# GreenTray® TIS bioreactor as an effective *in vitro* culture system for the micropropagation of *Prunus* spp. rootstocks and analysis of the plant-PGPMs interactions

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**Abstract**

The use of the GreenTray® TIS bioreactor for the *in vitro* analysis of the interaction between plantlets and two Plant Growth-Promoting Microorganisms (PGPMs) (*Pseudomonas oryzihabitans* PGP01 and *Cladosporium ramotenellum* PGP02) is reported herein. This *in vitro* culture system improved micropropagation of *Prunus* Rootpac 20® rootstock, showing greater shoot length and fresh weight compared with culture in semisolid agar-containing medium. Plant responses in co-culture with the PGPMs, their ability to control endophytes growth in the culture media and hormonal changes associated to plant growth were studied in the GreenTray® culture system. Inoculation with *P. oryzihabitans* PGP01 had no significant effects, whereas *C. ramotenellum* PGP02 considerably reduced endophytes population after 5 days post inoculation. However, the fungi overgrew the plants roots seriously reducing their growth. These results suggested the effect of the medium pH as a factor to control microbial endophytic growth in the culture medium. A higher *P. oryzihabitans* PGP01 concentration was inoculated in culture media previously adjusted to pH 5.7 or 7, observing an increased number of roots when plantlets were co-cultured for 15 days with the bacterium in medium adjusted to pH 5.7. This was associated with changes in the levels of IAA in the culture medium, and higher leaf chlorophyll contents than in the non-inoculated cultured plantlets. *P. oryzihabitans* PGP01 was not able to reduce endophytes growth in either of both media pHs. Altogether, the GreenTray® bioreactor was shown as an efficient system to *in vitro* micropropagate RP-20 explants as well as to monitor its interaction with PGPMs.

**Keywords:** Auxins; GreenTray® Bioreactor; *In vitro* biological control; Liquid MS medium; Low pH; PGPMs.

## 1. Introduction

*In vitro* micropropagation has become a worldwide implemented technique in the plant clonal production as it allows obtaining a large quantity of free-disease plant material all-year round (Dobránszki and Teixeira da Silva, 2010). Conventional micropropagation methods involving the use of small flasks with semi-solid media represents the most used procedure (Pereira-Lima et al., 2012). Nevertheless, the intense manipulation of these cultures has made of *in vitro* micropropagation a very time-consuming technique. In this sense, further progress to improve the shoot propagation rates, its growth and quality, reducing the cost of the process have already been intended with the implementation of liquid cultures in bioreactors. Innovative approaches conducted during the 90s demonstrated the effectiveness of this system over the traditional micropropagation methods using agar-containing media (Alvard et al., 1993; Escalona et al., 1999). However, it has been reported that direct liquid immersion cause vitrification or hyperhydricity of plant material, leading to a disruption of *in vitro* growth and development (Akdemir et al., 2014). To avoid this issue, bioreactors with Temporary Immersion Systems (TIS) improve the benefits of liquid cultures to ensure the *in vitro* performance of explants (Godoy et al., 2017). In TIS bioreactors, the soaking process of *in vitro* explants with the culture medium only occurs during shorts periods of time, sufficient for plants to uptake nutrients and plant growth regulators (Georgiev et al., 2014). In addition, the forced aeration within the explants vessel provided the explants with an environment with a high gas exchange, resulting in a better shoot or plantlet growth and development (Pereira-Lima et al., 2012). In the last years, TIS bioreactors have been successfully implemented for the micropropagation of several plant species including pistachio (Akdemir et al., 2014), cherry (Godoy et al., 2017), plum (Damiano et al., 2005) or apple (Sota et al., 2021).

