

Optimization of immersion frequency in a temporary immersion bioreactor for the micropropagation of *Prunus persica* × *Prunus amygdalus*

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Abstract

In vitro propagation of recalcitrant materials, such as *Prunus persica* × *P. amygdalus* hybrid rootstocks (Garnem), requires strictly controlled culture conditions. Temporary immersion systems (TIS) represent an efficient alternative for the micropropagation of demanding genotypes; however, their performance depends on the fine-tuning of key variables, particularly immersion frequency. The objective of this study was to evaluate the effect of different immersion frequencies on the productive, physiological, and histological performance of 'Garnem' rootstock plantlets micropropagated in a twin-vessel temporary immersion system compared to a conventional semi-solid medium system. Temporary immersion systems promoted greater vegetative growth and biomass accumulation compared to the semi-solid medium; however, the responses were dependent on immersion frequency. Immersion every 6 hours showed the best overall performance, yielding higher plant height, multiplication rate, and dry biomass, along with low levels of hyperhydricity and a histological organization of the stem and leaf comparable to the *ex vitro* phenotype. This treatment also exhibited adequate vascular differentiation, higher stability of photosynthetic pigments, and less alteration in stomatal morphology. In contrast, immersion every 3 hours induced an increase in fresh weight but was associated with high hyperhydricity, tissue disorganization, and functional alterations, which resulted in lower survival during acclimatization. Multivariate analysis confirmed the association of the 6-hour regime with growth and physiological quality variables. These results demonstrate that adjusting the immersion frequency is key to improving the micropropagation efficiency of 'Garnem' and enhancing its *ex vitro* performance.

1. Introduction

The 'Garnem' rootstock (GXN 15) is an interspecific hybrid derived from a cross between almond (*Prunus amygdalus* cv. Garfi) and peach (*Prunus persica* cv. Nemared) at the Center for Agrifood Research and Technology of Aragon (CITA), Spain. It has established itself as one of the most widely used rootstocks in modern pomology due to its vigor, adaptability to diverse soils, nematode resistance, and tolerance to adverse conditions such as drought and calcareous soils (Justamante et al. 2022).

In vitro micropropagation has become a fundamental biotechnological tool for the multiplication of these rootstocks (Eliwa 2025), enabling the production of genetically uniform, pathogen-free plants within reduced timeframes (Vidal and Sánchez 2019). However, traditional systems based on semi-solid media present significant limitations, such as the accumulation of metabolic gases, particularly ethylene, which reach phytotoxic levels, inhibiting growth and promoting tissue senescence and necrosis. Furthermore, 'Garnem' has shown a marked sensitivity to hypoxia and root asphyxia, increasing the incidence of hyperhydricity, one of the primary challenges in the *in vitro* culture of this genotype (Al-Eqabi and Al-Amery 2025; Bulunuz Palaz et al. 2023).

Hyperhydricity in *Prunus* shoots is characterized by thickened stems, short internodes, and translucent, brittle, and deformed leaves. These symptoms are associated with physiological and anatomical alterations, such as low chlorophyll accumulation, excessive water in intercellular spaces, reduced

lignification, thinner cell walls, and weakened vascular tissues. Collectively, these factors compromise plantlet development and subsequent acclimatization (Polivanova and Bedarev 2022).

To overcome these limitations, temporary immersion systems (TIS) have been developed. This technological innovation cyclically exposes explants to liquid media, alternating between immersion and aeration phases under sterile conditions (Mirzabe et al. 2022). This system enhances nutrient uptake and optimizes the gas exchange of the plantlets (Méndez-Hernández and Loyola-Vargas 2024). One of the TIS mechanisms available is the twin-vessel system, which offers advantages such as precise control of immersion times, guaranteed gas exchange during each cycle, and simple, cost-effective construction. This system is among the most widely adopted approaches (Mirzabe et al., 2022), demonstrating substantial improvements in multiplication rates, physiological shoot quality, and rooting and acclimatization efficiency in various *Prunus* and woody species (Vidal and Sánchez 2019; García-Ramírez 2023; Gago et al. 2022; Sanson et al. 2024).

The primary adjustment in TIS is the immersion frequency, as an excessive number of cycles increases hyperhydricity, while infrequent exposure to the culture medium may limit nutrient availability and cause surface dehydration of the tissue. Determining optimal immersion and aeration parameters is, therefore, a critical factor for the success of micropropagation under this system (Mirzabe et al. 2022). In this context, published studies on 'Garnem' remain limited, and there are no specific studies evaluating how immersion frequency affects the morphophysiological and histological development of the plantlets. Consequently, the objective of this study was to evaluate the productive performance and the morphophysiological and histological quality of *Prunus persica* × *P. amygdalus* plantlets micropropagated in both conventional and temporary immersion systems considering different immersion frequencies to generate information applicable to the refinement of propagation protocols and to breeding and commercial production programs.

2. Materials and Methods

The experiment was conducted at the Plant Biotechnology Laboratory of the Faculty of Agricultural Sciences, National University of Córdoba (LBV-FCA) in Córdoba, Argentina. The development of *Prunus persica* × *P. amygdalus* (Garnem) plantlets was evaluated in a twin-vessel temporary immersion system (TIS) at immersion frequencies of 3 h, 6 h, and 12 h, and compared with the traditional in vitro system described by Rivata et al. (2010).

2.1 Initial Plant Material

Nodal segments of *Prunus persica* (L.) Batsch × *Prunus amygdalus* Batsch ('Garnem') were established in vitro from adult mother plants. Disinfection was performed following the protocol by Rezaei (2023). Two-node stem sections were then inoculated into test tubes containing 10 mL of semi-solid culture medium consisting of Woody Plant Medium (WPM) salts and vitamins (Lloyd and McCown 1980), supplemented with 2.22 µM 6-benzylaminopurine (BAP), 4.9 nM indole-3-butyric acid (IBA), 3% sucrose, and 0.8% agar-

agar (Abou Elyazid 2021). Explants were grown for 30 days in a growth chamber regulated at $21 \pm 2^\circ\text{C}$ with a 16-h photoperiod. Contamination-free explants were selected for the trials.

2.2 Experimental Design

A twin-vessel temporary immersion system (TIS) was employed, establishing three treatments based on immersion frequency: 1-min immersions every 3 h (TIS 3), 6 h (TIS 6), and 12 h (TIS 12). A conventional semi-solid culture system (SS) was used as a control. For all in vitro developmental stages, a culture medium formulated with WPM salts and vitamins modified according to Abou Elyazid (2021) was used. The SS medium was solidified with 0.8% (w/v) agar-agar, while no gelling agent was added to TIS 3, TIS 6, and TIS 12 media. The pH was adjusted to 5.7 prior to autoclaving at 120°C for 20 min for all treatments.

The trial consisted of three consecutive multiplication subcultures supplemented with $4.44 \mu\text{M}$ BAP and 49 nM IBA. Subsequently, a fourth rooting subculture was performed, supplemented with $4.92 \mu\text{M}$ IBA. During the subcultures, plantlets were grown in a growth chamber for 30 days at $21 \pm 2^\circ\text{C}$ under a 16-h photoperiod.

TIS treatments were initiated with 10 explants per experimental unit, using 720 mL vessels containing 100 mL of liquid culture medium, with four experimental units per treatment (Murthy et al. 2023). In the SS treatment, 360 mL vessels with 50 mL of culture medium were used, initiated with five explants per experimental unit and four replicates (Rivata et al. 2010).

For acclimatization, plantlets were transferred to 200 mL containers with a substrate composed of perlite, peat, and vermiculite (1:1:1 v/v/v). Environmental conditions during acclimatization were 65% relative humidity and $24 \pm 2^\circ\text{C}$ for 35 days (Ekinci et al. 2024).

2.3 Evaluation of Variables

2.3.1 Growth Variables:

Following each multiplication and rooting subculture, 10 plantlets were randomly selected per treatment. Plant height (Ht), number of shoots (NS), and hyperhydricity were recorded. Hyperhydricity was estimated as the percentage of affected plants (%HYP) and severity grade (HYP) according to Bethge et al. (2023), where grade 0 represents a normal plantlet and grade 5 indicates extreme morphological defects throughout the plantlet (Table 1). Additionally, fresh weight (FW) and dry weight (DW) were determined; the latter was measured after placing plantlets in a drying oven at 60°C for 48 h until constant weight was achieved. Relative water content (%H₂O) was determined according to Gantait and Mahanta (2022). The multiplication rate (MR) was calculated during the multiplication subcultures as the number of explants generated per plantlet. For the rooting subculture, rooting percentage (%Root) and root length (RL) were determined. During the acclimatization stage, survival (%SURV) and plant height (HtP) were recorded at 0, 5, 15, and 35 days post-transplant. A non-linear growth function was fitted for each treatment: $\text{HtP} = \alpha \cdot e^{(\beta \cdot \text{days})}$.

2.3.2 Photosynthetic Pigments:

For all subcultures, chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (Chl t) were determined following the protocol by Ramírez-Mosqueda et al. (2019). Absorbance values were recorded at 663 nm and 645 nm using a DeNovix DS-11 spectrophotometer. Subsequently, pigment concentration ($\mu\text{g}\cdot\text{g}^{-1}$ FW) was calculated using the formulas described by Harborne (1984), where:

- $\text{Chl } a \text{ } (\mu\text{g}\cdot\text{g}^{-1} \text{ FW}) = \frac{[(12.7)(A_{663}) - (2.59)(A_{645})](V)}{(1000)(W)}$
- $\text{Chl } b \text{ } (\mu\text{g}\cdot\text{g}^{-1} \text{ FW}) = \frac{[(22.9)(A_{645}) - (4.70)(A_{663})](V)}{(1000)(W)}$
- $\text{Chl } t \text{ } (\mu\text{g}\cdot\text{g}^{-1} \text{ FW}) = \frac{[8.20(A_{663}) - (20.2)(A_{645})](V)}{(1000)(W)}$

Where: A: Absorbance at the wavelength indicated by the subscript (645 and 663 nm). V: Final volume of the extract (dm^3). W: Sample fresh weight (g). 1000: Conversion factor.

