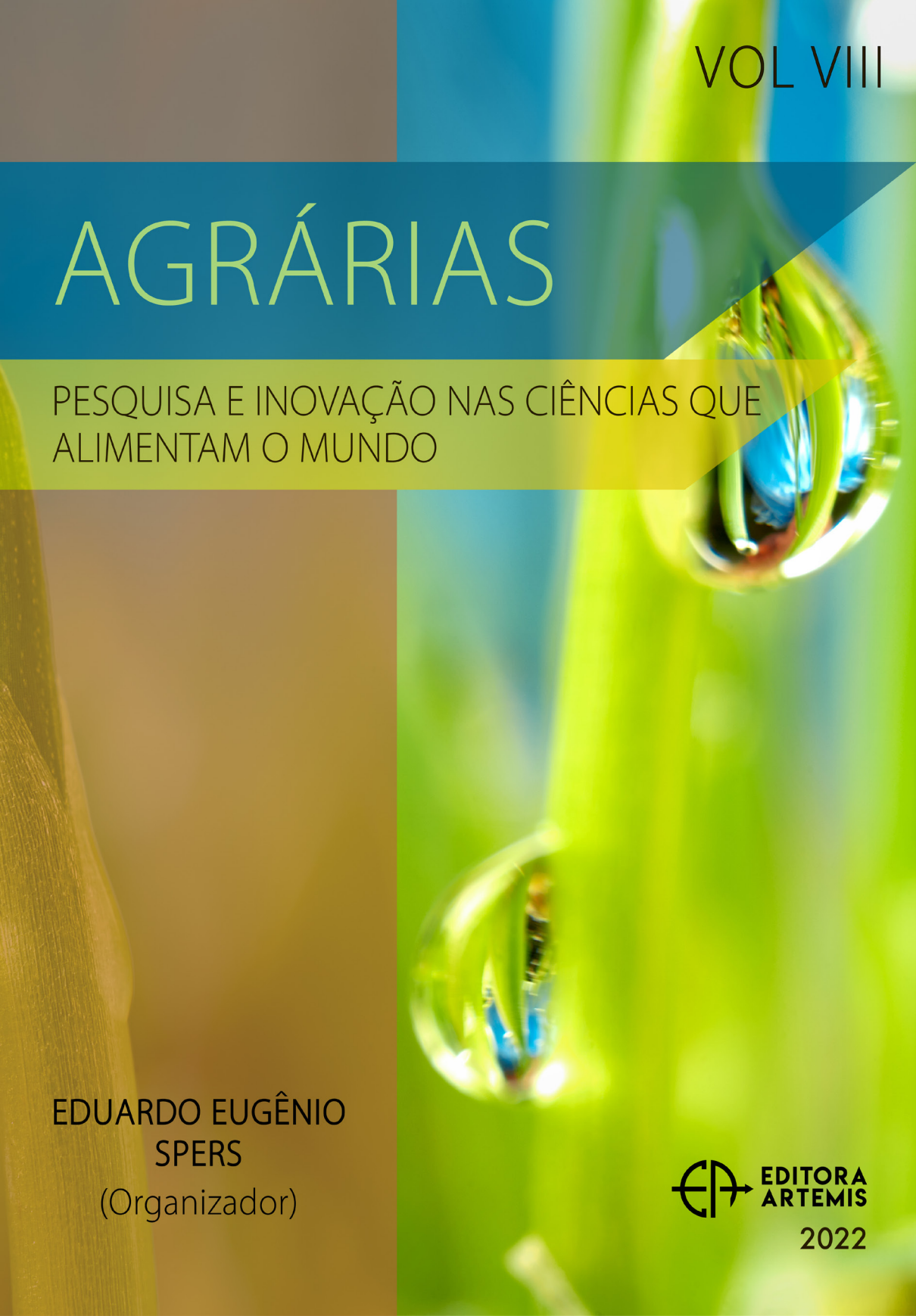
VOL VIII

AGRÁRIAS

PESQUISA E INOVAÇÃO NAS CIÊNCIAS QUE ALIMENTAM O MUNDO



EDUARDO EUGÊNIO SPERS
(Organizador)

