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MICROPROPAGATION

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

Sebastián Godoy[1] · Eduardo Tapia[1] · Paula Seit[1] · Diego Andrade[2] · Evelyn Sánchez[1] · Paola Andrade[1] · Andréa Miyasaka Almeida[2] · Humberto Prieto[1]

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Abstract Sweet cherry (*Prunus avium* L.) varieties and cherry rootstocks are an important part of the fruit industry, and difficulties associated with mass propagation provide an opportunity for the use of temporary immersion systems (TIS). We show the establishment of culture procedures for four genotypes: the rootstocks Maxma-14 and Colt and the varieties 'Van' and 'Rainier.' The starting explants were internodal segments from seedlings kept in solid propagation medium (PM) (Driver-Kuniyuki Walnut (DKW) base supplemented with indole butyric acid, benzyl amino purine; ascorbic acid, myo-inositol, and agar). Segments were cultured under TIS for 14 d and led to whole plant generation after 30 d of culturing in solid rooting media, which depended on whether they are varieties or rootstocks. A 15-d acclimatization phase led to establishment in greenhouse. The efficiency of TIS was specifically analyzed for the two best PM-derivative media and compared to cultures using solid medium. A number of shoots (P_x), biomass (Q_x), and sucrose consumption (SC) were evaluated for these purposes. The results showed that Maxma-14, Colt, and 'Van' TIS cultures had improved performance in comparison to solid cultures, whereas 'Rainier' showed no differences. The number of immersions influenced all of the productive parameters (P_x, Q_x, and SC), whereas genotype affected P_x, and the time of immersion influenced SC. The best Q_x and P_x values were obtained with the rootstocks Maxma-14 and Colt, as well as the variety Van; these showed no hyperhydration. Physiological studies show that 14-d TIS-produced shoots represented an intermediate stage between solid-derived and adult plants, although the photosynthetic efficiencies of these materials revealed a lack of autotrophic ability at this point.

Keywords *Prunus avium* · Bioreactor · Liquid media · Clonal propagation

Introduction

There are several difficulties associated with the vegetative propagation of genotypes that must be overcome for the important sweet cherry (*Prunus avium* L.) cultivars and rootstocks used in the fruit industry. The development of clonal propagation systems for elite scion and rootstock materials will sustain the high-quality growing standards required by this industry worldwide. Plant tissue culture techniques allow the rapid production of many genetically identical plants with a relatively small amount of space, supplies, and time. However, for fruit trees, particularly *Prunus* spp., these achievements are more complex and must overcome the intrinsic biological characteristics this species and its hybrids (Martínez-Gómez *et al.* 2005), as well as the complex effects of different nutrients used in for the propagation procedures (Alanagh *et al.* 2014).

Different approaches have been used to establish micropropagation techniques in *P. avium* cultivars and rootstocks. Potential strategies for cherry micropropagation include

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✉ Humberto Prieto
hprieto@inia.cl

[1] Biotechnology Laboratory, La Platina Research Station, Instituto de Investigaciones Agropecuarias, Santa Rosa 11610, La Pintana, Santiago, Chile

[2] Fruit Physiology and Genomic Laboratory, Plant Biotechnology Center, Universidad Andrés Bello, Avenida República 217, Santiago, Chile

internodal stem sections from 1-yr-old shoots (Feucht and Dausent 1976; Hartman and Brooks 1976), shoot tips (Németh 1979; Hammatt and Grant 1998; Muna *et al.* 1999; Sedlák and Paprštein 2008), embryos (Ivanička and Pret'ová 1980), and *in vitro* micrografting techniques (Özzambak and Schmit 1991; Amiri 2006). Whole plant formation has also been achieved using regeneration procedures with leaf explants (Bhagwat and Lane 2004) or mature cotyledons (Canli and Tian 2008) and calluses formed from both *in vitro* and orchard tree-derived explants (Feeney *et al.* 2007). In general, these works have demonstrated a strong genotype dependence for both propagation and regeneration capabilities (Zilkah *et al.* 1992; Feeney *et al.* 2007). Hence, the actual advantage of applying these tissue culture techniques to massive cloning within this species remains unclear and needs further development (Carrasco *et al.* 2013).

A successful micropropagation system is determined by the interaction of the explant and several complex factors, such as nutrients, light, temperature, and even the gas exchange rate of the culture vessels. The most common problems observed with these approaches are related to hyperhydricity, a process in which the leaves of micropropagated plants may be deficient in chlorophyll and contain more water than leaves grown *ex vitro*. Several processes involving hyperhydricity are related to altered leaf anatomy, which includes abnormal structures such as an irregular stomata and chloroplast degeneration (Chakrabarty *et al.* 2006). In addition, several works have revealed complications related to the use of gelling agents (Mahdavian *et al.* 2011). One potentially advantageous approach arose from the use of temporary immersion systems (TIS) (Harris and Mason 1983; Etienne and Berthouly 2002).

Previous studies carried out on wild pear (*Pyrus communis* var. pyraster L.) (Damiano *et al.* 2000) led to improved shooting from leaves in comparison to the conventional solid media approach (approximately 3.25-fold greater biomass production). More recent studies have used TIS, solid media, and stationary liquid techniques for culturing *Malus domestica* and *Prunus* spp. explants (Damiano *et al.* 2005). The liquid stationary approach led to detrimental development (primarily necrosis) of all the processed explants, while the obtained multiplication rates were quite similar between solid media and the two assayed immersion frequencies (30 and 60 min/24 h). In addition, slight differences in the carotenoid and total fructose contents (accumulated as sucrose) were observed in TIS-derived peach (cv. Yumyeong), cherry (cv. Bigarreau Burlat), and plum (cv. Adara) plantlets. Based on these results, further experimentation is necessary to determine the responses of plants cultured in TIS to different immersion frequencies, rooting, and stress of acclimation.