2.3.3 Anatomical Variables:

Anatomical analysis was performed on three plantlets from each treatment after each subculture. Additionally, anatomical analysis was conducted on tissues from adult ex vitro mother plants (MP). All samples were preserved in a 1:1:1 solution of formaldehyde, acetic acid, and 70% ethyl alcohol (v/v). Anatomical characterization of the stems was carried out on cross-sections of the tissue located at the first two fully expanded leaves. For leaf analysis, cross-sections of the middle region of the first fully expanded leaf were used. Samples were prepared following the protocol for fixation, dehydration, paraffin embedding, and double staining with Safranin and Fast-green, as described by Delbón et al. (2024). Slides were observed using an Arcano XSZ-107BNT optical microscope; images were captured at 10X and 40X magnifications with a Sony Hx400v camera. Image processing and data recording were performed using Image-J 1.52a software (Rueden et al. 2021). From the stem photographs obtained, the following parameters were recorded: xylem thickness (XiS) and phloem thickness (PhS) (μm) (García Osuna et al. 2011), stem xylem/phloem ratio (XiS/PhS), and stem epidermal tissue thickness (EpT, μm) (Majada et al. 2020; Castillo Ontaneda et al. 2020). For the leaf histological analysis, xylem thickness (XiL) and phloem thickness (PhL) in the leaf midrib (μm) (García Osuna et al. 2011), the XiL/PhL ratio, and Leaf blade thickness (LBT: μm) (Majada et al. 2020) were recorded. Additionally, hyperhydricity indices were determined for the stem vascular tissue (HYP-SVT) and leaf vascular tissue (HYP-LVT), stem epidermal tissue (HYP-SET) and leaf epidermal tissue (HYP-LET), and leaf blade (HYP-LB). For this purpose, a scale was established where grade 0 (zero) corresponds to normal tissue and grade 5 (five) indicates extreme morphological defects in the tissue (Polivanova and Bedarev, 2022) (Table 1).

Table 1

Visual and anatomical scale of hyperhydricity severity in in vitro cultured *P. persica* × *P. amygdalus* plantlets for the analyzed variables.

Grade	HYP*	HYP-SVT HYP-LVT**	HYP-LB ***	HYP-SET HYP-LET**
0 (Normal)	No visual symptoms	Well-defined, normal bundles; lignified xylem.	Organized mesophyll (palisade and spongy); compact cells.	Continuous cuticle; compact epidermis.
1 (Initial)	< 50% of leaves slightly curled	Minimal reduction in xylem cell wall thickness.	Slight increase in intercellular spaces; normal cell volume.	Slight cuticle thinning; functional epidermis.
2 (Moderate)	> 50% of leaves curled	Decreased xylem diameter; detectable hypolignification.	Palisade reduction; initial vacuolization.	Incipient cuticular fragmentation; partial loss of waxes.
3 (Substantial)	> 50% of leaves curled and thickened	Weak phloem–xylem connection; poorly developed phloem.	Disorganized mesophyll.	Cuticular gaps; epidermal hypertrophy.
4 (Severe)	Thick, translucent, and brittle leaves	Scanty and disorganized xylem and phloem; absence of procambium.	Loss of palisade; highly hypertrophied cells.	Epidermal discontinuity; giant epidermal cells.
5 (Extreme)	Apical necrosis or total shoot vitrification	Total absence of a functional vascular system.	Tissue necrosis; protoplasmic mass.	Epidermal collapse; loss of membrane integrity.

* Adapted and modified from Bethge et al. (2023).

** Adapted and modified from Polivanova and Bedarev (2022).

*** Adapted and modified from Kemat et al. (2023).

2.3.4 Stomatal Characterization

For each in vitro treatment (TIS 3, TIS 6, TIS 12, SS) and for the mother plants (MP), three fully expanded leaves were selected after the fourth subculture. Stomatal images were captured using a Confocal Laser Scanning Microscope (Olympus LEXT OLS4000). The images were analyzed with Olympus LEXT OLS4100 software (version 3.1.5). Stomatal length (StL), stomatal width (StW), pore length (PoL), and pore width (PoW) were recorded for subsequent analysis.

2.4 Statistical Analysis

The experiment followed a completely randomized design (CRD) with four replicates per treatment, considering each culture vessel (TIS or semi-solid container) as the experimental unit. All measured variables were expressed as the mean value per plantlet. Subcultures were analyzed as independent experimental cycles. Prior to analysis of variance (ANOVA), normality and homogeneity of variances were verified using the Shapiro–Wilk and Levene tests, respectively. Percentage data were arcsine square-root transformed to meet ANOVA assumptions. When ANOVA showed significant effects, means were compared using Fisher’s Least Significant Difference (LSD) test at a significance level of 5% ($p \leq 0.05$). Non-parametric variables were analyzed using the Kruskal–Wallis test. For multivariate analysis, all variables were standardized (mean-centered and scaled to unit variance) prior to Principal Component Analysis (PCA). Statistical analyses were conducted using InfoStat software (version 2020) (Di Rienzo et al. 2020).

3. Results

3.1 Productive Variables

In general terms, the use of temporary immersion systems (TIS) promoted greater vegetative growth and multiplication capacity in *P. persica* × *P. amygdalus* plantlets compared to those cultured on semi-solid medium (SS) (Table 2). Plantlet height (Ht) was significantly enhanced by the use of TIS compared to SS.

Treatment TIS 6 showed the highest vegetative growth, reaching an average height of 9.31 cm, which represented an increase of more than 100% compared to the control SS (4.44 cm). A similar trend was observed in the multiplication rate (MR), where the 6- and 12-hour immersion frequencies significantly outperformed the solid medium; however, the number of shoots (NS) was slightly higher in the SS treatment. The physiological quality of the shoots showed a critical dependence on immersion frequency. The TIS 3 treatment exhibited a notable deterioration in plant morphology, recording the highest levels of hyperhydricity incidence (31%) and severity (3.53), which correlated with the highest tissue water content (82.27%). In contrast, the 6 and 12-hour frequencies maintained significantly low hyperhydricity levels (6%), showing no statistically relevant differences between them. It should be noted that the semi-solid medium (SS) was the treatment that resulted in an average of 25% of plants with hyperhydricity, but with low severity (0.31) during the four subcultures evaluated. During the rooting phase, a differential behavior was observed between induction capacity and subsequent root development. The highest rooting percentage (%Root) was obtained in the SS treatment (88%), followed in decreasing order by TIS 12, TIS 6, and finally TIS 3 with only 50%. However, once rhizogenesis was initiated, the conditions in the TIS 12 and TIS 6 systems promoted greater root elongation (8.44 cm and 6.25 cm, respectively) compared to the semi-solid medium (5.63 cm). These findings indicate that while the solid medium facilitates the transition to the rooting phase, spaced immersion frequencies (every 12 h) significantly enhance the longitudinal growth of the root system.

Table 2

Productive variables in *P. persica* × *P. amygdalus* plantlets across immersion frequencies (TIS 3, TIS 6, TIS 12) and semi-solid control (SS) for four subcultures (30-day intervals) and overall average.

Variable	Treatment	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Average
	TIS 3	9.06 b	7.31 b	6.13 b	4.88 b	6.84 b
Ht	TIS 6	10.81 b	11.31 c	8.75 c	6.31 c	9.31 c
(cm)	TIS 12	9.56 b	10.50 c	7.00 bc	6.38 c	8.34 c
	SS	6.06 a	3.88 a	4.06 a	3.50 a	4.44 a
	TIS 3	2.25 a	1.13 a	1.5 a	1.25 a	1.53 a
NS	TIS 6	3.38 a	1.38 a	2.00 a	1.63 ab	2.09 ab
(shoots·plantlet⁻¹)	TIS 12	3.13 a	1.63 ab	1.88 a	1.38 a	2.00 ab
	SS	2.75 a	2.38 b	2.25 a	2.13 b	2.38 b
	TIS 3	3.38 a	2.38 a	2.88 bc	-	2.88 ab
MR	TIS 6	5.38 b	3.38 b	3.38 c	-	4.04 c
(explants·plantlet⁻¹)	TIS 12	4.88 b	3.13 b	2.50 ab	-	3.5 bc
	SS	3.25 a	2.25 a	2.00 a	-	2.50 a
	TIS 3	13 b	25 b	38 b	50 b	31 b
%HYP	TIS 6	0 a	0 a	13 ab	13 a	6 a
(%)	TIS 12	0 a	0 a	13 ab	13 a	6 a
	SS	13 a	25 b	25 a	38 a	25 b
	TIS 3	3.00 b	3.38 b	3.88 d	3.88 d	3.53 c
HYP	TIS 6	0.00 a	0.00 a	3.00 c	2.38c	1.34 b
	TIS 12	0.00 a	0.00 a	2.63 b	2.00 b	1.16 b
	SS	0.13 a	0.25 a	0.38 a	0.50 a	0.31 a
	TIS 3	82.59 b	83.50 b	78.97 bc	84.03 b	82.27 b
%H₂O	TIS 6	75.92 a	70.54 a	79.77 c	80.11 b	76.59 a
(%)	TIS 12	80.33 ab	73.76 a	72.13 a	81.15 b	76.84 a
	SS	79.87 ab	79.83 b	74.29 ab	74.52 a	77.13 a

Variable	Treatment	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Average
	TIS 3	9.06 b	7.31 b	6.13 b	4.88 b	6.84 b
	TIS 3	-	-	-	50 a	-
% Root	TIS 6	-	-	-	63 ab	-
(%)	TIS 12	-	-	-	75 ab	-
	SS	-	-	-	88 b	-
	TIS 3	-	-	-	2.25 a	-
RL	TIS 6	-	-	-	6.25 b	-
(cm)	TIS 12	-	-	-	8.44 b	-
	SS	-	-	-	5.63 b	-

Different letters indicate significant differences between treatments at $P \leq 0.05$ (Fisher's LSD test).

Abbreviations: **Ht**: Explant height (cm); **NS**: Number of shoots (shoots·plantlet⁻¹); **MR**: Multiplication rate (explants·plantlet⁻¹); **%HYP**: Percentage of hyperhydric plants (%); **HYP**: Hyperhydricity severity; **%H₂O**: Water content (%); **%Root**: Rooting percentage (%); **RL**: Root length (cm).

Biomass analysis reaffirmed the superiority of temporary immersion systems (TIS) over semi-solid medium (SS) in terms of productivity. Throughout the four subcultures, plant fresh weight (FW) was significantly higher in all TIS treatments compared to the SS control (Figure 1). The TIS 6 treatment consistently excelled, maintaining the highest FW values throughout the experiment (reaching nearly 1.0 g in subculture 1 and stabilizing at 0.66 g by subculture 4), which directly correlates with the greater height and multiplication rate previously observed in this treatment. In contrast, the SS treatment showed a decreasing trend in fresh matter accumulation, ending with the lowest values in the study (0.26 g).

Regarding plant dry weight (DW), the results revealed differences in the quality of the accumulated biomass. Although the TIS 3 treatment exhibited high FW in the initial subcultures, its dry matter accumulation (DW) in the fourth subculture was significantly lower (0.08 g) compared to TIS 6 (0.16 g) and TIS 12 (0.11 g). This discrepancy between fresh and dry weight observed in the TIS 3 treatment corroborates the previously detected hyperhydricity status (%HYP) and elevated water content (%H₂O; 82.27%). These findings indicate that a substantial proportion of the weight gain in this system results from excessive water accumulation in malformed tissues rather than from efficient nutrient assimilation.

Finally, it was observed that the stability of the TIS 6 system allowed for the maintenance of superior and constant dry matter production over time. In the final subculture, DW values in TIS 6 were double those of both the traditional system (SS) and the most frequent immersion system (TIS 3). These findings confirm

that an immersion frequency of every 6 hours not only maximizes plant size but also enhances the biosynthesis of structural compounds, resulting in plantlets with improved physiological robustness for subsequent culture stages.