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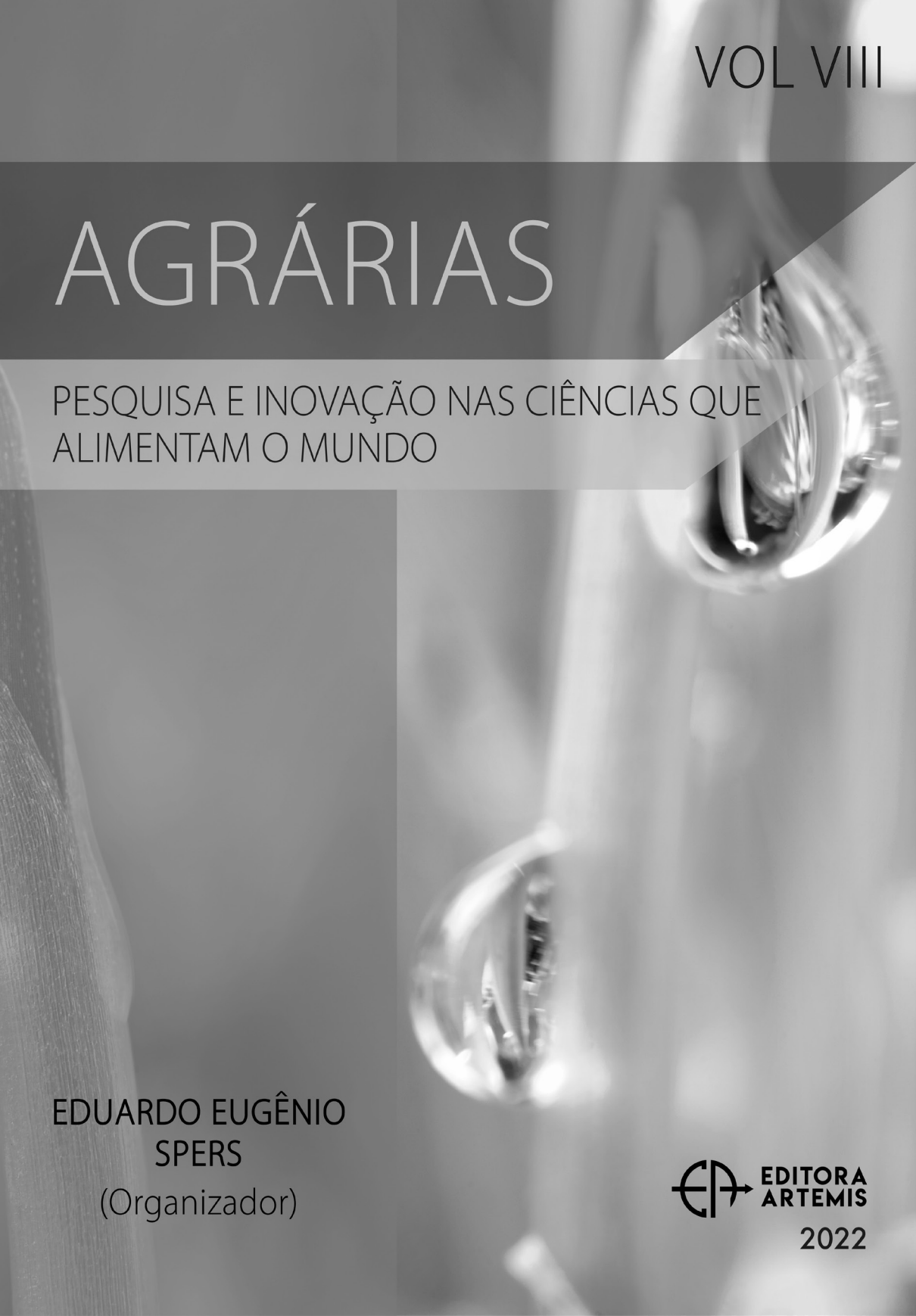

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APRESENTAÇÃO

As Ciências Agrárias são um campo de estudo multidisciplinar por excelência, e um dos mais profícuos em termos de pesquisas e aprimoramento técnico. A demanda mundial por alimentos e a crescente degradação ambiental impulsionam a busca constante por soluções sustentáveis de produção e por medidas visando à preservação e recuperação dos recursos naturais.

A obra **Agrárias: Pesquisa e Inovação nas Ciências que Alimentam o Mundo** compila pesquisas atuais e extremamente relevantes, apresentadas em linguagem científica de fácil entendimento. Na coletânea, o leitor encontrará textos que tratam dos sistemas produtivos em seus diversos aspectos, além de estudos que exploram diferentes perspectivas ou abordagens sobre a planta, o meio ambiente, o animal, o homem e a sociedade no ambiente rural.

É uma obra que fornece dados, informações e resultados de pesquisas tanto para pesquisadores e atuantes nas diversas áreas das Ciências Agrárias, como para o leitor que tenha a curiosidade de entender e expandir seus conhecimentos.

Este Volume VIII traz 26 artigos de estudiosos de diversos países, divididos em quatro eixos temáticos: *Cultura e Sociedade no Contexto Rural; Produção Sustentável; Produção Vegetal e Solos e Aquacultura, Produção Animal e Veterinária*.

Desejo a todos uma proveitosa leitura!

Eduardo Eugênio Spers

SUMÁRIO

CULTURA E SOCIEDADE NO CONTEXTO RURAL

CAPÍTULO 9

A TEMPORARY IMMERSION SYSTEM (TIS) BIOREACTOR USED FOR THE IN VITRO PROPAGATION OF *PRUNUS* AND *PYRUS* ROOTSTOCKS

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Carlos Rolando Mendoza
Plant in Vitro Culture Laboratory
Fruticulture Program
IRTA Fruitcentre
PCiTAL-Gardeny, 25003
Lleida, Spain

Ramon Dolcet-Sanjuan[1]
Plant in Vitro Culture Laboratory
Fruticulture Program
IRTA Fruitcentre
PCiTAL-Gardeny, 25003
Lleida, Spain
https://orcid.org/0000-0002-5817-7605

ABSTRACT: A new type of bioreactor, which has been patented (Dolcet-Sanjuan and Mendoza, 2018; Patent Pending ES201831164), is described herein and the results on the propagation of fruit tree rootstocks are detailed. The "GreenTray" (GT) bioreactor based on the temporary immersion system (TIS) principle, is a mid-sized unit reactor, from 1 to 4L capacity, with the distinction in the fact that, firstly, transforms glass, transparent and commercial flasks into a bioreactor; secondly, the flask containing the shoots is placed in horizontal position, and more importantly, its concept allows for the extraction and cut of all the shoots at once, making more efficient the explants culture and shoot extraction out of the bioreactor. As described here, the GT bioreactor consists of two flasks, one for the shoot explants and the other for the liquid culture media, joined through a perforated adaptor piece that permits the flow of the liquid media from one vessel to the other, driven by pressured air and gravity. The structural simplicity and the modular and independent nature of the bioreactor, helps its operation and reduces the amount of hand labor required for shoot transfers, thereby reducing the cost of the whole micropropagation process, when compared with other commercial TIS bioreactors or with the cultures in vessels with semisolid media. The system has been applied to propagate RootPack-20® (RP-20), a commercial *Prunus spp.* rootstock, and "Py170", an experimental *Pyrus spp.* hybrid rootstock. Compared with the conventional culture in semisolid medium, GT bioreactor improves by a 10% the multiplication rates of both rootstocks, and more significantly it improves shoot length by a 33% and 26%, and shoot fresh weight by a 21.9% and 30%, for RP-20 and "Py170" respectively. Shoot cultures produced with the GT bioreactor had better leaf development and less hyperhydricity, therefore improved shoot quality, than with the conventional cultures in semisolid media. Culture time required for

[1] Corresponding author: ramon.dolcet@irta.cat

plantlet development was reduced, hence costs to produce high quality commercial plants was 0.11€/plant with the GT bioreactor, significantly less than with the semisolid agar containing media.
KEYWORDS: **“**GreenTray”. GT bioreactor. IRTA-reactor. Temporary immersion system. TIS. Liquid culture. In vitro plant micropropagation.