In the present work, a TIS approach for massive micropropagation of two *P. avium* cultivars and two sweet cherry rootstocks was designed and characterized. The focus is on the plant responses in this *in vitro* strategy and the detrimental effects on the generated shoots. The best TIS conditions for each genotype were defined and compared to those of the solid media approach, and the application of these methods for clonal propagation in cherry is discussed.

Materials and Methods

Plant material Certified rootstock genotypes Maxma-14 (*Prunus mahaleb* × *P. avium*) and Colt (*P. avium* × *Prunus pseudocerasus*) were obtained from the Sweet Cherry Breeding Program, Rayentué Research Station (INIA, Los Choapinos-Rengo, Chile). Certified genotypes of *P. avium* L. 'Van' and 'Rainier' were obtained from the Cherry Breeding Program at La Platina Research Station (INIA, La Pintana-Santiago, Chile).

Experiment no. 1: establishment of genotypes in solid medium and preliminary assays for liquid micropropagation All explants and *in vitro* procedures were carried out under sterile conditions, with heat sterilized media.

a) Plant material introduction into in vitro culture. Woody sticks were water washed for 10 min, disinfected in 50% bleach/ 20 min, immersed in 0.2% (*w*/*v*) Captan plus 0.2% (*w*/*v*) benomyl/ 20 min, and stored at 4°C in darkness for 900 h. Before planting, sticks were immersed in 1000 ppm of indole butyric acid (IBA) (PhytoTechnology Laboratories, Overland Park, KS)/20 min and planted in soil at 24°C until budding (Fig. 1, step 1). The buds were collected, disinfected in 20% bleach/ 20 min, immersed in Captan/benomyl as above /5 min, water washed 3 × 5 min, and cultured *in vitro* using 75-mL glass jars containing 20 mL of solid propagation medium (PM) (Fig. 1, step 2).

PM solid medium was previously established in this laboratory and based on Driver-Kuniyuki Walnut (DKW) medium (Driver and Kuniyuki 1984) supplemented with 0.01 mg L^{-1} of IBA, 0.6 mg L^{-1} of benzyl amino purine (BAP) (Sigma-Aldrich, Saint Louis, MO), 500 mg L^{-1} of ascorbic acid (Calbiochem, Billerica, MA), 500 mg L^{-1} of myo-inositol (PhytoTechnology Laboratories), 25 g L^{-1} of sucrose (Sigma-Aldrich), and 7 g L^{-1} of agar (Sigma-Aldrich), with a pH 6.0. Explants were sub-cultured under these conditions every 30 d.

b) Preliminary TIS trials using liquid PM-derivatives. Preliminary TIS trials used PM-BAP and sucrose variants; Table 1) with five whole plants of each genotype derived from solid PM (Fig. 1, step 3). Inverted double-vessels were used (Fig. 1, step 4) with immersions of 1 min every 4 h, with a 16-h photoperiod, at 25 ± 1°C (Fig. 1, step 4). Results were judged on final plant quality (leaf color and

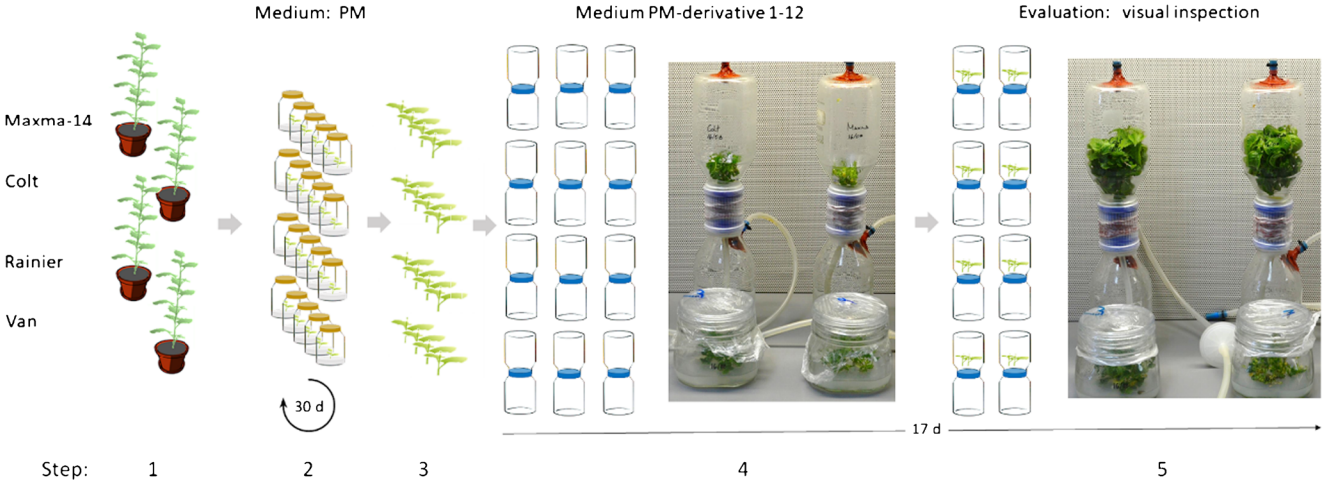

Fig. 1. Genotype introduction and temporary immersion system (TIS) preliminary trials. Generation of *in vitro* shoot populations was started by introduction of stem segments from young trees kept at greenhouse (step 1) and micropropagated in solid propagation medium (PM) (step 2). Five 30-d shoots (step 3) were used in the definition of improved PM media (step 4; PM-derivatives in Table 1) using primary TIS devices consisting of two inverted vessels. The quality of the final explants and amount of biomass produced were used as parameters to define the best PM-derivatives media after 17 d of culture (step 5). Genotypes of interest are indicated.

aspect) and biomass production after 17 d of culture (Fig. 1, step 5). The best performing media were selected for the next experiment.