The quality of the plantlets produced in vitro had a decisive impact on their performance during the acclimatization phase. Thirty-five days after transfer to ex vitro conditions, plantlets from the TIS 6 treatment exhibited the highest survival rate (75%), differing significantly from the TIS 3 and SS treatments (Figure 2). The SS treatment showed the lowest final survival rate (42%), similar to that of the hyperhydric TIS 3 treatment. This result suggests that the low vigor and limited biomass accumulation in the semi-solid medium restrict the plant's ability to respond to transplant stress. In contrast, the balance between growth and physiological robustness achieved in TIS 6 ensures successful establishment during the acclimatization stage.

Plant height (HtP) during the 35 days of acclimatization followed an exponential model of the type $HtP = \alpha \cdot e^{(\beta \cdot \text{days})}$ (Figure 3). This indicates a constant and sustained relative growth rate throughout the adaptation process, maintaining the growth hierarchy established during the in vitro phase of the TIS treatments. Plants from the TIS 6 treatment exhibited the highest vegetative development, reaching a final average height of 5.8 cm, followed by TIS 12 (5.0 cm) and TIS 3 (4.6 cm). The SS treatment showed the lowest growth throughout the period, ending with an average height of 3.5 cm. These results confirm that using temporary immersion systems with a 6-hour frequency not only maximizes plant viability after transplanting but also promotes superior growth kinetics during transfer to controlled environmental conditions.

3.2 Photosynthetic Pigments

Concentrations of chlorophyll a (Chl a), chlorophyll b (CHL b), and total chlorophyll (Chl t), expressed as $\mu\text{g}\cdot\text{g}^{-1}$ in fresh leaves of *P. persica* $\times$ *P. amygdalus*, showed significant variations in response to both immersion frequency and the progression of subcultures (Table 3). A sustained decreasing trend in the content of all photosynthetic pigments was determined from the first to the fourth subculture across all evaluated treatments. The TIS 12 treatment consistently stood out, recording the highest averages for Chl a ($6.63 \mu\text{g}\cdot\text{g}^{-1}$), Chl b ($3.75 \mu\text{g}\cdot\text{g}^{-1}$), and Chl t ($10.62 \mu\text{g}\cdot\text{g}^{-1}$), being statistically different from the other groups ($p < 0.05$). In contrast, the TIS 3 treatment systematically exhibited the lowest pigment levels, with a final average Chl t of only $6.16 \mu\text{g}\cdot\text{g}^{-1}$. Notably, while the TIS 6 immersion treatment and the conventional system (SS) showed intermediate behaviors, the 12-hour regime (TIS 12) was the only one capable of maintaining Chl t levels above $7 \mu\text{g}\cdot\text{g}^{-1}$ toward the end of the fourth subculture. In the remaining treatments, values dropped drastically, suggesting better physiological stability of the plant material under more spaced immersion periods.

Table 3: Photosynthetic pigment concentration in leaves of *P. persica* $\times$ *P. amygdalus* under TIS 3 h, TIS 6 h, TIS 12 h, and SS treatments after 30 days during four in vitro growth subcultures.

Variable	Treatment	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Average
Chl a	TIS 3	7.04 a	4.40 a	3.42 a	1.24 a	4.03 a
	TIS 6	9.70 c	6.69 b	3.94 a	2.08 a	5.35 b
($\mu\text{g}\cdot\text{g}^{-1}$ FW)	TIS 12	8.93 b	6.74 c	6.10 b	4.74 b	6.63 c
	SS	9.10 bc	6.31 bc	3.83 a	4.11 b	5.83 bc
Chl b	TIS 3	3.71 a	1.95 a	1.70 a	0.58 a	1.99 a
	TIS 6	6.91 c	2.93 b	2.08 a	1.06 ab	3.25 b
($\mu\text{g}\cdot\text{g}^{-1}$ FW)	TIS 12	5.22 b	3.52 b	3.56 b	2.70 c	3.75 b
	SS	5.21 b	3.62 b	1.72 a	1.56 b	3.03 b
Chl t	TIS 3	11.00 a	6.51 a	5.25 a	1.87 a	6.16 a
	TIS 6	16.97 c	8.83 b	6.17 a	3.21 a	8.79 b
($\mu\text{g}\cdot\text{g}^{-1}$ FW)	TIS 12	14.48 b	10.51 b	9.89 b	7.61 c	10.62 b
	SS	14.64 b	10.16 b	5.69 a	5.82 b	9.08 b

Different letters indicate significant differences between treatments $P \leq 0.05$ (Fisher's LSD test).

Abbreviations: **Chl a**: Chlorophyll a ($\mu\text{g}\cdot\text{g}^{-1}$ FW); **Chl b**: Chlorophyll b ($\mu\text{g}\cdot\text{g}^{-1}$ FW); **Chl t**: Total chlorophyll ($\mu\text{g}\cdot\text{g}^{-1}$ FW); **FW**: Fresh Weight.

3.3 Histological Variables

3.3.1 Stems

The histological evaluation determined the differential impact of the culture systems on the cauline tissue architecture, establishing the mother plant (MP) as the morphological reference standard. As shown by the cross-section analysis (Figure 4) and morphometric data (Table 4), a marked structural divergence between treatments was evident. The MP exhibited the most balanced tissue organization, characterized by the thickest epidermal cells (9.25 μm) and an absence of anomalies (0.00 for both HYP-SVT and HYP-SET). Visually, the MP showed compact tissues with clear delimitation between the cortex and the vascular system, serving as the benchmark for anatomical normality.

In the TIS treatments, it was observed that the frequency of contact with the liquid medium proportionally altered the stem anatomy. The TIS 3 treatment showed the greatest vascular development, with an average phloem thickness of 29.31 μm and xylem peaks of 39.36 μm in the fourth subculture. However, this dimensional growth did not translate into structural quality; Figure 4 reveals a loss of cellular continuity for this treatment. This correlates with the highest levels of vascular (2.50) and epidermal (2.56) hyperhydricity, in addition to presenting the thinnest epidermal cells in the study (3.47 μm), which visually results in a less defined and more lax epidermis.

In contrast, the TIS 6 and TIS 12 treatments showed a more conserved structure. Although TIS 12 presented the lowest xylem (24.68 μm) and phloem (20.37 μm) development among the TIS systems, it maintained the lowest hyperhydricity indices (0.94 and 0.93). This is reflected in the photomicrographs by a more orderly arrangement of the xylem and cambium, similar to the MP.

The Traditional System (SS) was distinguished by the highest xylem/phloem ratio (1.56), resulting from significantly lower phloem development (18.44 μm) compared to the immersion systems. Despite being a semi-solid medium, the SS treatment showed epidermal hyperhydricity levels (1.37) higher than those of TIS 12, which can be observed in Figure 4 as a slight detachment or irregularity in the chlorenchyma and epidermis toward the final subcultures. Overall, the data indicate that while frequent immersion systems (TIS 3) stimulate the growth of conducting tissues, they also induce progressive histological disorganization that intensifies with the number of subcultures.

Table 4

Histological results and hyperhydricity indices in stems of *P. persica* × *P. amygdalus* plantlets for TIS 3 h, TIS 6 h, TIS 12 h, and SS treatments across four subcultures, including the average and Mother Plant (MP).

Variable	Treatment	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Average
XiS	TIS 3	29.28 a	23.01 a	23.25 a	39.36 b	28.73 b
	TIS 6	26.25 a	28.36 a	22.87 a	29.45 a	26.81 ab
(µm)	TIS 12	25.25 a	23.64 a	26.36 a	23.45 a	24.68 a
	SS	26.97 a	23.99 a	27.22 a	29.18 a	26.84 ab
	MP	25.24 a	25.24 a	25.24 a	25.24 a	25.24 ab
PhS	TIS 3	18.68 a	27.52 c	34.51 b	36.52 c	29.31 d
	TIS 6	15.16 a	23.38 bc	23.96 a	26.59 b	22.27 bc
(µm)	TIS 12	16.83 a	18.96 ab	21.97 a	23.72 b	20.37 ab
	SS	19.99 ab	17.41 a	18.56 a	17.82 a	18.44 a
	MP	24.29 b	27.52 c	24.29 a	24.29 b	24.29 c
XiS/PhS	TIS 3	1.53 ab	0.82 a	0.86 a	1.11 a	1.08 ab
	TIS 6	1.99 b	1.25 bc	0.98 ab	0.98 a	1.30 b
	TIS 12	1.49 ab	1.28 bc	1.35 bc	1.00 a	1.28 bc
	SS	1.44 ab	1.58 c	1.59 c	1.64 b	1.56 c
	MP	1.04 a	1.04 ab	1.04 ab	1.04 a	1.04 a
HYP-SVT	TIS 3	1.25 c	1.50 b	3.50 c	3.75 c	2.50 c
	TIS 6	0.50 b	0.75 ab	1.50 b	1.75 b	1.13 b
	TIS 12	0.50 b	1.00 b	0.75 ab	1.50 b	0.94 b
	SS	0.75 bc	1.00 b	0.75 ab	1.50 b	1.00 b
	MP	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
EpT	TIS 3	3.24 a	3.90 a	3.39 a	3.34 a	3.47 a
	TIS 6	6.99 b	6.10 b	5.63 b	4.10 ab	5.70 b
(µm)	TIS 12	7.03 b	6.44 b	6.77 b	4.75 bc	6.25 b

Different letters indicate significant differences between treatments at $P \leq 0.05$ (Fisher's LSD test). Abbreviations: **XiS**: Stem xylem thickness (µm); **PhS**: Stem phloem thickness (µm); **XiS/PhS**: Stem xylem/phloem thickness ratio; **HYP-SVT**: Stem vascular tissue hyperhydricity index; **EpT**: Stem epidermal cell thickness (µm); **HYP-SET**: Stem epidermal tissue hyperhydricity index.

Variable	Treatment	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Average
	SS	7.56 bc	6.04 b	5.54 b	5.34 c	6.12 b
	MP	9.25 c	9.25 c	9.25 c	9.25 d	9.25 c
HYP-SET	TIS 3	1.00 b	1.38 b	3.72 c	4.16 c	2.56 c
	TIS 6	0.75 b	1.00 b	1.38 b	2.00 b	1.28 b
	TIS 12	0.22 ab	0.63 ab	1.28 b	1.59 b	0.93 b
	SS	0.25 ab	1.06 b	1.94 bc	2.22 b	1.37 b
	MP	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
Different letters indicate significant differences between treatments at $P \leq 0.05$ (Fisher's LSD test). Abbreviations: XiS : Stem xylem thickness (μm); PhS : Stem phloem thickness (μm); XiS/PhS : Stem xylem/phloem thickness ratio; HYP-SVT : Stem vascular tissue hyperhydricity index; EpT : Stem epidermal cell thickness (μm); HYP-SET : Stem epidermal tissue hyperhydricity index.						