1 INTRODUCTION

Micropropagation has great commercial impact and potential due to the speed of propagation, the high quality of plants that are obtained and the possibility of producing plants free of pathogens (Steward et al., 1970; Ammirato, 1985). Traditional micropropagation methods on semisolid media are limited by the high labor cost. In vitro culture systems based on liquid culture medium are considered to be more effective than culture systems in semisolid medium due to better accessibility of medium components by the plant tissue (Martínez, 2019), ease handling, and possibility of better scaling up and automation. However, as direct liquid immersion of some plant material causes vitrification or hyperhydricity, and sometimes inhibits growth and development of the plant material, TIS bioreactors have emerged as a workable alternative, capturing the benefits of liquid medium and semisolid support. In comparison with both solid and liquid culture systems, TIS bioreactors have been shown to offer technological and quantitative benefits such as higher multiplication rates and reduction of production cost (Etienne, H. and Berthouly, 2002). or explant quality improvements (Tisserat and Vandercook, 1985; Aitken-Christie and Davies, 1988; Etienne and Berthouly, 2002).

The development of propagation systems to produce affordable certified, true-to-type and disease-free fruit tree rootstocks or self-rooted varieties, is a worldwide objective in particular in the Mediterranean areas, such as in Catalonia, leader in the production of stone and pit fruits (MAPAMA, 2016). In order to meet these market demands, it is proposed to develop a technology that maximizes productivity by optimizing the use of resources, guaranteeing the quality of production and innovating in the design.

Unlike traditional micropropagation, bioreactors use liquid media based on the principles of temporary immersion (TIS). This principle is simply immersing the plant material into liquid growth media for short periods of time, sufficient for the plant to uptake nutrients. TIS technology profits from the advantages of liquid cultures, while growing the plants under high gas- exchange environments. Bioreactors have been considered as a promising alternative to the semi-solid culture system. However, there are no profitable bioreactors available for commercial production of fruit tree rootstocks.

There are various TIS bioreactor conformations, which have been described by Georgiev and Schumann (2014). From those, Rita® and Setis® are the most widely used in commercial and research propagation, although they need improvements to facilitate plant manipulation and reduce hyperhidricity in some plant materials. The GT bioreactor described herein, in comparison with the commercial ones, is cheaper, easy to assemble, occupying materials that can be found anywhere in the World. Its adaptability, practicality and low cost make it a good option to make escalations for mass production of plants and to carry out research works.

Herein it's described and evaluated the performance of the GT bioreactor, developed and patented by IRTA, previously called "IRTA-reactor" (Mendoza and Dolcet-Sanjuan, 2017) in comparison with the conventional in vitro culture in vessels. with semisolid medium, through the micropropagation process of the commercial *Prunus spp.* rootstock "RootPack-20®" (RP20), and a new *Pyrus spp.* hybrid pear rootstock "Py170", which is a clonal selection of an IRTA breeding program (Clavería and Asin, 2012).