Experiment no. 2: temporary immersion system design and comparative study between solid media and TIS The two best media, medium A (MA) and medium B (MB) (Table 1), were used in the comparative study (Fig. 2). As starting material, buds from rooted sticks of each genotype were sub-cultured in media A and B every 30 d (Fig. 2, step 1), keeping a set of 100 shoots per genotype and medium.

Table 1. Assays carried out for propagation medium (PM) improvement

Run	Block	BAP (mg L^{-1})	Sucrose (g L^{-1})
1	1	0.4	15
2	1	0.8	15
3[x] (A)	1	0.4	25
4[x] (B)	1	0.8	25
5	2	0.4	15
6	2	0.8	15
7	2	0.4	25
8	2	0.8	25
9	3	0.4	15
10	3	0.8	15
11	3	0.4	25
12	3	0.8	25

PM is Driver-Kuniyuki Walnut (DKW) medium plus 0.01 mg L^{-1} of IBA, 0.6 mg L^{-1} of BAP, 500 mg L^{-1} of ascorbic acid, 500 mg L^{-1} of myo-inositol, 25 g L^{-1} of sucrose, pH 6.0. Evaluated after 17 d of culture using five plants of each genotype

[x] Best performing PM-derivatives: medium A and medium B

a) Temporary immersion system design. The TIS consisted of separated explant and medium vessels, of 2 L and 1 L respectively, connected by silicon pipes (Fig. 2, step 3; details in Fig. S1). Pressure compensation filters (0.22 μm) were used between each vessel and between the vessels and the environment. Multiple systems with 10 in-line bioreactors were arranged in parallel (Fig. 1, step 3).

b) Culture initiation. TIS and solid medium systems contained internodal segments isolated from 30-d shoots from the best media (Fig. 2, step 2). The explants typically covered two nodes and were 1.5 cm long (black bars in step 2). Bioreactor and solid media batches were started using 1.5 g of explants (approximately 28–32 explants; Fig. 2, step 3), yielding one 2-L TIS vessel or 5- to 6100-mL solid medium containers.

c) Culture development. The culture batches were evaluated after 14 d of cultivation for both the solid and liquid approaches (Fig. 2, step 3). A 16-h photoperiod was used, and the temperature was maintained at 25 ± 1°C. In addition, the photosynthetically active radiation (PAR) was 21.90 ± 3.76 μmol s^{-1} m^{-2}.

d) Additional temporary immersion system settings. Immersion conditions were additionally varied using different values for number of immersion (*N*) and time of immersion (*T*) (Fig. 2, step 3). Two, three, and four immersions per d were analyzed, which lasted for 1 or

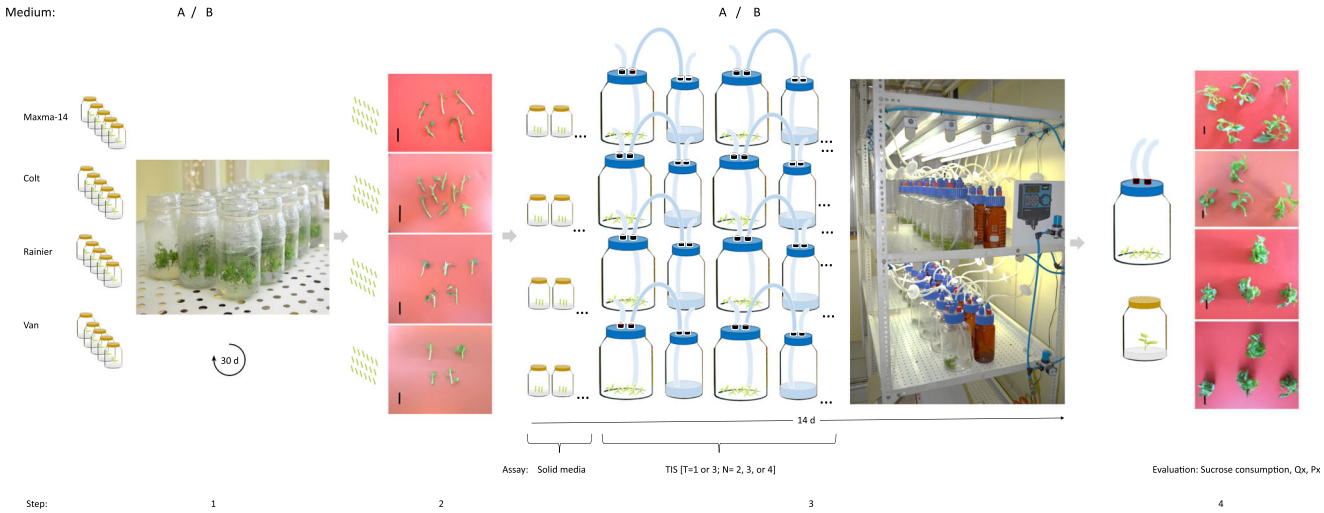

Fig. 2. Experimental design for the evaluation of a temporary immersion system (TIS) for sweet cherry varieties and cherry rootstocks. *In vitro* shoot populations of each genotype were established in medium A and medium B (best PM-derivatives) and used for the comparative evaluation between TIS and solid culture. Stem segments (1.5 cm in length) from these shoots (step 2) were used as explants for both shoot maintenance (step 1; every 30 d) and comparison of solid propagation/TIS (step 3). Assays for the latter were carried out during 14 d (step 3). Different TIS conditions [immersion number (*N*) and times (*T*)] were also included in these assays. Evaluations (step 4) included determinations of number of shoots (P_x), biomass (Q_x), and sucrose consumption. The *black bars* in steps 2 and 4 represent approximately 1.5 cm. A single TIS unit consisted of two vessels [medium and explant containers] joined by silicon pipes. Group of several units was installed in series to perform this work.