3.3.2 Leaves

Analysis of Garnem's leaf anatomy revealed significant structural variations influenced by the culture system type and immersion frequency, as detailed in Table 5 and illustrated in Figure 5. It was observed that the development of conducting tissues showed a plastic response to the in vitro environment; the TIS 3 treatment reached maximum values for xylem width ($70.69 \mu\text{m}$) and phloem width ($39.60 \mu\text{m}$) by the fourth subculture, statistically surpassing both the traditional system (SS) and the mother plant (MP). This hypertrophy of the vascular bundles (xi, ph) is clearly visible in the cross-sections shown in Figure 5, displaying a laxer and more expanded organization compared to the compact and defined structure of the MP. Likewise, leaf blade thickness (LBT) increased proportionally with immersion frequency, with TIS 3 reaching an average of $95.05 \mu\text{m}$, representing a statistical difference compared to the $59.66 \mu\text{m}$ of the mother plant.

The characterization of hyperhydricity through histological indices confirmed that frequent contact with the liquid medium induces progressive degradation of the cellular architecture. In Figure 5, red arrows highlight critical areas where the palisade parenchyma (pp) and spongy parenchyma (sp) lose their typical morphology, presenting rounded cells and large air gaps. This coincides with the high hyperhydricity indices in the leaf blade (HYP-LB) and the leaf vascular tissue (HYP-LVT) reported for the TIS 3 treatment. This vitrification phenomenon intensifies as subcultures progress. In contrast, the TIS 12 treatment showed intermediate values and a less disorganized tissue structure than TIS 3, while the traditional system (SS) maintained a greater similarity to the mother plant's organization, albeit with significantly reduced vascular development. Overall, these results indicate that while temporary immersion systems enhance the growth of conducting tissue, high immersion frequencies compromise the anatomical integrity of the leaf, distancing it from the normal phenotype required for successful acclimatization.

Table 5

Histological results and hyperhydricity indices in leaves of *P. persica* × *P. amygdalus* plantlets for TIS 3 h, TIS 6 h, TIS 12 h, and SS treatments across four subcultures, including the average and Mother Plant (MP).

Variable	Treatment	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Average
XiL (μm)	TIS 3	45.23 c	55.39 c	57.88 d	70.69 d	57.30 c
	TIS 6	33.94 b	43.87 b	51.34 bc	50.87 c	45.01 b
	TIS 12	31.78 ab	41.96 b	47.98 ab	49.22 c	42.73 b
	SS	25.47 a	26.90 a	31.40 a	36.27 a	30.01 a
	MP	42.20 c	42.20 b	42.20 b	42.20 b	42.20 b
PhL (μm)	TIS 3	32.68 d	38.57 c	40.08 d	39.60 d	37.73 c
	TIS 6	24.68 bc	30.34 b	35.66 cd	35.40 cd	31.52 b
	TIS 12	19.87 ab	32.26 b	33.44 bc	34.57 c	30.04 b
	SS	17.43 a	17.98 a	21.45 a	25.13 a	20.49 a
	MP	29.78 cd	29.78 b	29.78 b	29.78 b	29.78 b
XiL/PhL	TIS 3	1.39 a	1.44 ab	1.45 a	1.81 b	1.52 b
	TIS 6	1.39 a	1.45 ab	1.44 a	1.43 a	1.43 ab
	TIS 12	1.61 b	1.31 a	1.43 a	1.45 a	1.45 ab
	SS	1.45 ab	1.50 b	1.47 a	1.44 a	1.47 ab
	MP	1.42 a	1.42 ab	1.42 a	1.42 a	1.42 a
HYP-LVT	TIS 3	1.75 b	2.88 b	3.75 c	4.00 c	3.09 d
	TIS 6	1.63 b	1.50 b	1.50 ab	1.75 abc	1.59 bc
	TIS 12	1.00 ab	1.25 ab	1.25 ab	1.00 ab	1.13 b
	SS	1.00 ab	1.75 b	2.88 bc	3.13 bc	2.19 cd
	MP	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
LBT (μm)	TIS 3	77.93 c	93.97 d	97.97 c	110.31 c	95.05 c
	TIS 6	58.63 b	74.22 c	87.02 bc	86.29 b	73.54 c
	TIS 12	51.66 ab	74.23 c	81.44 b	83.81 b	72.78 c

Different letters indicate significant differences between treatments at $P \leq 0.05$ (Fisher's LSD test). Abbreviations: **XiL**: Leaf xylem width (μm); **PhL**: Leaf phloem width (μm); **XiL/PhL**: Leaf xylem/phloem width ratio; **HYP-LVT**: leaf vascular tissue hyperhydricity index; **LBT**: Leaf blade thickness (μm); **HYP-LB** leaf blade hyperhydricity; **HYP-LET**: Leaf epidermal tissue hyperhydricity index.

Variable	Treatment	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Average
	SS	42.92 a	44.89 a	52.86 a	59.66 a	50.52 a
	MP	59.66 b	59.66 b	59.66 a	61.41 a	59.66 b
HYP-LB	TIS 3	1.75 b	3.75 c	3.88 c	4.13 c	3.38 c
	TIS 6	1.25 b	1.88 bc	1.88 ab	1.75 ab	1.69 b
	TIS 12	0.88 ab	1.50 ab	1.75 ab	1.88 ab	1.50 b
	SS	2.00 b	1.75 bc	2.88 bc	3.25 bc	2.47 c
	MP	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
HYP-LET	TIS 3	1.75 b	2.25 c	2.88 b	4.13 c	2.75 c
	TIS 6	1.25 ab	1.50 bc	1.75 b	2.13 bc	1.66 b
	TIS 12	0.75 ab	1.25 abc	1.75 b	1.25 ab	1.25 b
	SS	0.50 a	0.75 ab	1.75b	2.13 bc	1.28 b
	MP	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
Different letters indicate significant differences between treatments at $P \leq 0.05$ (Fisher's LSD test). Abbreviations: XiL : Leaf xylem width (μm); PhL : Leaf phloem width (μm); XiL/PhL : Leaf xylem/phloem width ratio; HYP-LVT : leaf vascular tissue hyperhydricity index; LBT : Leaf blade thickness (μm); HYP-LB leaf blade hyperhydricity; HYP-LET : Leaf epidermal tissue hyperhydricity index.						

3.4 Stomata

Micrometric analysis revealed that in vitro culture conditions induce significant alterations in the dimensions of the stomatal apparatus compared to the ex vitro reference ($P < 0.05$). The Mother Plant (MP) exhibited the smallest stomata, recording the minimum values for both the length and width of the pore and the total stomatal complex (Table 6). This compact morphology corresponds to the functionality observed in Figure 6 (A), where the ability to close the ostiole is evident.

Among the in vitro treatments, the traditional solid medium culture system (SS) and the bioreactor with the highest immersion frequency (TIS 3) induced the greatest morphological alterations, exhibiting generalized hypertrophy. The SS treatment recorded the absolute maximum values for all measured variables, such as stomatal length ($33.09 \mu\text{m}$), differing significantly from the MP and the less frequent immersion treatments. Stomata developed under these conditions were visually characterized by their large size and the presence of permanently open pores with wide apertures (Figure 6, B-E).

In contrast, reducing the immersion frequency in temporary systems favored a less altered stomatal morphology. The TIS 12 treatment showed intermediate values, achieving a significant reduction in total stomatal size compared to SS. Notably, regarding the Pore Width variable, the TIS 12 treatment ($4.53 \mu\text{m}$)

showed no statistical differences from the Mother Plant (4.12 μm), suggesting that this immersion regime attenuates the excessive ostiole opening typical of the in vitro condition.

Table 6: Morphological characteristics of *P. persica* $\times$ *P. amygdalus* stomata in leaves from the mother plant (MP) and those propagated under temporary immersion systems every 3 h (TIS 3), 6 h (TIS 6), 12 h (TIS 12), and traditional in vitro system (SS).

Treatment	StL (μm)	StW (μm)	PoL (μm)	PoW (μm)
TIS 3	26.17 c	11.85 b	21.10 c	7.08 c
TIS 6	19.81 ab	9.10 a	16.02 ab	5.64 b
TIS 12	22.71 b	9.18 a	18.70 bc	4.53 ab
SS	33.09 c	13.34 b	21.78 c	7.69 c
MP	17.9 a	9.22 a	13.51 a	4.12 a

Different letters indicate significant differences between treatments at $P \leq 0.05$ (Fisher's LSD test).

Abbreviations: **StL:** Stomatal length (μm); **StW:** Stomatal width (μm); **PoL:** Pore length (μm); **PoW:** Pore width (μm).

3.5 Results of Principal Component Analysis

Principal Component Analysis (PCA) synthesized the variability associated with the morpho-physiological and anatomical variables evaluated in *P. persica* $\times$ *P. amygdalus* plantlets cultivated under different temporary immersion systems and a traditional system (Figure 7). The first two principal components together explained 89.4% of the total variation, with 52.0% corresponding to PC1 and 37.4% to PC2.

PC1 was primarily and positively associated with variables linked to the growth and general performance of the plantlets, such as Ht, FW, DW, %SURV, LBT, and leaf vascular system characteristics (XiL, PhL, and the XiL/PhL ratio). The TIS 6 treatment was located along this axis, indicating an association with greater vegetative development and superior physiological quality indicators. In the opposite direction, PC1 was negatively correlated with hyperhydricity indicators (%HYP, HYP, and %H₂O) and anatomical variables associated with hyperhydric tissues (HYP-SVT, HYP-LVT, HYP-SET, HYP-LET, HYP-LB). This allowed for a clear discrimination of the treatments with the highest expression of this physiological disorder.

PC2 was dominated by variables related to organogenesis (NS) and the root system, such as %Root and RL, as well as photosynthetic pigment content (Chl a, Chl b, and Chl t). Along this axis, the TIS 12 treatment was positioned in the positive quadrant, showing an association with higher values for photosynthetic and root variables. In contrast, the traditional system (SS) was located in the upper-left quadrant, primarily associated with stomatal characteristics (StL, StW, PoL) and stem anatomical variables (EpT, and the PhS/XiS ratio).

The TIS 3 treatment was located in the lower-left quadrant of the biplot, showing a strong association with variables related to leaf and stem hyperhydricity, as well as higher water content and anatomical

alterations, evidencing a differential response compared to the other evaluated systems.

Discussion

The results of this study demonstrate that the efficiency of the temporary immersion system (TIS) for the micropropagation of *Prunus persica* × *P. amygdalus* depends critically on the immersion frequency, which modulates not only vegetative growth but also the anatomical and functional quality of the tissues. The integration of productive, histological (stem and leaf), stomatal, and multivariate parameters confirms that protocol optimization should not rely solely on increasing in vitro growth. Instead, it should prioritize the production of anatomically well-developed and physiologically competent plantlets for ex vitro performance.