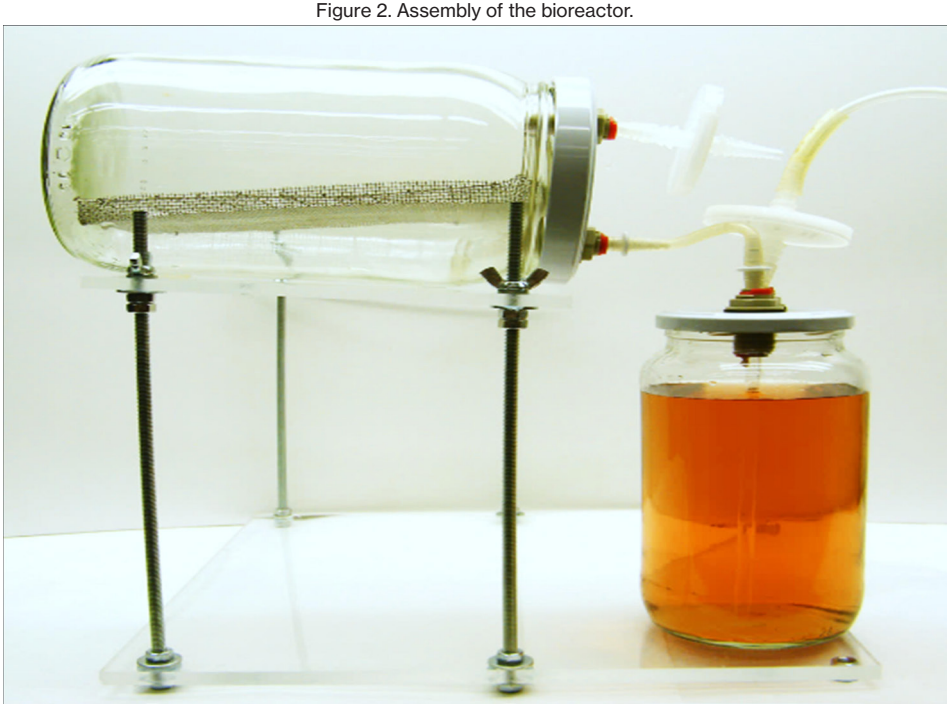
2 MATERIALS AND METHODS

2.1 PARTS, ASSEMBLY, AND LIQUID FLOW IN THE GTBIOREACTOR

Two commercial glass flasks are adapted to be used as two different parts of the bioreactor. A first one, placed in its upright position, containing the liquid medium. The other, in horizontal position, having the explants inside through the whole growth process. The reactor is assembled using the following parts (Figure 1): one 2 liter and one 850 mL glass flask and their corresponding metallic lids; four hose plastic fitting; two 0.2 µm PTFE Midisart® 2000 air filters; one 6 mm external diameter glass elbow; 6 mm external diameter silicone hose to connect all parts; one 6 mm external diameter glass tubing; a stainless steel 2-mm-opening mesh and a stainless steel 15-mm-opening grid. These parts are assembled as indicated in Figure 2 to get one bioreactor unit. The bioreactor, without the medium, was autoclaved at 120°C during 30 minutes, and sterile liquid culture media poured in the vertical 850 mL glass flask after opening it under a flow hood.

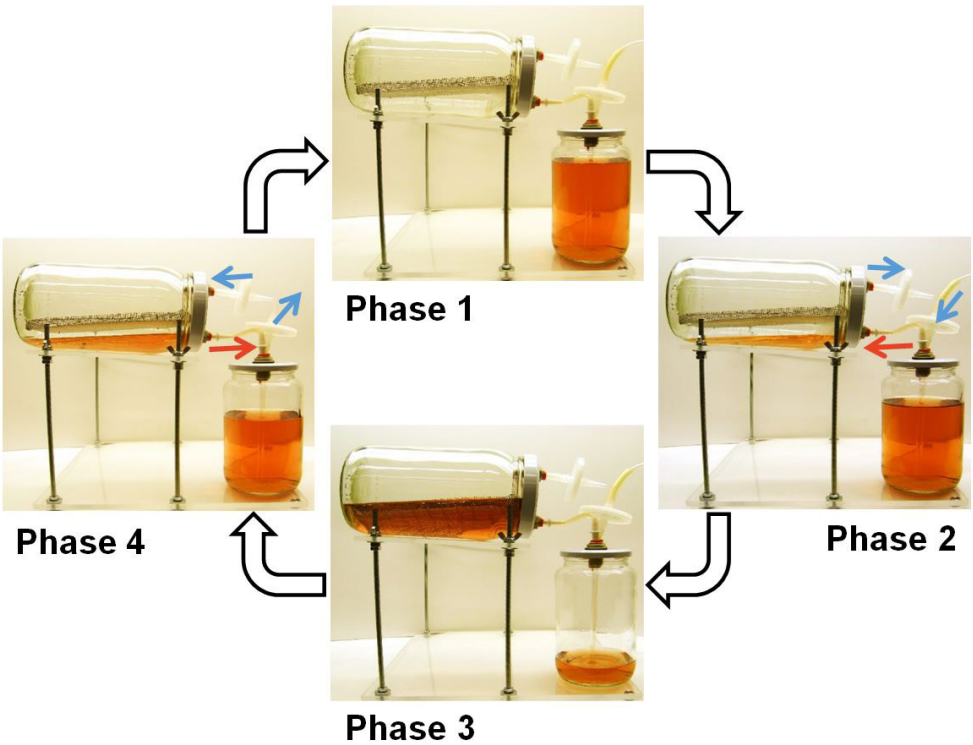
Figure 1. Parts of the bioreactor: (1) metallic cap with 2 hose plastic fittings for the 2-liter-capacity glass flask (2); (3) Metallic cap with 2 hose plastic fittings for the 850-mL-capacity glass flask (4); (5) 0.2 µm PTFE Midisart® 2000 filter; (6) Glass elbow; (7) Silicone hose; (8) glass tubing; (9) stainless steel 2-mm-opening mesh; and (10) 15-mm-opening stainless steel grid.

Figure 2. Assembly of the bioreactor.



The liquid medium passes from the fist flask to the second flask using air under pressure. The incoming air is supplied by a 4 bar line and passes through a 0.2 µm Millex-FG50PTFE Midisart® 2000 filter (Merck KGaA, Darmstadt, Germany), to avoid contamination (Figure 3). The explants were placed in a stainless steel grid which is inserted into the horizontal flask. The immersion frequencies were controlled by a timer that kept the explants in contact with the culture medium for 1 minute, every 3 hours.

Figure 3. Liquid media (←) and air (←) flow in the bioreactor. (Phase 1) media rests in the vertically oriented 850-mL-capacity glass flask, until pressured air pushes the media to the horizontally oriented 2-Liter-capacity glass flask (Phase 2), while air gets out of the latter. Pressured air stops flowing (Phase 3) when stainless steel mesh and grid, holding the explants, are completely immersed in the liquid medium. Media returns by gravity to the original flask moving the air out, while atmospheric air flows into the horizontally oriented flask.



2.2 PLANT MATERIALS

Rootstocks of commercial interest in fruit production were used in this study. A *Prunus* rootstock marketed by the industry, named Rootpac®20 (RP-20) (Agromillora Group), and a *Pyrus* rootstock, named "Py170", under agronomic evaluation by IRTA,. RP-20 is a natural hybrid between the "Myrobalan" plum (*Prunus cerasifera Ehr*.) and an almond (*Prunus dulcis*) (Pinochet et al., 2011), can be used as a rootstock for Japanese plums,

peaches, nectarines, almond and some apricot cultivars (Pinochet, 2010). The Py170 is a hybrid between "OH11" (Simard and Michelesi, 2002) and *Pyrus amygdaliformis*, which is in the last phase of selection in an IRTA breeding program, oriented to have tolerance to iron chlorosis and reduced vigor. Shoot tip cultures of both clones were established in vitro as follows.

