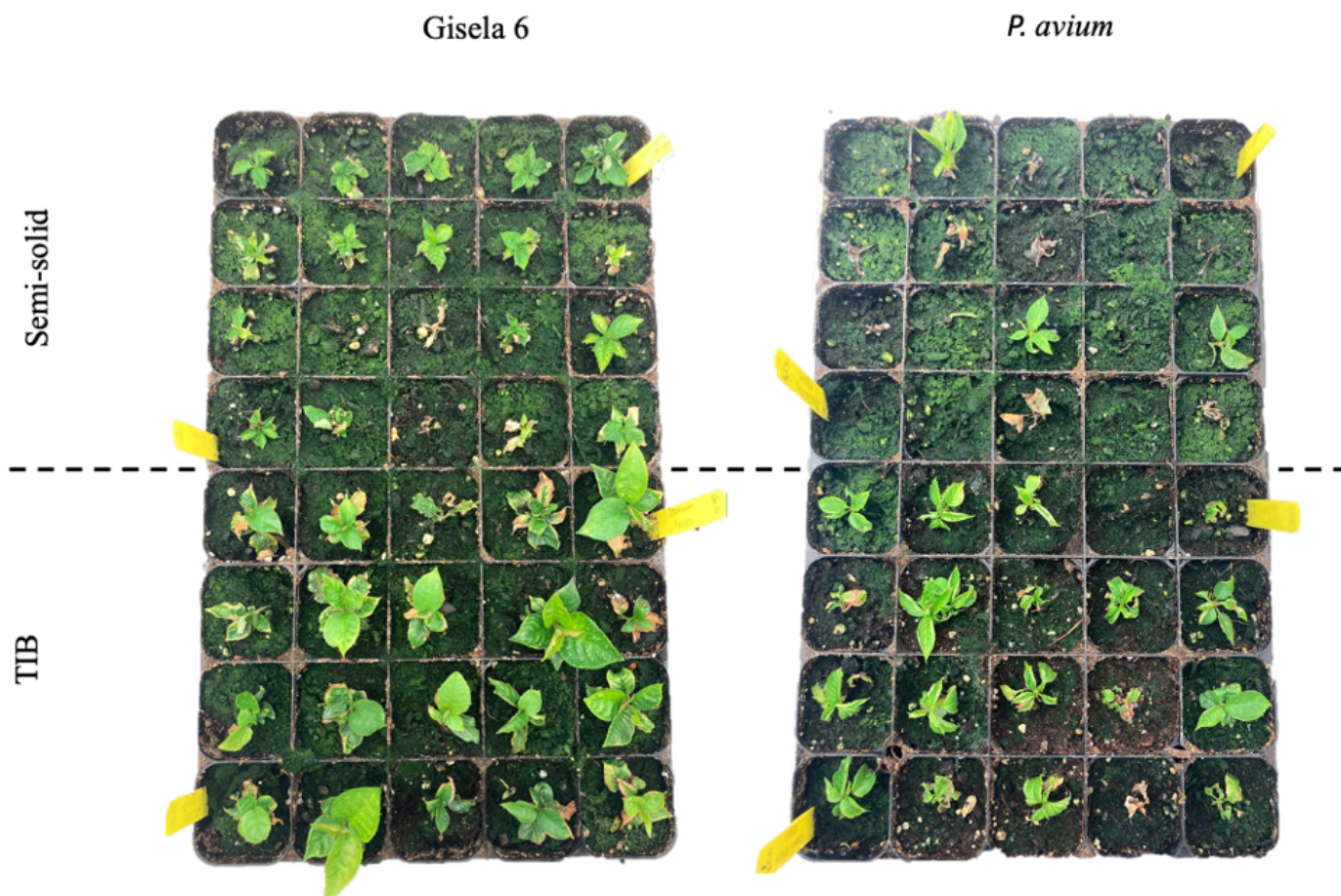


Figure 4

Gisela 6 and *P. avium* plantlets propagated in semi-solid media and TIB in the acclimatation stage in plastic trays with 80 cm³ per cell after 28 days of root induction