On the other hand, microorganisms and plants coexisting in their natural environment establish parasitic, competitive, mutualistic or commensals relations with their hosts (Senthilkumar et al., 2011). In *in vitro* cultures, the occurrence of contaminant microorganisms (especially bacteria) has been frequently associated to an inappropriate explant manipulation, as *in vitro* tissues must be maintained sterile (Orlikowska et al., 2017). Nonetheless, it has been assumed since many years that, in spite of the surface sterilization process, *in vitro* cultures are unexpectedly contaminated by microorganisms that colonize the internal tissues (Quambusch et al., 2016). Those microorganisms are commonly known as endophytes. Several authors have defined the term “endophyte” as referring to microorganisms including bacteria or fungi that are capable of living within plants without expressing disease symptoms (Petrini, 1991; Wilson,

1995). In this regard, endophytes have been defined as non-pathogenic bacterial strains with the ability to colonise plant tissue through the natural openings available on plant surface (Cassells and Tahmatsidou, 1997). Although not pathogenic, microorganisms in *in vitro* cultures may alter the behavior of tissues, negatively affecting propagation, growth and development (Cassells, 2012; Leifert and Cassells, 2001; Tsao et al., 2000). Some authors proposed the addition of antimicrobial compounds to the culture medium as strategy to eliminate endophytes and thereby obtain clean plant *in vitro* cultures (Khan et al., 2018; Lotfi et al., 2020; Shehata et al., 2010). However, some endophytic bacteria may reappear after transference to a medium without antibiotic (Bunn and Tan, 2002). No reports concerning the use of plant growth-promoting microorganisms (PGPMs) to suppress the growth of *in vitro* endophytes, while promoting plant growth and development, are available in literature. It is well established that PGPMs are able to suppress the growth of antagonistic microorganisms through multiple mechanisms (Morales-Cedeño et al., 2021), and their introduction to control endophytes growth in plant *in vitro* cultures might represent a more sustainable approach to mitigate the high losses of plant material associated to this issue. On the other hand, other mechanisms used by PGPMs to promote plant growth have been described such as the production of phytohormones that could be used for plants to complete their growth and functionality (Calvo et al., 2014). In this context, auxins, cytokinins (CKs) and gibberellins (GAs), among others, could be synthetized by PGPMs (Arkhipova et al., 2005; Hamayun et al., 2010; Zamioudis et al., 2013), altering the hormone status of plants and favouring numerous physiological processes including cell division, root initiation, root elongation, senescence or shoot elongation (Lugtenberg and Kamilova, 2009; Vejan et al., 2016).

Although much evidences concerning the benefits of the application of PGPMs in plants have been provided in the last few years, very little of them have been conducted using *in vitro* culture systems. The TIS bioreactor used in the study, patented (Patent No. ES201831164; PCT WO 2020/109637 A1) and registered as GreenTray® (Dolcet-Sanjuan and Mendoza, 2018 and 2020), is a mid-sized unit bioreactor, from 1 to 4L capacity, with the distinction in the fact that, firstly, transforms glass, transparent or other commercial flasks into a bioreactor. Other advantages of the GreenTray® bioreactor are: (1) the high illumination of the plant material, since the lid is not shading it; (2) the elimination of all the liquid medium and condensed water in the recipient walls closer to the leaves, since the flask surface is curved and the liquid moves to the bottom from where it returns by gravity to its external original container; (3) atmospheric air renewal in the recipient holding the plant material each time there is a movement of the

liquid media, or alternatively when needed though and independent circuit, and (4) it needs small volumes of media (150 to 250 mL) to temporally immerse the cultured explants or plantlets. Such GreenTray® particular characteristics enhance leaf development, shoot elongation, and avoids vitrification or hyperhydricity observed with the plant materials cultured in flasks with semisolid media or in other TIS bioreactors (Dolcet-Sanjuan and Rolando-Mendoza, 2018 and 2020). Herein, the present study shows the first application of a TIS bioreactor as a niche for the study of the *in vitro* plant-microbe interactions between two microorganisms (*Pseudomonas oryzihabitans* PGP01 and *Cladosporium ramotenellum* PGP02) with plant growth promoting ability and the *Prunus* commercial rootstock Rootpac 20® (RP-20). In this interaction, our attempts were destined to study if these microorganisms were able to control the growth of endophytic contaminants existing in plants without negatively affecting growth. In addition, their effect on the hormonal status was also considered in order to elucidate the mechanism of action of these microorganisms.