2.3 ESTABLISHMENT OF SHOOT IN VITRO CULTURES

New actively growing shoots, collected from greenhouse potted RP-20 plants or from field grown Py170 trees, were used as source of explants. Explants were 1 to 2-cm-long nodal segments with one to three axillary buds each. Explants were washed for 1 h in stirring distilled water, and surface-sterilized in 70% ethanol for 1 minute, followed by 0.5 % (w/v) NaOCl with 0.1 % (v/v) Tween-20 for 10 minutes. Each explant was rinsed individually three times with sterile distilled water, trimmed, and cultured in a tube containing 15 mL of MS with 5 µM BA. Shoots that appeared uncontamined were screened further for bacterial contamination. Explants that showed bacterial growth on this medium were discarded (Iglesias et al., 2004).

2.4 CULTURE MEDIA

Micropropagation in flasks with semisolid agar-containing media were used as the standard culture conditions described earlier (Iglesias et al., 2004). MS (Murashige and Skoog, 1962) supplemented with 3% sucrose and 5mM 6-Benzylaminopurine (BAP), pH to 5.7,agar (7 g/L), autoclaved at 121 ° C for 20 minutes, The same MS+5BAP media, but without agar, was used in the GT bioreactor culture system. . In both culture systems, explants were kept during 3 weeks in MS+5BAP medium, and then moved to MS, without BAP medium, to promote shoot elongation for an additional 3-week-long period.

2.5 GREEN TRAY VERSUS CONVENTIONAL IN VITRO CULTURE CONDITIONS

Nodal segments, 1 to 2-cm-long, derived from shoot tip cultures in the multiplication phase, were used as explants to initiated new cultures in the GT bioreactor or in glass flasks. 70 explants per bioreactor and 10 explants per flask, with three replicates per clone, were used. All cultures were kept at 26±1°C under a photoperiod of 16h of cool-white fluorescent light (70 $umol \cdot m^{-2} \cdot s^{-1}$), and 8h darkness.

The TIS bioreactor was set at an immersion frequency of 1 minute every 6 hours. After 3 weeks of culture, the culture medium in the bioreactors was replaced with fresh

medium of the same composition but without BAP. In the case of flasks, 50ml of the same medium was dispensed to each flask.

2.6 SHOOT MULTIPLICATION EVALUATION

In the case of flasks, with semisolid media, shoot clumps were moved out with the help of forceps, and in the case of the GT bioreactor, were all moved at once by pooling out the tray holding the whole plant material. Individual shoots were separated from the clumps with the help of scapel and forceps. The number of new shoots from each initial explant, their length, fresh weight, and multiplication rate was determined for each container. Additionally, the incidence of vitrification or hyperhydricity was scored for each shoot clump.

2.7 ACCLIMATION TO SOIL IN THE GREENHOUSE

Ninety shoots per clone, 4 to10-cm-long, were removed from the GT bioreactors, rinsed with tap water and planted in trays with 28 wells, of 90-mL-capacity each, containing a mixture peat moss and vermiculite (2:1, v:v). The peat moss Pro Start (Castillo Arnedo S.L.) contained perlite (10%). Plantlets were acclimated to low atmospheric humidity was done gradually from 100 to 60%, in a 3-week-long period, in which soil temperature was kept above 20°C, and air temperature of 26-30°C. The photoperiod was supplemented to 14h light, with LED light (AlternativaLED, Valencia, Spain) to 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Another ninety shoots per clone were treated with 1000 ppm indole-3-butyric acid (IBA) and planted in the same conditions.

The percent survival of acclimated plantlets was determined.

2.8 COST EVALUATION AND OCCUPIED IN VITRO GROWTH CHAMBER AREA

The needed in vitro growth chamber area (*CAb*) and the cost to produce new shoots (*CS*) were separately analyzed for the micropropagation in flasks or in the bioreactor.

The occupied chamber area per shoot produced (*CAb*) was calculated by dividing the area, in cm^2, needed to hold each container (*Ab*) by the number of new explants produced (*NE*).

$$CAb = \frac{Ab}{NE}$$

The cost to produce each plant, in € per plant (*CP*) was obtained after adding the cost of each container (*CC*), the costs of all the culture media used (*CM*), and the workforce cost needed to complete the culture process (*CWF*), and dividing the sum by the number of new explants produced (*NE*).

$$CP = \frac{CC + CM + CWF}{NE}$$

2.8 STATISTICAL ANALYSIS

The experiment was set up as a completely randomized design, with two culture systems, two plant materials, and three containers per treatment. Data was statistically analyzed with JMP Statistics software (version 13.1.0, SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) followed by Tukey's test ($p \leq 0.05$) was performed.

3 RESULTS

3.1 ASSEMBLY AND OPERATION OF THE GREEN TRAY BIOREACTOR

The principal advantage of the GT bioreactor operation is the facility to move the plant material out of the recipient, or placing it back into it after is being cut, since it is held in a tray easily moved with a pair of forceps. This is the main claim of the patented bioreactor (Dolcet-Sanjuan and Mendoza, 2018; Patent Pending ES201831164). This operation is always done with the recipient in horizontal position, avoiding placing the hands above or close to the flask entrance, and this way reducing the possibilities to contaminate the cultures.