3 min. All these assays involved the liquid versions of MA or MB. The same indicated photoperiod was used.

e) Nomenclature assigned for TIS regimes. A nomenclature system for the different TIS regimes was defined by three terms in the form a.b.c., e.g, (medium: A or B).*T* (immersion time 1 or 3 min).*N* (no. of immersions 2–4 immersions), e.g, B.3.3 denotes MB, $T = 3$, and $N = 3$.

f) Evaluated parameters

- Sucrose consumption (SC) of the explants. Residual sucrose during TIS culture was measured using a sucrose assay kit (Sigma-Aldrich) according to manufacturer's instructions, in triplicate.
- Biomass determination (Q_x). The fresh material produced using both *in vitro* approaches was weighed in triplicate.
- Number of shoots (P_x). A number of shoots similar in length to the original explants were determined. After weighing, shoots from each cultured batch were segmented along their main stems in sections of two nodes (1.5 cm) and counted. Each two-node segment was considered to be a new explant, and all determinations were made in triplicate.

g) Statistical analyses. In the case of TIS assays, the variables considered were as follows: medium (MA and MB), *N* (2–4 per day), *T* (1 and 3 min), and genotype ('Van' and 'Rainier' varieties; Maxma-14 and Colt rootstocks). A factorial design 2 × 2 × 3 × 4 (*T*, PM, *N*, and genotype) was generated with a total of 48 experiments run with three independent repetitions. In the case of solid media, a 2 × 4 (medium and genotype) design led to eight experiments with five independent repetitions. Responses for Q_x, P_x, and SC were obtained and the data were subjected to ANOVA, and averages were separated by Tukey's SD multiple comparison at a significance level of 5% using Statgraphics Centurion XV.

Experiment no. 3: fitness analysis of micropropagated plants

a) Confocal microscopy. Images of leaves from plants in experiment no. 2 (*i.e.*, 14 d solid and TIS) were acquired using a Leica HCS LSI microscope (Leica Microsystems Inc., Buffalo Grove, IL). The images were recorded using 1024 pixel definition at a scanning speed of 400 Hz. The laser intensity was set at 40% with an offset of − 3%. The gain was set at 800. Leaf images were obtained using a magnification of × 3.53 and a gain of 950. Control leaves were from young greenhouse trees (Fig. 1, step 1) .

b) Scanning electron microscopy (SEM). Leaf pieces from the same plants were processed as described by Silveira

(1998). Leaf fragments were observed using a HITACHI TM 3000 scanning electronic microscope. The images were obtained using a magnification of × 1200.

c) Chlorophyll a fluorescence measurements. Leaves from 14-d TIS-produced plants in experiment no. 2 were used. The leaf chlorophyll fluorescence was measured on the adaxial side of dark-acclimated leaves (30 min) using a FluorPen FP 100 fluorometer (Photon Systems Instruments, Drasov, Czech Republic). After dark adaptation (30 min), the leaf was exposed to increasing PAR with light intensity values varying from 100 to 1000 μmol m^{-2} s^{-1} in five steps of 30 s. At the end of each light intensity step, a saturation pulse was applied to measure the minimal fluorescence (F_0), maximal fluorescence (F_m), light-acclimated minimal fluorescence ($F_{0'}$), and light-acclimated maximal fluorescence ($F_{m'}$). These parameters were used to calculate the fluorescence parameters as described previously (Baker 2008; Klughammer and Schreiber (2008).
d) Chlorophyll a and b quantification. Chlorophylls a and b were quantified according to the procedure described by Wellburn (1994). For all analyses, leaves from three 14-d TIS-produced plants were used.

Experiment no. 4: whole plant generation from TIS

a) Rooting. Rootstock shoots were cultured for 30 d in solid rooting medium [RM; DKW medium without hormones supplemented with 250 mg L^{-1} of ascorbic acid, 25 g L^{-1} of sucrose, and 1 g L^{-1} of activated charcoal (Sigma-Aldrich)]. For varieties, rooting was achieved by a first culture during 15 d in MW medium [2.58 g L^{-1} of McCown's Woody Plant Basal Salt Mixture (Sigma-Aldrich); 40 mg L^{-1} of phloroglucinol (Sigma-Aldrich); 0.01 μM of kinetin (Sigma-Aldrich); 5 μM naphthaleneacetic acid (Sigma-Aldrich); 25 g L^{-1} of sucrose; and 3.2 g L^{-1} of phytagel (Sigma-Aldrich)] and then transference into RM, without activated charcoal, for additional 15 d. All procedures were carried out at 25 ± 1°C and photoperiod as described.
b) Acclimatization. Rooted plantlets were transferred to 300-mL black plastic bags containing 200 g of a soil/perlite/peat (1:1:1) mixture and covered with transparent plastic bags. The upper bags were gradually opened *via* corner perforation to avoid excessive loss of humidity during the first 15–20 d. The upper bags were then removed. The greenhouse temperature was set between 26 ± 2°C.

Results

Introduction of sweet cherry variety and rootstock genotypes into in vitro culture (experiment no. 1) The use of woody sticks of the genotypes Maxma-14, Colt, 'Van' and 'Rainier' and PM-derivatives led to the successful introduction of buds into an adequate micropropagation system based on PM solid media and shoot sub-cultures every 30 d (Fig. 1, step 2). Improvements to these micropropagation procedures were started by the use of liquid PM-derivatives and preliminary TIS devices designed as shown in Fig. 1 (step 4). By this approach, the best conditions were found for shoot subcultures every 30 d (Fig. 2, step 1) in media derivatives A and B (Table 1). These regimes were the baseline to define a final TIS system (Fig. S1) and for TIS comparison with the solid micropropagation technique.