## 2. Material and methods

### *2.1. In vitro plant material*

The experiments of the study were conducted using shoot-tip cultures of the *Prunus* rootstock Rootpac®20 (RP-20) (Agromillora Group, Spain). This rootstock constitutes a natural hybrid between the "Myrobalan" plum (*Prunus cerasifera* Ehr.) and an almond (*Prunus dulcis*). RP-20 rootstock was selected for this study due to the presence of natural endophytic contaminants. In the present research, RP-20 rootstock *in vitro* micropropagated explants were destined to two independent experiments: a study on the use of GreeTray® bioreactor for the micropropagation of RP-20 explants, as well as its use as a system to monitor the interaction between two PGPMs (*P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02) and RP-20 plantlets. In this experiment, the evolution of the two PGPMs in this micropropagation system, and how their co-culture influenced the development of RP-20 plantlets were studied. In addition, these two PGPMs were proven for its ability to control the growth of endophytic contaminants. Finally, the hormonal changes in the culture medium mediated by the inoculation with the two microorganisms were considered.

### *2.2. Micropropagation of RP-20 in GreenTray® bioreactor*

In this experiment, the main goal was to compare the efficiency of the GreenTray® bioreactor with the conventional micropropagation method in agar-containing medium. For that reason,

132 micropropagation in 770 mL glass flasks with polypropylene plastic lid (Apiglass, Barcelona, Spain)
133 containing semisolid medium were used as the standard culture conditions described earlier (Iglesias et
134 al., 2004). Murashige and Skoog (MS) (Murashige and Skoog, 1962) supplemented with 3% sucrose and
135 5 μM 6-Benzylaminopurine (BAP), pH to 5.7, agar (7 g $L^{-1}$) and autoclaved at 121 °C for 20 minutes was
136 used for flasks experiments. In the GreenTray bioreactor culture system, the same MS+5BAP medium,
137 but without agar, was used. In both culture systems, explants were kept during 3 weeks in MS+5BAP
138 medium, and then in MS without BAP medium, to promote shoot elongation, for an additional 3-week-
139 long period.

140 Two-cm-long RP-20 nodal segments, with one or two axillary buds each, derived from shoot tip
141 cultures in the multiplication phase, using semisolid medium, were used as explants to initiate new
142 cultures in the GreenTray® bioreactor or in glass flasks. Twenty-five explants per bioreactor and 10
143 explants per flask, with three replicates per treatment were used. All cultures were kept at 24±1 ºC under
144 a photoperiod of 16h of cool-white fluorescent light (140 μmol $m^{-2}$ $s^{-1}$), and 8h darkness. The
145 GreenTray® bioreactor was set at an immersion frequency of 2 minutes every 6 hours. Three weeks later,
146 the culture medium in the bioreactor was replaced with fresh medium of the same composition but
147 without BAP. In the case of flasks, 50 mL of this liquid medium without BAP was dispensed to each
148 flask, using a sterile pipette, containing the shoots growing in 100 mL semisolid medium with BAP. For
149 shoot multiplication evaluation, in the case of flasks with semisolid medium, shoot clumps were moved
150 out with the help of forceps, and in the case of the GreenTray® bioreactor, were all moved at once by
151 pooling out the tray holding the plant material. Individual shoots were separated from the clumps with the
152 help of scalpel and forceps. The number of new shoots from each initial explant, their length in cm, fresh
153 weight in grams, and multiplication rate were determined for each container.

154 *2.3. GreenTray® bioreactor as a system for the plant-PGPMs interaction study*

155 As previously said the GreenTray® bioreactor, based on the principle of TIS, was used in this
156 research as a system to evaluate the biological control activity of two fruit tree PGPMs, *P. oryzihabitans*
157 PGP01 and *C. ramotenellum* PGP02. Both microorganisms were selected for their ability to promote *in*
158 *vitro* plantlet growth and development of different *in vitro* fruit tree plant material (Cantabella et al.,
159 2021). On the other hand, since in the GreenTray® bioreactor, micropropagated explants and culture
160 medium are placed in separated vessels, this system allowed an easy sampling process. Firstly, RP-20