Other advantages of the GT bioreactor are (1) the high illumination of the plant material, since the lid is not shading it, (2) the elimination of all the medium and condensed water in the recipients walls closer to the leaves, since the flask surface is curved and the liquid moves to the bottom where it returns by gravity to the original, and (3) atmospheric air renewal in the recipient holding the plant material each time there is a movement of the liquid media. Such GT bioreactor characteristics enhance leaf development, shoot elongation, and avoids vitrification or hyperhydricity of the plant tissues observed with the plant materials used herein, when cultured in flasks with semisolid media o in other TIS bioreactors.

3.2 SHOOT PROLIFERATION IN THE GREEN TRAY BIOREACTOR VERSUS CONVENTIONAL CULTURES IN FLAKS

As shown in Figure 4 and 5, leaf number and stem thickness of shoots produced from both plant materials were comparable after culture in the two culture systems. Although differences in the multiplication rates were observed, after the 6-week-long culture process, no statistically significant differences were found, for Py170 or RP-20, between cultures in flaks with semisolid agar-containing medium or in the temporal immersion system with the GT bioreactor (Table 1). However, significant statistical differences were observed on the shoot length and the fresh weight per shoot between both culture systems. Both rootstocks, Py170 and RP-20, were 35% and 49% longer respectively, when grown in the GT bioreactor than in flasks with semisolid media. This was in concordance with a 43% and a 28% increase in the shoot fresh weight per new shoot, for Py-170 and RP-20 respectively.

Figure 4. Shoot growth after 42 weeks of culture in conventional semisolid agar-containing media (A and C), and culture in the bioreactor (B and D), of RP-20 (A and B) and Py170 (C and D) clones.

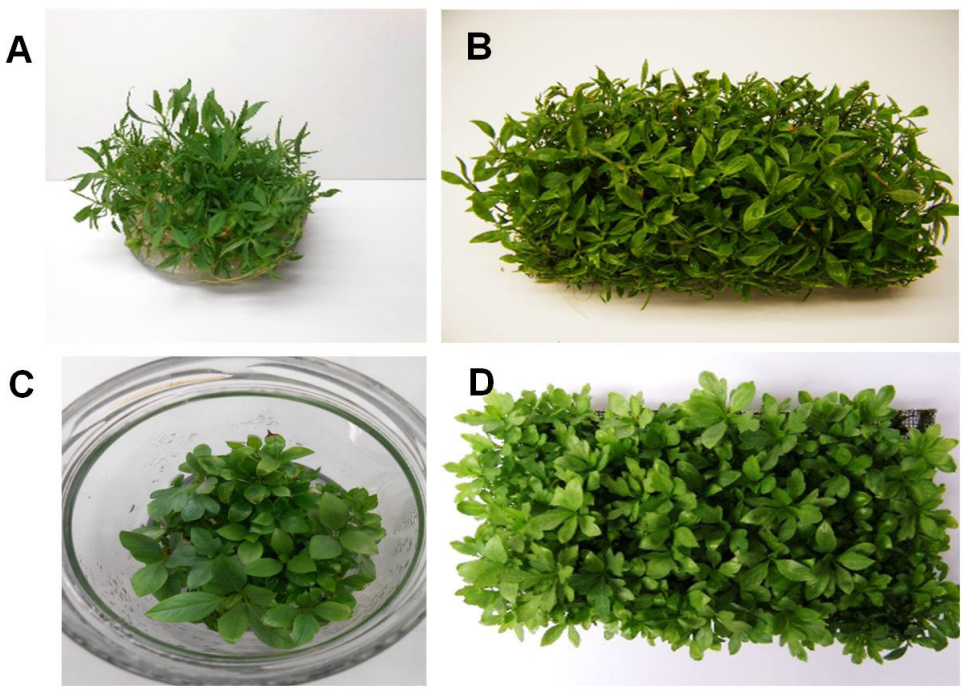


Figure 5. Evolution of the RP-20 and Py170 clones in the bioreactor. Initial explants (0), after 3 weeks of immersion in 5mM BAP (21), after 3 weeks of immersion in 0mM BAP (42), and after direct rooting of shoots in acclimation tunnels.