From a productive standpoint, TIS showed clear advantages over the traditional semi-solid medium system, particularly regarding height, biomass accumulation, and general vigor. These findings align with numerous reports highlighting the superiority of TIS in woody species (Gago et al. 2022; Pérez-Caselles et al. 2023; García-Ramírez 2023; Gómez-Cruz et al. 2024), attributed to greater nutrient availability, improved explant hydration, and a reduction in the diffusive limitations typical of gelled media (Méndez-Hernández and Loyola-Vargas 2024; Vidal and Sánchez 2019). However, this work demonstrates that these productive benefits can be compromised if the immersion frequency is excessive.

The TIS 3 treatment (immersion every 3 h) promoted rapid growth and a significant increase in fresh weight; however, this effect was associated with high levels of hyperhydricity in both stems and leaves. At the histological level, this treatment induced an apparent hypertrophy of cauline and foliar vascular tissues, characterized by increased xylem and phloem thickness accompanied by tissue disorganization, thin cell walls, and reduced structural differentiation. This pattern matches descriptions of hyperhydric tissues, where excessive water absorption and intercellular hypoxia alter the differentiation of the procambium and leaf mesophyll (Barua et al. 2025), generating voluminous but functionally inefficient tissues (Majada et al. 2000; García Osuna et al. 2011; Polivanova and Bedarev 2022). In the stem, the observed phloem-xylem disproportion, along with a reduced xylem/phloem ratio in certain subcultures suggests an alteration in the normal balance of vascular differentiation. Previous studies in *Prunus* and other woody species indicate that these alterations are linked to changes in hormonal signaling, particularly auxin distribution and ethylene accumulation under water-excess conditions (Majada et al. 2000; Abou Elyazid et al. 2021). From an applied perspective, this vascular architecture is unfavorable as it compromises the efficient conduction of water and photoassimilates during acclimatization.

Furthermore, TIS 3 leaves exhibited increased blade thickness and vascular tissue expansion, accompanied by a marked loss of mesophyll organization, poorly defined palisade parenchyma, expanded spongy parenchyma, and large intercellular spaces. These leaves also recorded low levels of chlorophyll a, b, and total chlorophyll, possibly due to oxidative damage to the chloroplasts (Gantait and Mahanta 2022; García-Ramírez 2023), which hinders the heterotrophic-to-autotrophic transition necessary for ex vitro survival (Eliwa et al. 2025). Combined with the lack of a waxy cuticle and the stomatal inability to close observed in this treatment, plantlets undergo massive transpirational water loss when transferred to lower

relative humidity conditions (Ekinici et al. 2023). These factors result in very low survival during acclimatization (47%), with losses potentially reaching 70% of the plant material according to Ramírez-Mosqueda and Cruz-Cruz (2024). Therefore, the larger leaf size in this treatment is not a productive advantage but rather a symptom of a physiological disorder induced by the culture system.

In contrast, the TIS 6 and TIS 12 treatments exhibited a more favorable balance between growth and anatomical quality. These plantlets maintained an orderly vascular organization with cambial continuity, well-defined mesophyll differentiation, less epidermal alteration, and lower hyperhydricity indices, reflecting characteristics similar to those of the mother plant. In this regard, Dolcet-Sanjuan et al. (2024) determined an optimal immersion frequency of 30 seconds every 6 h for *Prunus* hybrid rootstocks (Rootpac series and GF677), while Pérez-Caselles et al. (2023) found an optimal frequency of 2 minutes every 6 h for apricot (*Prunus armeniaca* L.). Both studies ensured plantlet development during acclimatization similar to the results found under TIS 6, suggesting that this treatment achieves an optimal compromise between vegetative vigor and anatomical stability. García-Ramírez et al. (2023) state that higher chlorophyll content, as found in TIS 6 and TIS 12, boosts the photosynthetic capacity required for post-in vitro survival. Likewise, Abou Elyazid et al. (2021) suggest that a well-developed root system is vital for immediate water and nutrient uptake from the substrate, leading to significantly higher survival rates (94%). Furthermore, a well-developed cuticle and an epicuticular wax layer are essential to prevent excessive water loss through transpiration (Ekinici et al. 2023). Leaves must possess a well-structured mesophyll and a robust, lignified vascular structure to provide the necessary mechanical support to withstand external environmental conditions (Majada et al. 2000, Barua et al. 2025). Greater overall vigor—reflected in superior biomass (fresh and dry weight), thick stems, and notable leaf expansion—positively correlates with an improved capacity for establishment and subsequent growth in the greenhouse (Lawson et al. 2023).

The traditional system (SS) showed intermediate behavior. Although it presented lower growth rates and biomass accumulation, it initially preserved a more stable anatomical organization, particularly in the stem. However, the progression of subcultures revealed a gradual increase in foliar and epidermal hyperhydricity indices, indicating that semi-solid media do not completely eliminate physiological disorders, especially in vigorous genotypes like 'Garnem'. These results coincide with reports indicating that limited aeration, ethylene accumulation, and restricted nutrient availability negatively affect the quality of material propagated in traditional systems, even if the incidence of severe vitrification is lower than in poorly adjusted liquid systems (Vidal and Sánchez 2019; Mirzabe et al. 2022). While lower survival is generally recorded during the acclimatization of plants from traditional systems compared to TIS (Castillo Ontaneda et al. 2020), sources warn that if TIS is not optimized and causes hyperhydricity, the traditional system may prove superior (Murthy et al. 2023).

Conclusion

Immersion frequency was a decisive factor in the productive, anatomical, and physiological responses of *Prunus persica* × *P. amygdalus* cultured in vitro under a temporary immersion system. The 6-hour immersion frequency achieved the best balance between vegetative vigor, tissue organization, and physiological functionality, which was reflected in superior survival and growth during acclimatization, making it the most efficient condition in this study. In contrast, the 3-hour immersion frequency promoted rapid growth and high fresh biomass but induced severe hyperhydricity, histological disorganization in both stems and leaves, and alterations in stomatal functionality, thereby compromising ex vitro performance. The 12-hour immersion frequency favored greater histological stability and a stomatal morphology closer to the ex vitro phenotype, albeit with lower in vitro yield. Taken together, these results demonstrate that the fine-tuning of immersion frequency is a key factor in optimizing the in vitro propagation of *Prunus* rootstocks for commercial and productive applications.

Declarations

Author Contribution

Delfino, P. M.: Conceptualization, Methodology, Formal Analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization. Bima, P. J. I.: Conceptualization, Writing – Review & Editing, Project Administration, Resources, Funding Acquisition, Supervision.

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

References

1. Abou Elyazid, D. M., Gawish, M. S., & Eliwa, G. I. (2021). Optimized protocol for micropropagation of Cadaman and Garnem peach rootstocks. *Journal of Plant Production*, 12(7), 731–735. <https://doi.org/10.21608/jpp.2021.83432.1038>
2. Barua, R., Kundu, S., Igamberdiev, A. U., & Debnath, S. C. (2025). Hyperhydricity-induced physiological changes and catechin accumulation in blueberry hybrids (*Vaccinium corymbosum* × *V. angustifolium*). *Horticulturae*, 11(4), 418. <https://doi.org/10.3390/horticulturae11040418>
3. Bethge, H., Mohammadi Nakhjiri, Z., Rath, T., & Winkelmann, T. (2023). Towards automated detection of hyperhydricity in plant in vitro culture. *Plant Cell, Tissue and Organ Culture*, 154(3), 551–573. <https://doi.org/10.1007/s11240-023-02477-0>
4. Bulunuz Palaz, E., Ugur, R., & Yaman, M. (2023). Micropropagation protocols of new *Prunus* hybrids with significant rootstock potential in fruit breeding and cultivation. *Erwerbs-Obstbau*, 65(5), 1359–1364. <https://doi.org/10.1007/s10341-023-00908-6>

5. Castillo Ontaneda, A. L., Moreno Herrera, A., & García Batista, R. M. (2020). Eficiencia del sistema de inmersión temporal frente al método de propagación convencional in vitro. *Revista Metropolitana de Ciencias Aplicadas*, 3(2), 173–182. <https://doi.org/10.62452/9dq60d52>
6. Delbón, N., Machado, A. S., Cabrera, V. A., Wiemer, A. P., Matesevach Becerra, A. M., Chiarini, F. E., & Saur Palmieri, V. (2024). Técnicas de histología vegetal: Manual teórico-práctico. Editorial de la Universidad Nacional de La Plata.
7. Dolcet-Sanjuan, R., Casanovas, M., Franquesa, S., Alsina, E., Carrasco-Cuello, F., Torres, E., & Teixidó, N. (2024). GreenTray®, a TIS bioreactor for plant micropropagation and abiotic or biotic stress bioassays. *Applied Sciences*, 14(10), 4051. <https://doi.org/10.3390/app14104051>
8. Ekinici, H., Saskin, N., Ak, B. E., & Dogan, B. D. (2024). Effects of different healing agents on acclimatization success of in vitro rooted Garnem (*Prunus dulcis* × *Prunus persica*) rootstock. *In Vitro Cellular & Developmental Biology – Plant*, 60(3), 309–317. <https://doi.org/10.1007/s11627-023-10385-5>
9. Eliwa, G. I., El Dengawy, E. R. F., Gawish, M. S., & Yamany, M. M. (2025). Comprehensive study on in vitro propagation of some imported peach rootstocks: In vitro rooting and acclimatization. *Scientific Reports*, 15(1), 17905. <https://doi.org/10.1038/s41598-025-00848-z>
10. Al-Eqabi, F. A., & Al-Amery, L. K. J. (2025). Micropropagation of *Prunus* Sp., Factors, Hormones: A Review. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1487, No. 1, p. 012070). IOP Publishing. <https://doi.org/10.1088/1755-1315/1487/1/012070>
11. Gago, D., Sánchez, C., Aldrey, A., Christie, C. B., Bernal, M. Á., & Vidal, N. (2022). Micropropagation of plum (*Prunus domestica* L.) in bioreactors using photomixotrophic and photoautotrophic conditions. *Horticulturae*, 8(4), 286. <https://doi.org/10.3390/horticulturae8040286>
12. Gantait, S., & Mahanta, M. (2022). Hyperhydricity-induced changes among in vitro regenerants of gerbera. *South African Journal of Botany*, 149, 496–501. <https://doi.org/10.1016/j.sajb.2022.06.038>
13. García Osuna, H. T., Benavides Mendoza, A., Escobedo Bocardo, L., Villarreal Quintanilla, J. A., & Cornejo Oviedo, E. (2011). Hyperhydricity control of in vitro shoots of *Turbinicarpus valdezianus*. *Phyton – Revista Internacional de Botánica Experimental*, 80, 175–182. <https://doi.org/10.32604/phyton.2011.80.175>
14. García-Ramírez, Y. (2023). Temporary immersion system for in vitro propagation via organogenesis of forest plant species. *Trees*, 37(3), 611–626. <https://doi.org/10.1007/s00468-022-02379-w>
15. Harborne, J. B. (1984). Nitrogen compounds. En J. B. Harborne (Ed.), *Phytochemical methods* (pp. 37–99). Springer. https://doi.org/10.1007/978-94-009-5570-7_5
16. Justamante, M. S., Mhimdi, M., Molina-Pérez, M., Albacete, A., Moreno, M. Á., Mataix, I., & Pérez-Pérez, J. M. (2022). Effects of auxin (Indole-3-butyric Acid) on adventitious root formation in peach-based *Prunus* rootstocks. *Plants*, 11(7), 913. <https://doi.org/10.3390/plants11070913>
17. Kemat, N., Visser, R. G. F., & Krens, F. A. (2023). Physiological and morpho-anatomical analyses of hyperhydric *Arabidopsis thaliana* influenced by media components. *Journal of Plant Biotechnology*, 50(1), 255–266. <https://doi.org/10.5010/JPB.2023.50.032.255>