Comparison between TIS and solid culture (experiment no. 2)

i.- Productive parameters. Biomass production (Fig. 3*a*; values provided in Tables S1, S2, S3, and S4) was improved in both rootstock genotypes (Maxma-14 and Colt) using the TIS in comparison to solid micropropagation. The results highlight the use of MB as having the best nutrient conditions for these genotypes, which preferred higher *N*, regardless of *T* (Pareto charts of the standardized effect estimates are shown in Fig. S2*a*). The top yields were seen for Maxma-14 using B.3.3 and B.1.4 (see Nomenclature assigned for TIS regimes), which produced Q_x = 23.85 ± 1 g, and for Colt the regimes B.3.3 and B.1.4, which showed Q_x = 17.3 ± 1 g. Both tested varieties showed the best results preferably using MA, and also higher *N* were preferred; 'Van' reached Q_x = 26.2 ± 1 g under regimes A.3.3, A.1.4, B.3.3, and B.1.4, and 'Rainier' produced Q_x = 18.1 g under A.3.3 and A.1.4.

In terms of P_x (Fig. 3*b*; Tables S1, S2, S3, and S4), MB with TIS resulted in the best nutrient combination for three of the evaluated genotypes (*i.e.*, Maxma-14, Colt, and 'Van'), which showed statistical differences from the solid micropropagation procedures. P_x also slightly depended on the medium and more markedly on N and the genotype used (Fig. S2b). In the case of Maxma-14, the best values were 117 ± 10 shoots (regimes B.1.4; B.3.3; B.3.4); Colt explants responded with the largest number reaching 79 ± 10 shoots (B.3.2; B.3.3); and 'Van' showed the highest yield of 59 ± 10 shoots using conditions A.1.4, A.3.3, A.3.4, B.3.3, and B.1.4. The fourth genotype, 'Rainier,' showed slight differences in TIS cultures from the solid approach, and MA and MB conditions, at regimes A.1.4, A.3.3, A.3.4, B.1.3, and B.3.2, yielded the best results.