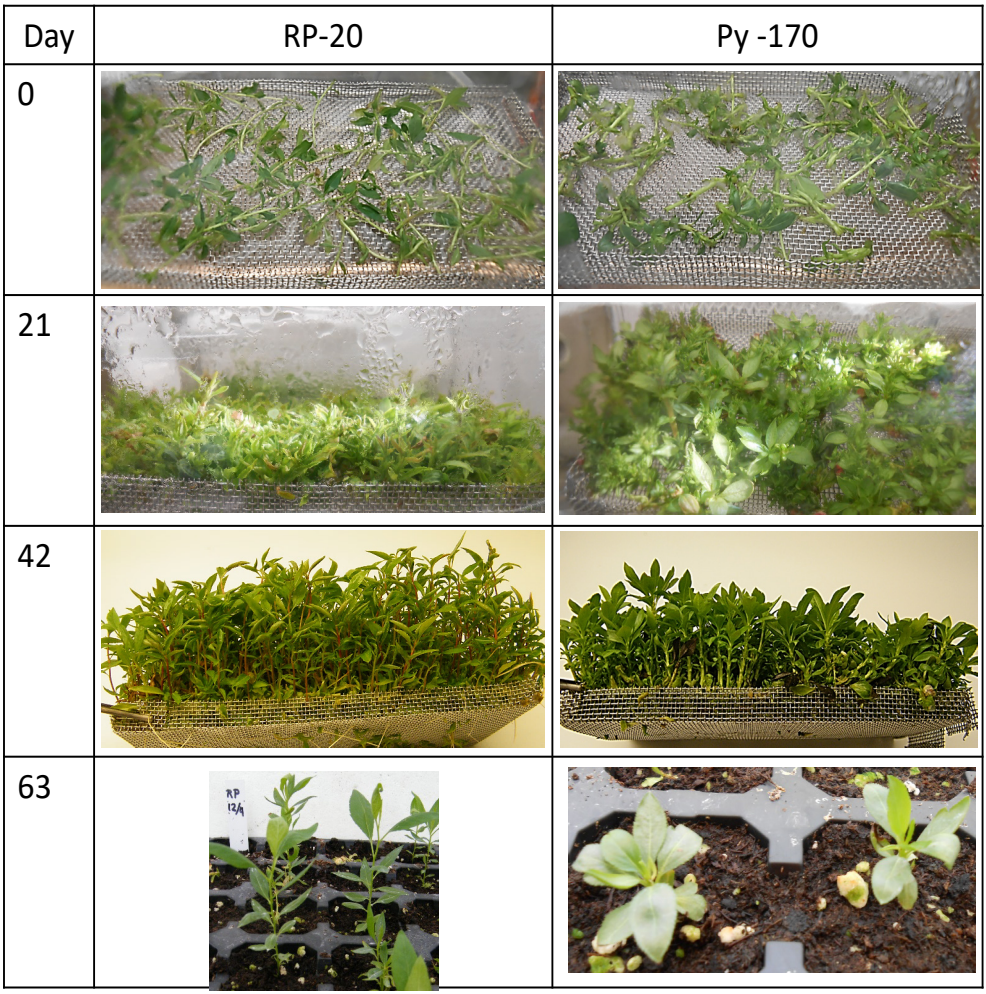


Table 1. Shoot multiplicatión rates and growth, of RP-20 and Py170 clones, after 6 weeks of culture in semisolid agar-containig medium or in the temporal immersion system with GT IRTA bioreactor.

Clon	Culture System	Multiplication Rate (Shoots / Explant)		Shoot Length (cm)		Fresh weight (grs / explant)	
Py170	Semisolid medium	5.03 ± 0.44	a*	16.90 ± 0.82	a	0.42 ± 0.03	a
Py170	GT bioreactor	5.59 ± 0.45	a	22.84 ± 0.68	b	0.60 ± 0.05	b
RP-20	Semisolid medium	7.29 ± 0.79	a	26.60 ± 0.80	a	0.57 ± 0.06	a
RP-20	GT bioreactor	8.10 ± 0.48	a	39.70 ± 0.69	b	0.73 ± 0.04	a

Values represent mean ± SE (Standard Error) out of three repetitions. *Means with different letter, within each measured variable, are significantly different according to Tukey HSD (P=0,05)

3.3 EVALUATION OF GT BIOREACTOR IN THE ACCLIMATION PHASE

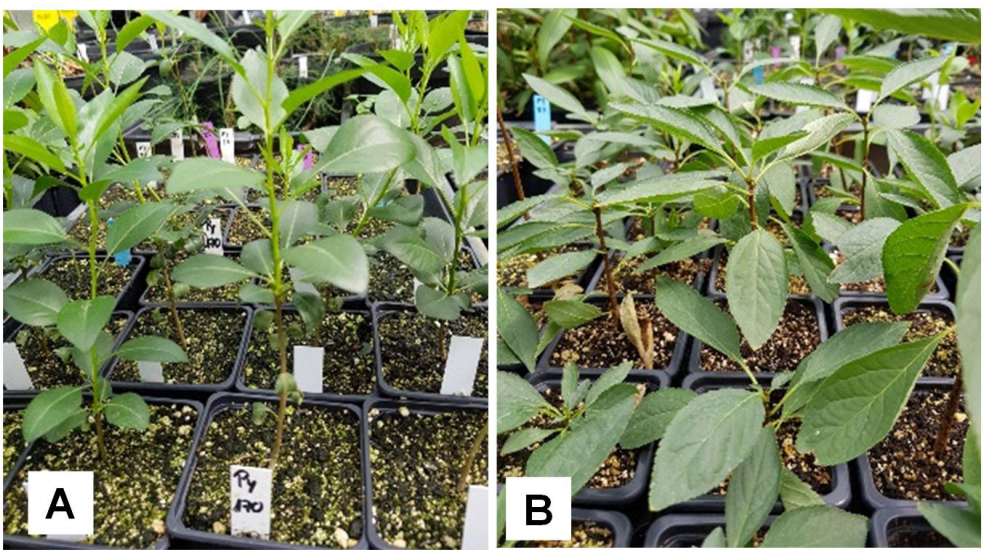
Shoot acclimation and rooting to the greenhouse conditions (Figure 5) and further plant growth and endurance (Figure 6) were highly successful. And 81% or 78% of shoots, Py170 and Rp-20 respectively, derived from the GT bioreactor directly rooted in the acclimation tunnels and survived in greenhouse conditions, without IBA treatment. Indeed the percentage of rooting dropped to 60% and 59%, for Py170 and Rp-20 respectively, when the shoot basis was dipped in IBA before planting, though the root development was better when the explants were treated with IBA (Table 2).

Table 2. Rooting of RP-20 and Py170 shoots derived from the GT IRTA bioreactor, treated or not with IBA, after 3 weeks of acclimation and 4 additonal weeks of culture in the greenhouse.

Clon	IBA (ppm)	Rooting (%)
Py170	0	81.1 ± 4.0
Py170	1000	60.0 ± 8.8
RP-20	0	78.3 ± 7.3
RP-20	1000	58.6 ± 8.3

Values represent mean ± SE (Standard Error) out of three repetitions.

Figure 6. Actively growing 3-month-old Py170 (A) and RP-20 (B) rooted plants derived from shoots produced in the bioreactor.