18. Lawson, J. D., Bridges, W. C., & Adelberg, J. W. (2023). IBA delivery technique and media salts affected in vitro rooting and acclimatization of eight *Prunus* genotypes. *Plants*, 12(2), 289. <https://doi.org/10.3390/plants12020289>
19. Lloyd, G., & McCown, B. H. (1980). Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot-tip culture. *Combined Proceedings – International Plant Propagators' Society*, 30, 421–427.
20. Majada, J. P., Tadeo, F., Fal, M. A., & Sánchez-Tamés, R. (2000). Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation. *Plant Cell, Tissue and Organ Culture*, 63(3), 207–214. <https://doi.org/10.1023/A:1010650131732>
21. Méndez-Hernández, H. A., & Loyola-Vargas, V. M. (2024). Plant micropropagation and temporary immersion systems. En *Plant cell culture protocols* (pp. 35–50). Springer. https://doi.org/10.1007/978-1-0716-3954-2_3
22. Mirzabe, A. H., Hajiahmad, A., Fadavi, A., & Rafiee, S. (2022). Temporary immersion systems (TISs): A comprehensive review. *Journal of Biotechnology*, 357, 56–83. <https://doi.org/10.1016/j.jbiotec.2022.08.003>
23. Murthy, H. N., Joseph, K. S., Paek, K. Y., & Park, S. Y. (2023). Bioreactor systems for plant micropropagation: Current status and future prospects. *Frontiers in Plant Science*, 14, 1159588. <https://doi.org/10.3389/fpls.2023.1159588>
24. Pérez-Caselles, C., Burgos, L., Sánchez-Balibrea, I., Egea, J. A., Faize, L., Martín-Valmaseda, M., ... Alburquerque, N. (2023). The effect of silver nanoparticle addition on micropropagation of apricot cultivars (*Prunus armeniaca* L.) in semisolid and liquid media. *Plants*, 12(7), 1547. <https://doi.org/10.3390/plants12071547>
25. Polivanova, O. B., & Bedarev, V. A. (2022). Hyperhydricity in plant tissue culture. *Plants*, 11(23), 3313. <https://doi.org/10.3390/plants11233313>
26. Ramírez-Mosqueda, M. A., Cruz-Cruz, C. A., Cano-Ricárdez, A., & Bello-Bello, J. J. (2019). Assessment of different temporary immersion systems in the micropropagation of anthurium (*Anthurium andreaeanum*). *3 Biotech*, 9(8), 307. <https://doi.org/10.1007/s13205-019-1833-2>
27. Rezaei, A., Gharaghani, A., Shekafandeh, A., et al. (2023). Development of a promising micropropagation method for drought-tolerant wild and domesticated almond genotypes. *Erwerbs-Obstbau*, 65, 1463–1477. <https://doi.org/10.1007/s10341-023-00866-z>
28. Rueden, C., Dietz, C., Horn, M., Schindelin, J., Northan, B., Berthold, M., & Eliceiri, K. (2021). ImageJ Ops [Software]. <https://imagej.net/Ops>
29. Sanson, D., de Almeida Garrett, A. T., Rodrigues, I. C., et al. (2024). Alternative system for micropropagation of *Prunus campanulata*. *In Vitro Cellular & Developmental Biology – Plant*, 60, 333–343. <https://doi.org/10.1007/s11627-024-10424-1>
30. 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>

Figures

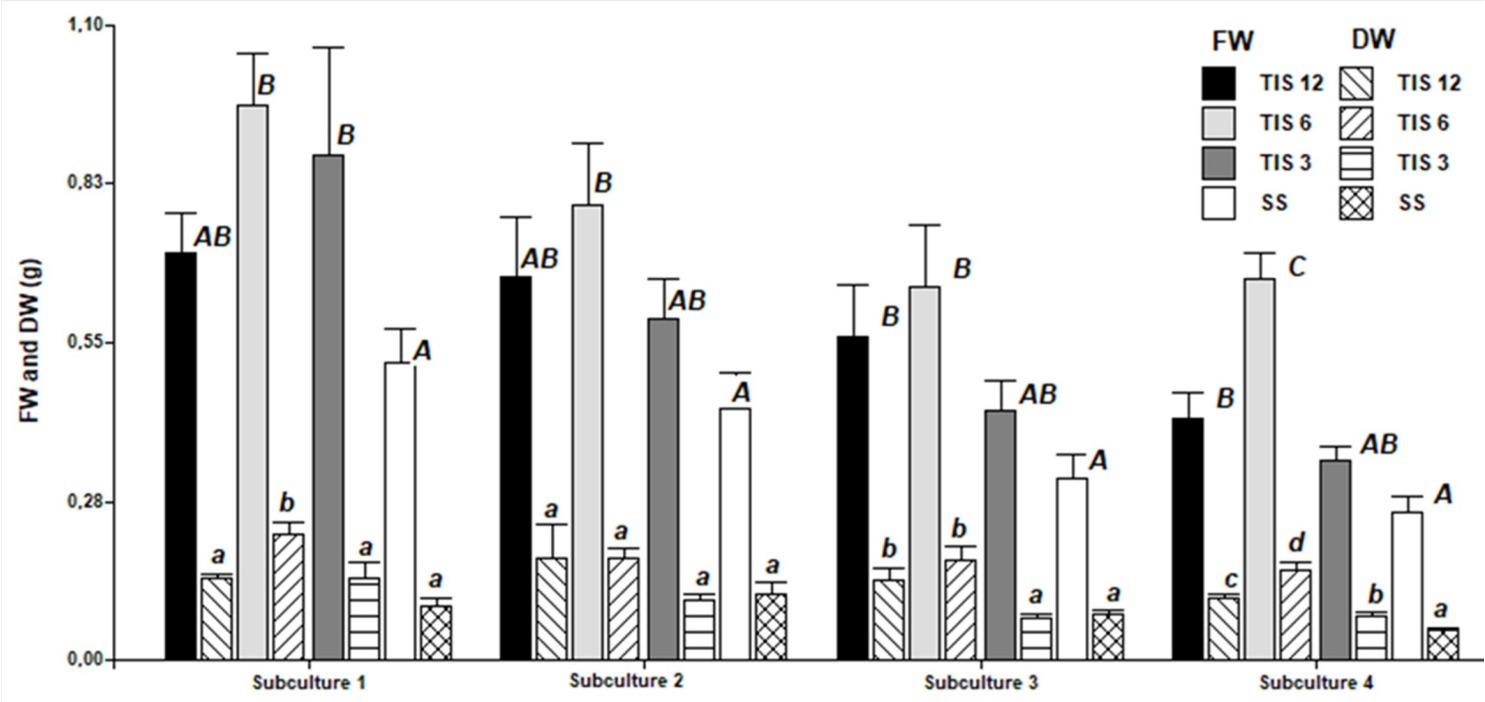


Figure 1

Plant fresh weight (FW) (g) and plant dry weight (DW) (g) of plantlets propagated of *P. persica* × *P. amygdalus* in a temporary immersion system every 3 h (TIS 3), 6 h (TIS 6), 12 h (TIS 12), and in a traditional system (SS) after 30 days of growth across four subcultures. Different uppercase and lowercase letters indicate significant differences within each subculture for FW and DW, respectively ($P \leq 0.05$, Fisher's LSD test).

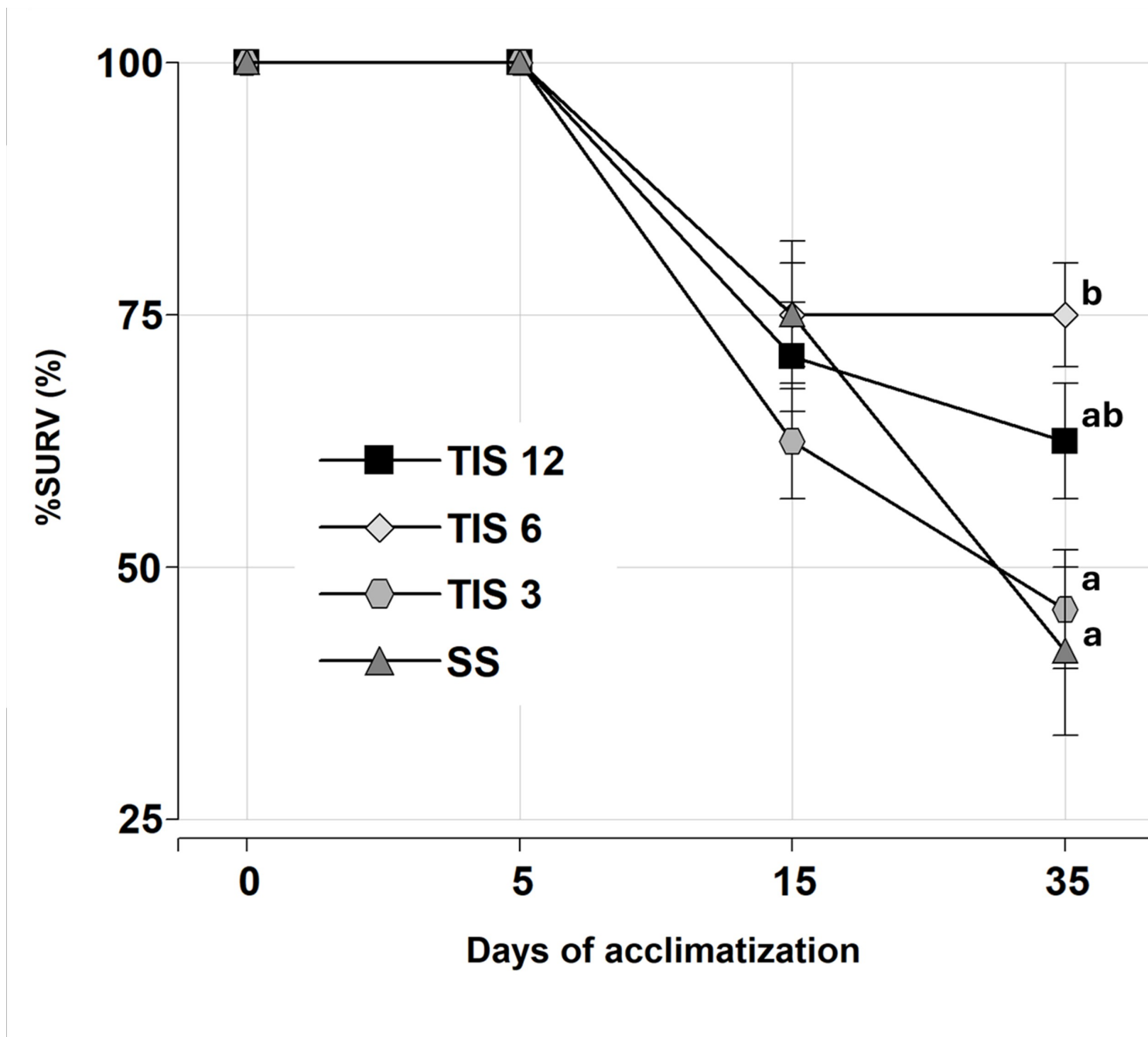


Figure 2

Survival rate (%SURV) of *P. persica* × *P. amygdalus* plants at 0, 5, 15, and 35 days of acclimatization from TIS 3, TIS 6, TIS 12, and SS treatments. Different letters indicate significant differences between treatments ($P \leq 0.05$, Fisher's LSD test).