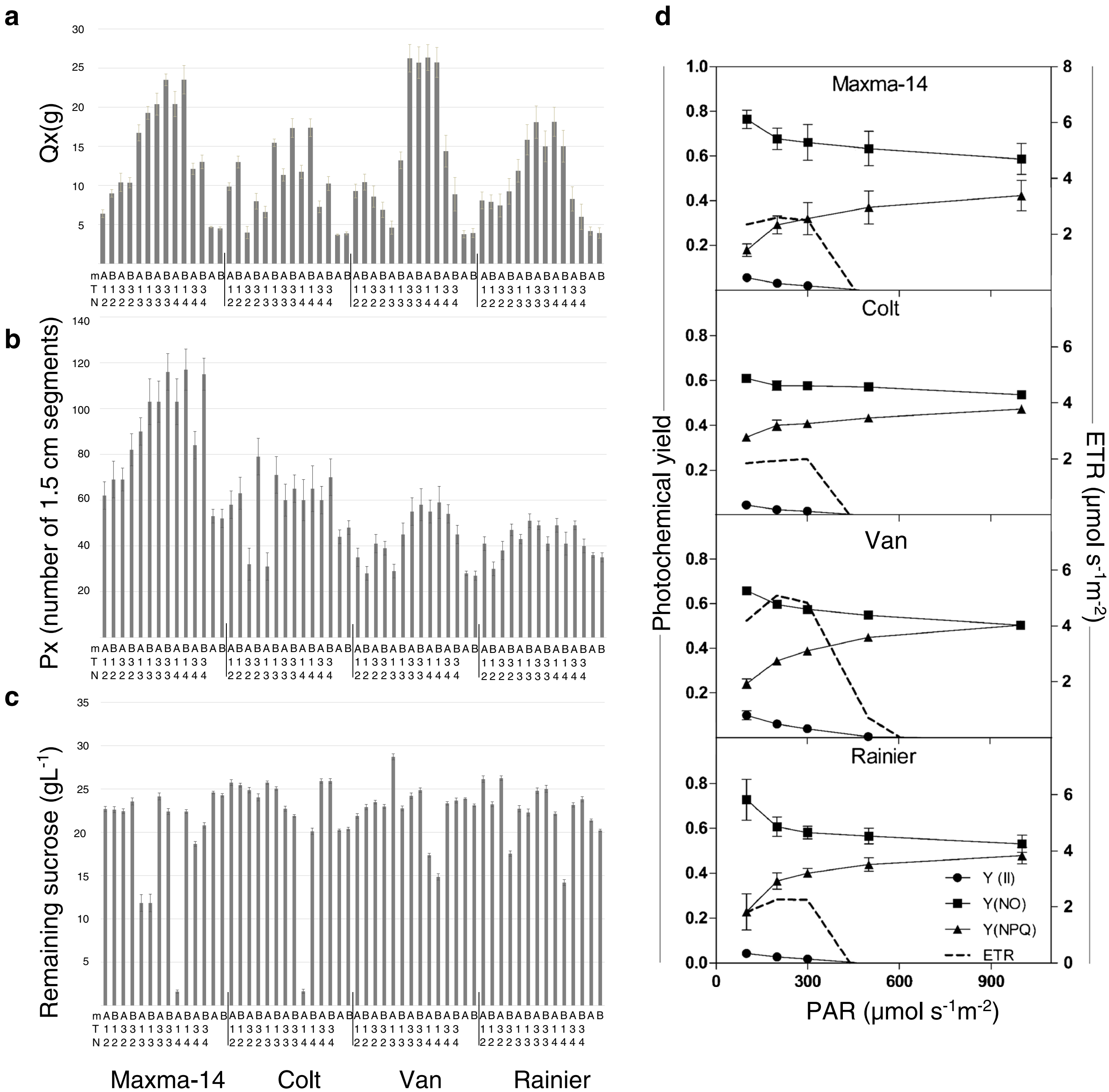

Fig. 3. Productive parameters and physiological behavior of TIS resulting shoots. Explants of different varieties (Van, Rainier) and rootstocks (Colt and Maxma-14) were evaluated for biomass Q_x (*a*), number of shoots P_x (*b*), and sucrose consumption (*c*) after 14 d of culturing in medium (m) A or B (*a*, *b*) with different numbers (*N*) and times (*T*) of immersions. The shoot elongation was determined based on the ability of the resulting explants to generate new 1.5-cm internodal segments. The biomass was determined based on the fresh weight. The sucrose plot (*c*) shows the sucrose remaining in the medium after culturing. The time of immersion is shown in minutes. Chlorophyll a fluorescence analyses (*d*) for TIS-produced plants (Van, Rainier, Colt, Maxma-14) showing the electron transport rate (ETR, *dashed lines*), the effective quantum yield in PS II [Y (II), *circle curves*], the quantum yield of regulated non-photochemical energy loss in PS II [Y (NPQ), *triangle curves*], and the quantum yield of non-regulated non-photochemical energy loss in PS II [Y (NO); *square curves*]. PAR, photosynthetic active radiation. Leaves were selected from 14-d TIS-produced shoots with rootstocks and Van under MB, $T = 3$ and $N = 3$ regime and Rainier under MA under $T = 3$ and $N = 3$ regime. *Bars* indicate mean standard deviation.

ii.- Sugar consumption and physiological parameters. Both varieties and rootstocks showed comparable SC, measured as remaining sucrose in the medium, between solid culture and the best TIS conditions (Fig. 3*c*; Tables S1, S2, S3, and S4). In addition, SC showed a marked dependence on *N* and a minor dependence on *T* (Fig. S2c). Interestingly, MA and MB yield both improved and diminished SC values. For instance, whereas in Maxma-14 SC values in

the best TIS performances resulted as low as in solid cultures, Colt explants responded better than solid under A.1.2, A.1.3, A.3.4, B.1.2, B.1.3, and B.3.4 regimes. On the contrary, remarkable SC in both rootstock genotypes was obtained by TIS with A.1.4 configuration. In the case of varieties, a similar effect was observed in 'Van' explants, which showed the same SC as solid cultures in most of the TIS conditions. Improved culture using TIS was observed under A.1.3, and also A.3.3 and B.3.3 regimes, which led to better performances than the solid counterpart. On the contrary, A.1.4 and B.1.4 had higher SC than the observed under solid culture for this genotype. In the case of 'Rainier,' several treatments led to lower SC than the solid culture (*e.g.*, A.1.2, A.3.2, A.3.3, A.3.4, B.3.3, and B.3.4), and two TIS settings (B.1.4 and B.3.2) had higher SC values.

Fitness capacity in shoots derived from TIS (experiment no. 3) A preliminary physiological characterization of the plants from the best trials was achieved by measuring chlorophyll fluorescence (Fig. 3*d*) at this point of plant development (*i.e.*, 14 d of culture; rootstocks and 'Van' using B.3.3 and 'Rainier' using A.3.3). It is important to note that plants developed under solid conditions did not produce leaves of adequate size to carry out chlorophyll fluorescence analyses. The photochemical responses of leaves from Maxma-14, Colt, 'Van,' and 'Rainier' were assessed using a light curve to estimate the saturating photochemical yield (Y(II)) and the electron transport rate through photosystem II (ETR). A general similar trend to incident PAR was seen in the evaluated genotypes. The maximum ETRs were reached at around 300 PAR and then started to decrease in the four genotypes, showing that the electron transport in the thylakoids is saturated at this light intensity (Fig. 3*d*, dashed curves). The photochemical yields of all genotypes showed the same behavior, with values under 0.2 for the quantum yield of photochemical energy conversion in PS II [Y (II); Fig. 3*d*, circle curves], which reached zero at the saturation point (300 μmol m^{-2} s^{-1}). The quantum yield of non-regulated non-photochemical energy loss in PS II [Y (NO); Fig. 3*d*, square curves] started between 0.6 and 0.8 for the four genotypes and decreased slightly to 0.5 and 0.6 with increasing PAR. Chlorophyll quantification showed that TIS-produced plants possessed significantly less of these pigments and lower chlorophyll a/b ratio than adult plants grown in greenhouse conditions (Table 2). Interestingly, some differences can be highlighted in 'Van,' which showed the best ETR between 100 and 300 PAR and a slightly best photochemical yield; this behavior was also correlated with the higher chlorophyll a/b ratio.

In addition, microscopy stomatal analyses of TIS-produced plants (Fig. S3) showed different stomata apertures using both SEM and CM techniques between solid- and TIS-produced materials. The TIS-produced plants represented an intermediate state in terms of stomata condition between solid and greenhouse-grown plants (Fig. S3; control).

Whole plant production in TIS (experiment no. 4) Routine batching using TIS and the best conditions for each genotype led to the production of whole plants in a workflow that required a total of 65 to 70 d (Fig. 4). After culturing in the TIS for 14 d (Fig. 4*a*), the plants were rooted in RM for 30 d (Fig. 4*b*; rootstocks (upper pictures) and varieties (lower pictures)) and acclimated using plastic bags for gradual humidity adaptation to the greenhouse conditions (Fig. 4*c*). Whole plants were obtained after this period (Fig. 4*d*), with 100% in the efficiency for all the genotypes. In comparison, the solid approach allowed only for the generation of eventual rooted plants during the same time period (not shown).