3.4 COST EVALUATION AND OCCUPIED CHAMBER AREA

Following the protocol described here the cost to produce an acclimated plant of RP-20 with the GT bioreactor was significantly lower than with the conventional method in flasks with semisolid medium (Table 3). While the cost of the container and materials to assemble a GT bioreactor, is sixty times more expensive than that of the flask with semisolid medium, the hand labor is less than one half since the time to produce and acclimated plant is reduced to 9 weeks. In consequence, the cost to produce one plant with the GT bioreactor is 0,11€, seven times less than with the semisolid medium. In addition, the in vitro growth chamber surface needed to get one shoot of RP-20 with the GT bioreactor is 0,32 cm^2, two and a half times less than with the conventional culture in agar containing medium.

Table 3. Comparative costs between the micropropagation in semisolid agar-containig media and the temporal immersion with GT bioreactor, of the RP-20 clone.

Plant growth requirements for each container	Semisolid medium	GT bioreactor
Number of initial explants	15	70
Total time needed for plant production (weeks)	14	9
NE: Number of shoots produced	100	700
Ab: Growth chamber area needed for each container (cm^2)	81	230
CAb: Growth chamber area needed for each shoot (cm^2/shoot)	0.81	0.32
Costs for each container		
CC: Container cost (€)	0.45	47.23
CM: Culture media cost (€)	0.28	1.38
CWF: Workforce cost (€)	75.00	30.00
Total Costs (€)	75.73	78.61
CP: Total Cost / Plant (€)	0.76	0.11

4 DISCUSSION

The results show that this newly developed Green Tray or GT bioreactor is suitable for mass production of fruit tree species, with survival rate and plant quality similar to those from semisolid medium. Similar results have been obtained in different species such as Mentha x piperita L. (Vaidya, 2018), red currant (J. Hautsaloa, 2018), sugarcane (Carrillo-Bermejo, 2018) using liquid media in different temporary immersion systems. The multiplication rate was either similar to or better than that from semisolid medium. Depending on the rootstock, between 400 and 700 shoots could be obtained per bioreactor, whereas in agar containing medium, over 100 shoots per flask were obtained. The average

shoots length was higher in the GT than in the semisolid medium. In addition, while shoots produced had the same quality in both systems, the time needed was shortened two weeks when cultures were done in the GT. In this reactor, shoot hyperhydricity was controlled and eliminated, when immersion time and frequency were corrected for each rootstock. Overall, the analysis of the culture systems results show that the GT is stable, reliable and with greater productivity that the culture in semisolid media.

The results of this study demonstrated the utility of the GT for the micropropagation of the two clones studied, obtaining more plants in a shorter time, occupying less chamber area per explant and reducing costs. The high plantlet survival rate obtained with this system assured the success of the micropropagation process because the in vitro rooting phase could be eliminated.

It is reported in the literature that the positive effects of TIS are due to a better aeration and renewal of chemical components at each immersion, otherwise limited in solid condition by the agar matrix. In this way the risk of anoxia and hyperhydricity, frequently observed in constant immersion as well as in solid culture, is significantly reduced (Etienne et al., 1997). The results obtained in the present work are consistent with those obtained in other micropropagation studies using temporal immersion systems in other species (Ramírez et al 2016; Escolana et al 1999, Bernal et al 2008, Niemenak et al 2008; Roels et al 2005) however most of these studies did not make the comparison between different temporal immersion system, or with the culture in semisolid media as here presented.

Microshoots under in vitro culture conditions can have a negative net photosynthetic rate and depend on sucrose uptake from the medium for their growth. Few results are available on the mechanisms of sugar uptake in shoot tissue culture systems. It is accepted that sugar uptake is an energy dependent process. Most publications dealing with sucrose uptake by cell cultures assume that sucrose molecules supplied via the culture medium are hydrolysed by cell wall. The use of LED lamps to improve the development of explants in the different stages of micropropagation in temporary immersion systems should be studied in greater depth, always seeking to improve the systems, some works show it that way J. Hausaloa (2018). In our experience (data not shown) the higher light intensity available with the LED lights increases the fixation of CO2 available the inner atmosphere of the 0GT bioreactor. One of the main drawbacks of the establishment of a micropropagation protocol in a temporary immersion system are the morphological and fisilogical abnormalities that occur in the shoots and explants (Ruffoni and Savona 2013). In temporary immersion systems, hyperhydricity problems usually occur, but in the GT bioreactor there were no symptoms of vitrification.

The main advantages of this reactor are, first, the plant material can be easily manipulated, and second, the shoots produced have well developed leaves, which can be easily acclimated in the greenhouse. The production capacity of explants in the GT bioreactor can be 4 to 7 times greater than in semi-solid medium, with the same plant quality, and at lower costs when compared to the conventional micropropagation in semi-solid medium. Consequently, the bioreactor GT is stable and reliable and with higher productivity, and has possibilities to be employed in large scale plant production.

5 ACKNOWLEDGEMENTS

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SOBRE O ORGANIZADOR

EDUARDO EUGENIO SPERS realizou pós-doutorado na Wageningen University (WUR), Holanda, e especialização no IGIA, França. Possui doutorado em Administração pela Universidade de São Paulo (USP). Foi Professor do Programa de Mestrado e Doutorado em Administração e do Mestrado Profissional em Comportamento do Consumidor da ESPM. Líder do tema Teoria, Epistemologia e Métodos de Pesquisa em Marketing na Associação Nacional de Pós-Graduação e Pesquisa em Administração (ANPAD). Participou de diversos projetos de consultoria e pesquisa coordenados pelo PENSA e Markestrat. É Professor Titular no Departamento de Economia, Administração e Sociologia, docente do Mestrado em Administração e Coordenador do Grupo de Extensão MarkEsalq no campus da USP/Esalq. Proferiu palestras em diversos eventos acadêmicos e profissionais, com diversos artigos publicados em periódicos nacionais e internacionais, livros e capítulos de livros sobre agronegócios, com foco no marketing e no comportamento do produtor rural e do consumidor de alimentos.

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