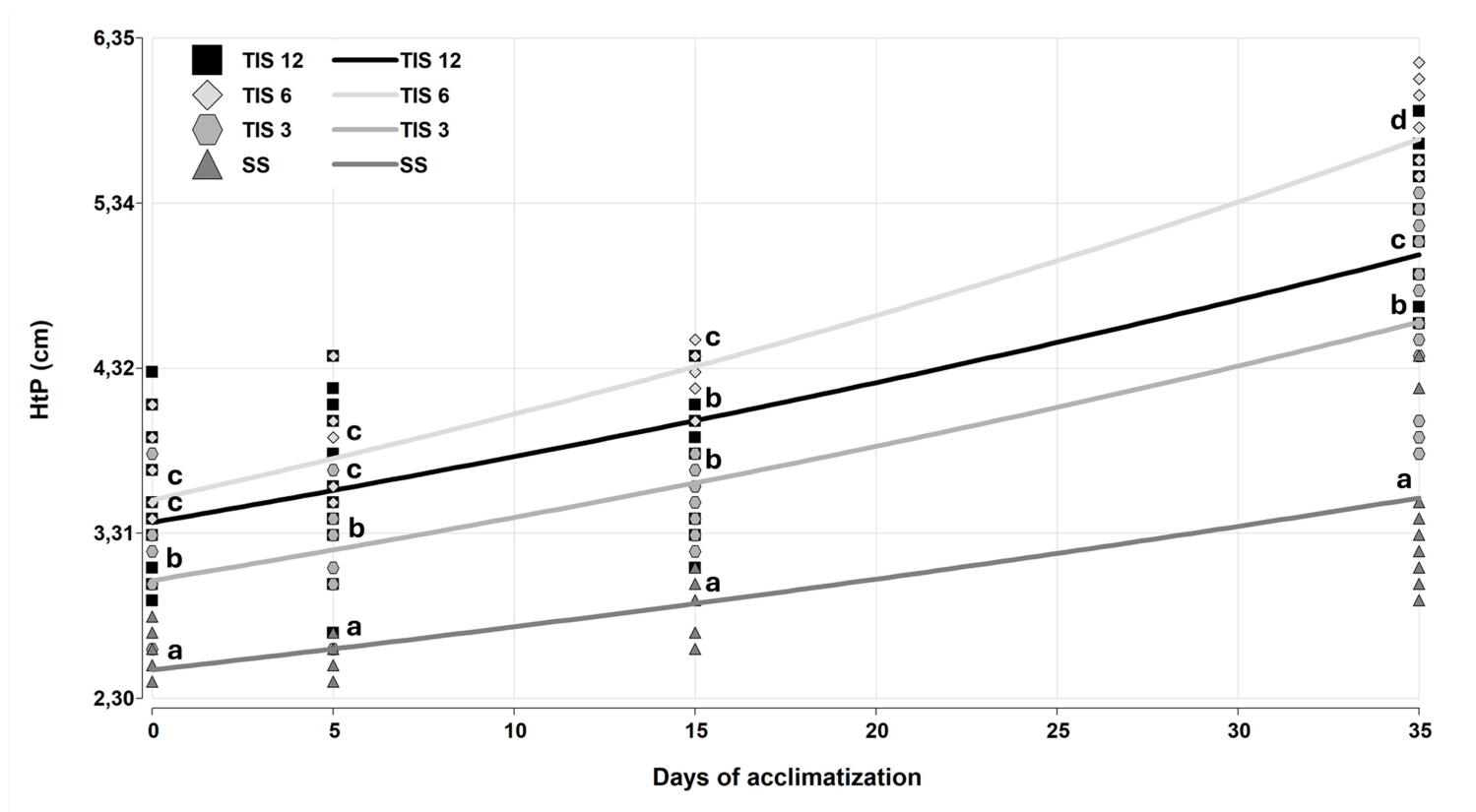


Figure 3

Height of *P. persica* × *P. amygdalus* plants (HtP; cm) during 35 days of acclimatization for explants propagated under temporary immersion systems every 3 h (TIS 3), 6 h (TIS 6), 12 h (TIS 12), and the traditional system (SS). Growth was fitted to an exponential model: $HtP = \alpha \cdot e^{(\beta \cdot \text{days})}$, where TIS 3 ($\alpha = 3.02$ and $\beta = 0.01$; MSE = 0.13), TIS 6 ($\alpha = 3.52$ and $\beta = 0.01$; MSE = 0.08), TIS 12 ($\alpha = 3.38$ and $\beta = 0.01$; MSE = 0.25), and SS ($\alpha = 2.47$ and $\beta = 0.01$; MSE = 0.09). Different letters indicate significant differences between treatments ($P \leq 0.05$, Fisher's LSD test).

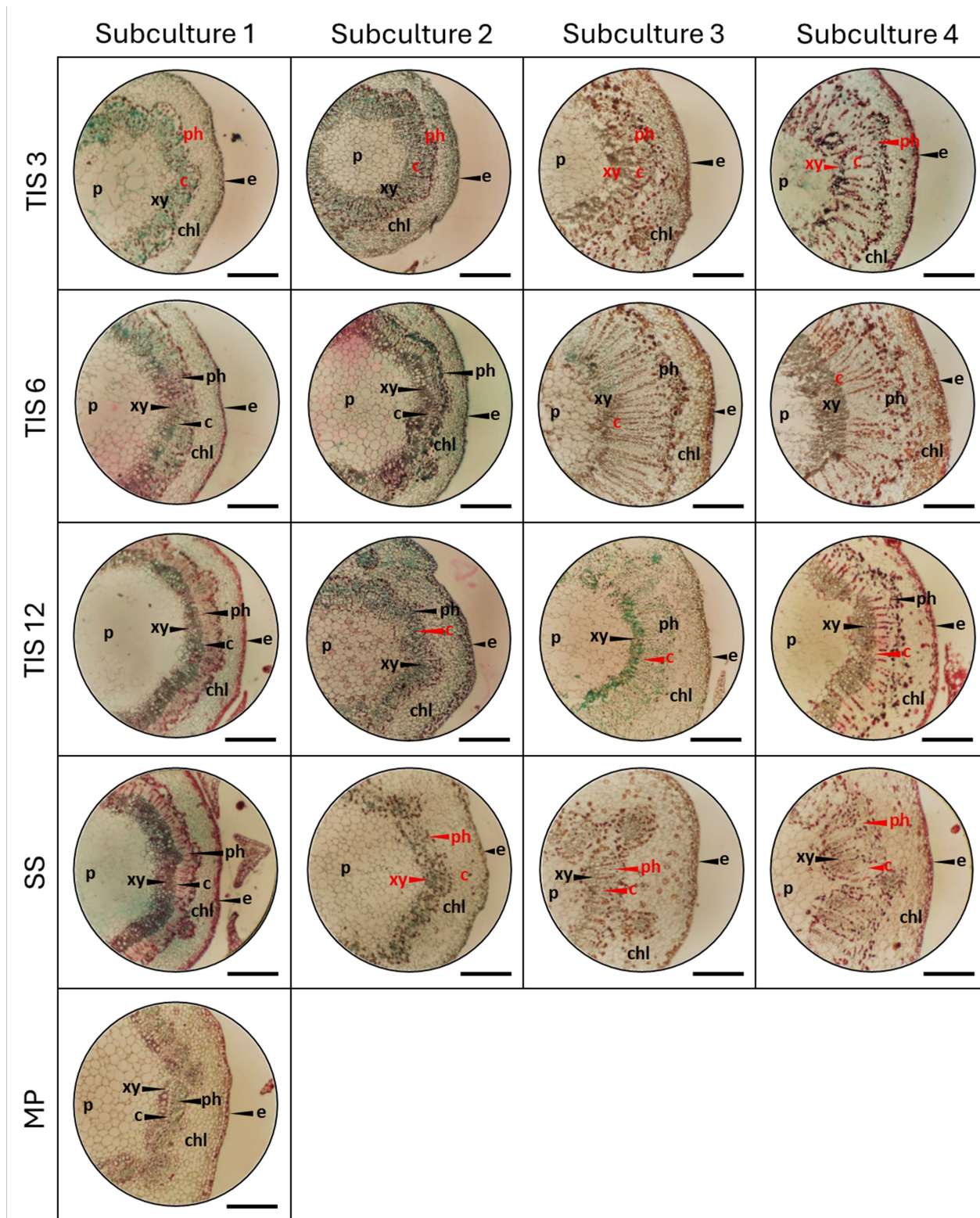


Figure 4

Stem cross-sections of *P. persica* × *P. amygdalus* (Garnem) after 30 days of growth across four subcultures from temporary immersion systems every 3 h (TIS 3), 6 h (TIS 6), 12 h (TIS 12), traditional system (SS), and mother plant (MP). Abbreviations: **p**: pith, **xy**: xylem, **ph**: phloem, **c**: cambium, **chl**: chlorenchyma, **e**: epidermis. Scale bars = 100 µm. Red arrows indicate hyperhydricity symptoms. Black arrows indicate normal tissues.