Discussion

The large-scale production perspective The use of solid systems in sweet cherry propagation has been reported to be difficult and genotype-specific (Sedlák and Papštein 2011; Carrasco *et al.* 2013; Druart 2013), and very specific studies have been generated (*e.g.*, for 'Lapins') (Ruzic and Vujovic 2008). In this work, we describe TIS procedures with potential for large-scale use to propagate specific, successful genotypes, and present improved nutrient conditions. Recently, Alanagh *et al.* (2014) described different improved protocols using neurofuzzy logic technology, which is a bioinformatics-assisted nutrient analysis technique, in order to optimize microshoot propagation of peach rootstock GF677 (*Prunus dulcis* × *Prunus persica*). In an exhaustive screening of multiplication and rooting conditions for different cherry cultivars and related *Prunus* species, Druart (2013) reported the use of MS (Murashige and Skoog 1962) and Quoirin-Lepoivre (Quoirin and Lepoivre 1977) macroelements, Nitsch (Nitsch 1969) and Quoirin-Lepoivre microelements, and vitamins such as thiamine and inositol.

The micropropagation workflow In our case, PM allowed for adequate solid maintenance and the base propagation of several varieties, including 'Van' and 'Rainier' and 'Bing' (not shown), as well as the rootstocks Colt, Maxma-14, and Gissella 6 (not shown). The PM is a modification of DKW medium that has a similar base to that used by Druart in terms of macro- and microelements, with the only difference being use of BAP (Table 1, MA and MB). Both media A and B allowed the establishment of genotype cultures in a liquid format, with no apparent effect on the final individuals.

Our results allow us to propose massive micropropagation pipelines by TIS in both rootstock and variety genotypes. A summary of the data for these purposes is presented in the Table S5; according to this, additional considerations must be taken. As seen in Fig. 3, several TIS regimes showed SC

Table 2. Comparison of the chlorophyll contents of the genotypes according to micropropagation technique

Genotype	Chlorophyll A + B			Chlorophyll A/B		
	Solid[x]	TIS[y]	Greenhouse[z]	Solid	TIS	Greenhouse
Maxma-14	116.3 ± 3.9cd	165.9 ± 13.2e	192.6 ± 9.3f	1.71	1.29	1.57
Colt	69.9 ± 2.3b	124.1 ± 5.1d	229.9 ± 10.3g	2.44	1.29	1.77
Van	28.6 ± 7.3a	49.5 ± 7.0ab	95.6 ± 4.8c	2.49	1.72	2.04
Rainier	49 ± 4.9ab	124.7 ± 6.2d	96.9 ± 4.0c	2.33	1.22	2.25

Pooled leaf samples from each individual ($n = 3$) were used. Values are indicated as mean of 3 determinations ± SD. *Letters* indicate groups after Tukey's HSD (5%)

[x] Measured using 30 d sub-cultured plantlets in solid medium

[y] Measured using 14 d temporary immersion system-propagated plantlets

[z] Measured using 6-mo growth plants under greenhouse conditions

values higher than those obtained during solid cultures; however, they did not involve improved yields (Q_x and/or P_x) and a global balance among these factors must be applied; for instance, in Colt explants, whereas the use of B.3.3 and B.1.4 yielded similar Q_x and P_x by a similar SC, the regime B.1.3 produced slightly lower Q_x and higher P_x at a lower SC cost. In addition, other examples showed that high SC and P_x values did not ensure high Q_x yields; in 'Van' explants, regimes B.3.3 and A.3.4 showed similar P_x and SC; however, the A.3.4 regime led to Q_x lower than under B.3.3. Similarly, 'Rainier' under A.3.3 and A.3.4 had similar SC and P_x; however, Q_x was considerably higher applying A.3.3.

From this, a general balance indicated that both rootstock genotypes and 'Van' performed well by the use of MB, a high sucrose and cytokinin formulation which led to promising productive parameters under a regime $T = 3$ and $N = 3$. In addition, results showed that 'Rainier,' a variety that had a certain trend to hyperhydricity, behaved better under the same high sucrose content and the lower the cytokinin supplementation, conditions given by the regime A.3.3 (Fig. 3). This balance also encouraged us for the fitness trials carried out in experiment no. 3.

The TIS advantages for sweet cherry genotypes Etienne and Berthouly (2002) addressed *T* and *N* as the most important factors for successful TIS adaptation to plant propagation and production under culture. In our results, these factors were the most relevant for SC, which could also indicate the best autotrophy status of resulting shoots. These authors also highlighted that the capacity of vessels and the immersion volumes are relevant factors. In our system, the selection of vessels derived from preliminary TIS prototypes with smaller containers (Fig. 1, steps 4 and 5) led to hyperhydricity (especially for the varieties; data not shown). Hyperhydricity has been reported in woody species cultured in TIS, such as apple (Chakrabarty *et al.* 2003 and 2006; Zhu *et al.* 2005), eucalyptus (McAlister *et al.* 2005), and pistachio (Akdemir *et al.* 2014). In addition, several types of abnormal structures have been observed in this process, including irregular stomata and