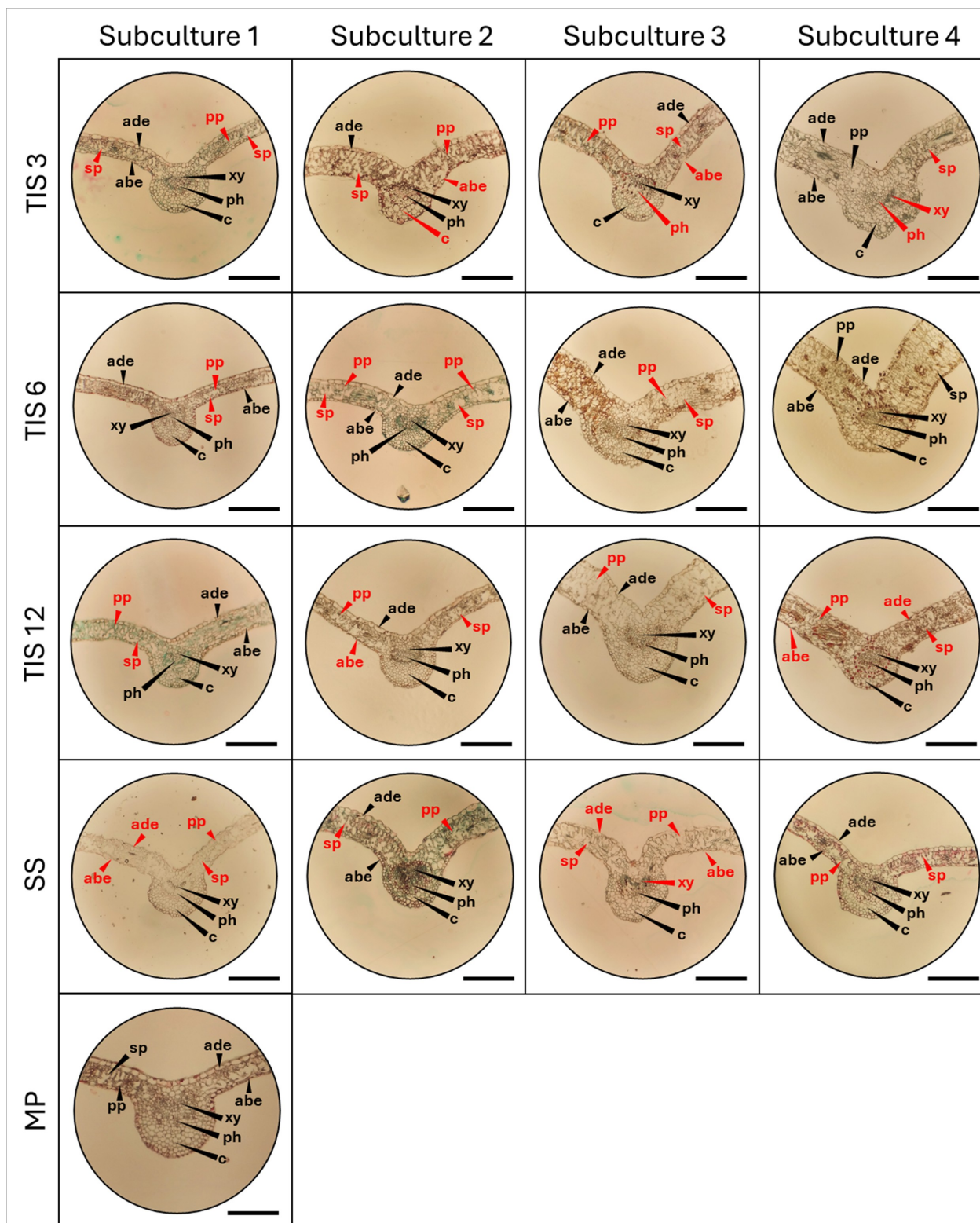


Figure 5

Leaf cross-sections of *P. persica* × *P. amygdalus* after 30 days of growth across four subcultures (Subculture 1, 2, 3, and 4) from *temporaryimmersion* systems every 3 h (TIS 3), 6 h (TIS 6), 12 h (TIS 12), traditional system (SS), and mother plant (MP). Abbreviations: **xy**: xylem, **ph**: phloem, **c**: cambium, **sp**: spongy parenchyma, **pp**: palisade parenchyma, **ade**: adaxial epidermis, **abe**: abaxial epidermis. Scale bars = 100 µm. Red arrows indicate hyperhydricity symptoms. Black arrows indicate normal tissues.

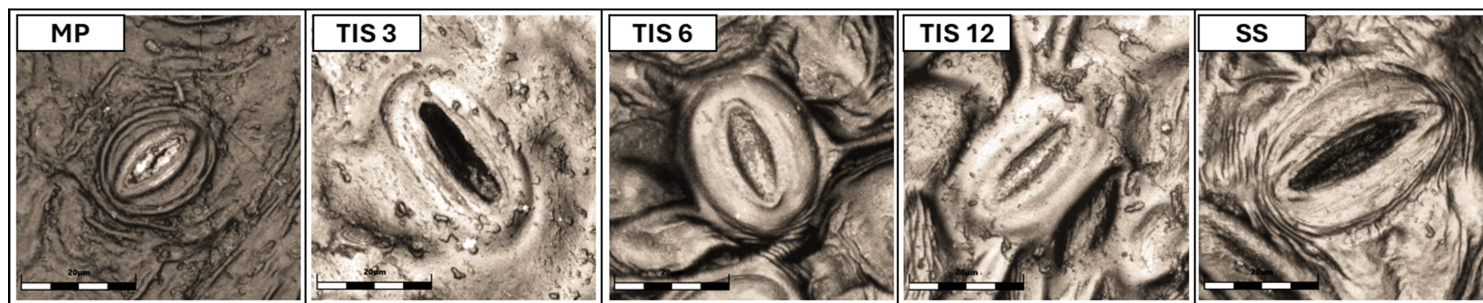


Figure 6

Topographic images of *P. persica* × *P. amygdalus* stomata in leaves from the mother plant (MP) and those propagated under temporary immersion systems every 3 h (TIS 3), 6 h (TIS 6), 12 h (TIS 12), and the traditional in vitro system (SS). Scale bars indicate a total length of 20 μm.

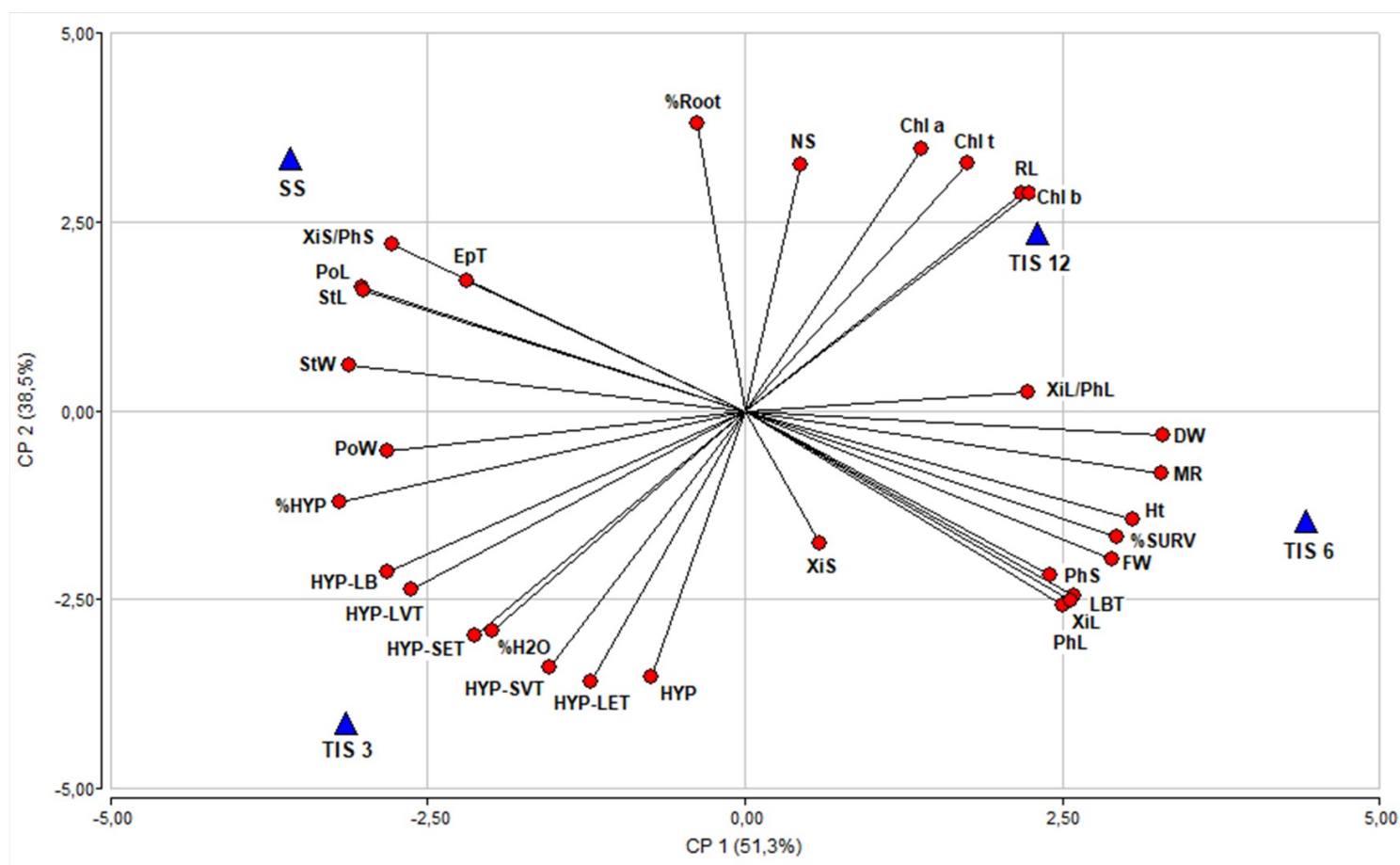


Figure 7

Principal Component Analysis (PCA) biplot of morpho-physiological and anatomical variables in *P. persica* × *P. amygdalus* plantlets. The PC1 and PC2 axes explain 52.0% and 37.4% of the variance, respectively. Blue triangles represent the treatments: traditional system (SS) and temporary immersion systems with frequencies of 3 h (TIS 3), 6 h (TIS 6), and 12 h (TIS 12). Variables (red vectors) are abbreviated as: **Ht**: height; **%Root**: rooting percentage; **RL**: root length; **NS**: number of shoots; **MR**: multiplication rate; **FW/DW**: plant fresh/dry weight; **%H₂O**: water percentage; **%SURV**: ex vitro survival; **Chl a/b/t**: chlorophyll a,

b, and total; **LBT**: leaf blade thickness; **Ph** and **Xi (S/L)**: phloem and xylem thickness in stem (S) or leaf (L); **XiL/PhL** and **XiS/PhS**: xylem/phloem ratios; **EpT**: stem epidermal cell thickness; **%HYP** and **HYP**: hyperhydricity percentage and severity; **HYP-(S/L)VT**: hyperhydricity in the vascular tissue of stem or leaf; **HYP-(S/L)ET**: hyperhydricity in epidermal tissue of stem or leaf; **HYP-LB**: leaf blade hyperhydricity; **StL/StW**: stomatal length/width; **PoL/PoW**: pore length/width.