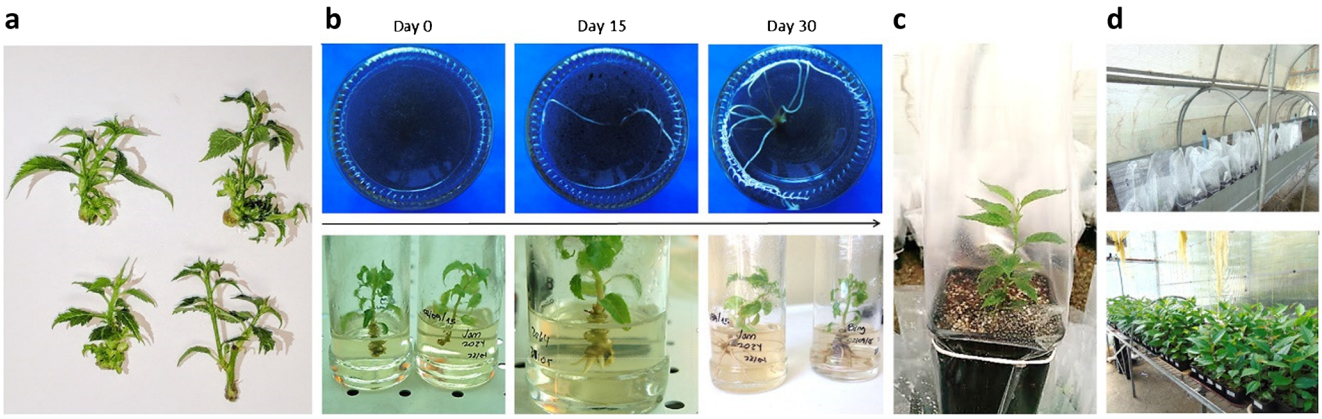

Fig. 4. Rooting and acclimatization workflows for sweet cherry and cherry rootstock TIS-derived explants. Sweet cherry and cherry rootstocks were cultured using TIS for 14 d (*a*) and rooted in their corresponding rooting media for 30 d (*b*; rootstocks (*upper pictures*) and varieties (*lower pictures*)) and progressively acclimatized using plastic bags (*c*, *d*, *upper picture*) for 15 d. Complete plants were obtained after 60 d (*d*, *lower picture*).

chloroplast degeneration (Chakrabarty *et al.* 2006; Shin *et al.* 2013). In spite of the observation of physiological malformation in 'Rainier' shoots in some TIS conditions, our results based on 14-d cultures showed no major alterations in both leaf structure and functionality (Fig. 3*d*; Fig. S3). Also, and in general terms, these results emphasize the relevance of space availability in the TIS containers for achieving adequate gas/liquid exchange surface and proper size for good explant elongation. Recently, new vessel systems have been proposed for TIS use with several species, which improved the size and weight restriction of current devices (Welander *et al.* 2014).

Physiology considerations It has been established that TIS-produced plants survive the acclimation stage more successfully than plants produced using the solid technique, and these differences consider the physiological status of the produced plants (Etienne and Berthouly 2002; Watt 2012). Additional evidence indicates that the TIS environment prepares the plantlets for the stress of acclimatization (Watt 2012). The growth and physiological changes in several plant species under *in vitro* propagation microenvironments have been described (Shin *et al.* 2013; Pérez *et al.* 2015; Pérez *et al.* 2015), but very little information is available for sweet cherry varieties and rootstocks.

In general terms, the high sugar content of micropropagation media has been indicated as a negative component regarding plant fitness and autonomy for *ex vitro* adaptation (Arigita *et al.* 2010; Pérez *et al.* 2015). Although no abnormal organization of leaves was observed in our assays, the intermediate stomatal opening in TIS-produced plants, which is an intermediate condition between solid and greenhouse plants, could be experimental evidence that supports these suggestions (Fig. S3). Sugar supplementation of nutrients in the micropropagation medium and the scarce CO_2 in culture vessels leads to inadequate operation of the photosynthetic machinery (Park *et al.* 2011; Xiao *et al.* 2011). Interestingly, several treatments derived from TIS evaluation also resulted in low SC by the explants (Fig. 3*c*) and this fact could indicate that these conditions could lead to more competitive explants and adequate for whole plant generation. In other cases, such as observed in both rootstocks under A.1.4 (Fig. 3), high Q_x values had high SCs, indicating that TIS could require further optimization and these improvements will probably depend on the genotype being cultured.

Consequently, there was evidence of a lack of autotrophic capability in measurements of photosynthetic efficiencies. The low Y (II) rates (Fig. 3*d*) showed that TIS-produced individuals were not capable of efficient photochemistry. The low Y(II) reflects the low chlorophyll a/b ratio on these plants (Table 2), which probably resulted from cultivation under low irradiance (approximately 20 μmol m^{-2} s^{-1}). Also, the chlorophyll status of these materials (Table 2) could reflect an effort by these explants in order to improve photosystem assembly. From this, the chlorophyll a/b ratio obtained in all genotypes under the TIS regime would allow us to think of an accommodation to the shade condition, since a high content of chlorophyll b is related to a higher concentration of light-harvesting complexes (Wang *et al.* 2007). Additionally, Y(NO) values higher than the Y(NPQ) values indicate that these individuals cannot manage the excess energy in PS II and exhibit chronic photoinhibition. Regardless these antecedents, optimization steps can be derived from including progressive light regimes in the TIS culturing step and avoiding photoinhibition episodes during batching (PAR higher than 150 μmol m^{-2} s^{-1}), which could also play a role in the gradual reduction of sugar in the media.

Conclusions

The TIS system presented for sweet cherry varieties and cherry rootstocks represents a feasible, more efficient, and faster propagation approach than solid media culture. The stronger growth observed with the TIS was probably caused by the increased availability of nutrients in the liquid medium, as well as the improved exchange rate of involved gases due to the major volume available in 2-L vessels, as suggested for other reactor systems (Adelberg and Fári 2010).

In addition, the different SC values observed with different TIS treatments (Fig. 3*c*) could reinforce the hypothesis that the TIS-produced plants have a partial autotrophic capability after 14 d of culture. These findings agree with previous descriptions of *Prunus* spp., such as *Prunus serotonia* (Horsley and Gottschalk 1993) and *P. persica* (Marchi *et al.* 2008), in which improved photosynthetic rates were strongly related to the ontogenetic development of plantlets. For a range of perennial fruit crop species, it has been described that rates of photosynthesis are frequently lower for leaves on extension shoots compared to leaves on fruiting spurs (Buwalda and Noga 1994).

Finally, whereas maximum Q_x and P_x values could represent important goals for large scale micropropagation, SC must be also considered in these assumptions, and light effect on improved systems should be also considered.

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