micropropagated explants in MS+5BAP semisolid medium were transferred to the same medium without BAP to promote shoot elongation. In this case, the experiment was performed using *in vitro* rooted plantlets as some phytohormones such as auxins and CKs are involved in root events (Sokolova et al., 2011; Zamioudis et al., 2013). For this reason, 3-cm-long shoots were cultured in ½ MS medium supplemented with 10-µM indole-3-butyric acid (IBA) for one week in darkness for root induction. Before initiating root elongation, RP-20 IBA-treated shoots, with root primordia initiated in the shoot basis, were randomly transferred to each GreenTray® bioreactor 48 h prior microbial inoculation. Cultures in the bioreactor were kept 48 h with the presence of only MS medium, with 3% sucrose and pH adjusted to 5.7, to ensure the release of endophytes to the culture medium. After 48 h of culture, microbial inoculation took place following two independent repetitions. In a first trial, the inoculation of GreenTray® bioreactors containing RP-20 explants took place by adding 3 mL of *P. oryzihabitans* PGP01 or *C. ramotenellum* PGP02 suspensions at $1x10^3$ CFU $mL^{-1}$ and $1x10^5$ esp $mL^{-1}$, respectively. On the other hand, in a second trial, GreenTray® bioreactors were only inoculated with *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$. However, in the latter case, MS media at pH 5.7 (MS 5.7) and 7 (MS 7) were used to study how the pH may affect the interaction of *P. oryzihabitans* PGP01 and RP-20 *in vitro* plantlets. The pH of the medium was adjusted to 5.7 or 7 before autoclaving at 121ºC during 20 min. In the latter case the pH was buffered with the addition of 0.06% 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. All media were then autoclaved at 121ºC during 20 min, and cooled down before use. Three bioreactors per treatment were set up containing fifteen RP-20 shoots each, and the co-culture in the presence of the two PGPMs was maintained during 15 days.

### *2.4. Inocula preparation*

Two microorganisms, *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 in the first trial, and *P. oryzihabitans* PGP01 in the second trial were used to inoculate the bioreactors containing RP-20 shoots. Bacterial inoculum was prepared from 48 h-old *P. oryzihabitans* PGP01 plates grown in nutrient yeast dextrose agar (NYDA: nutrient broth, 8 g $L^{-1}$; yeast extract, 5 g $L^{-1}$; anhydrous glucose, 10 g $L^{-1}$; and agar, 15 g $L^{-1}$). *P. oryzihabitans* PGP01 cells were collected in phosphate buffer (70 mL $KH_2PO_4$ 0.2 M; 30 mL $K_2HPO_4$ 0.2 M; 300 mL of deionized water). Bacterial cell concentration was estimated by measuring the absorbance at 420 nm, and the final concentration was set up with sterile distilled water at $1x10^3$ colony forming unit per mL (CFU $mL^{-1}$) in the first trial, and $1x10^6$ CFU $mL^{-1}$ in the second trial.

For *C. ramotenellum* PGP02, a concentrated suspension from 14 d-old plates grown on potato dextrose agar (PDA: potato tissue, 200 mL; glucose, 20 g $L^{-1}$; and agar, 20 g $L^{-1}$) was used for the preparation of the fungal inoculum used in the first trial. Conidia of *C. ramotenellum* PGP02 of this suspension were measured using a haemocytometer, and the concentration was ultimately set to $1x10^5$ spores per mL (esp $mL^{-1}$) with sterile distilled water. The inoculation of GreenTray® bioreactors took place by the application of 3 mL of microbial inoculum to each bioreactor.

### *2.5. Microorganisms population dinamics*

As in GreenTray® bioreactor, explants and culture medium are placed in two separated compartments, the evolution of the microorganisms *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02, as well as endophytic contaminants naturally existing in RP-20 explants could be easily monitored. Regarding sampling, 2 mL were taken from the culture medium vessel at 0, 1, 2, 5, 7, 9, 12, 15 days post-inoculation (dpi), and the CFU $mL^{-1}$ for each microorganism was determined using dilution plate technique in NYDA medium. In addition, the endophytic microbe population was calculated in microbe-treated and non-treated bioreactors for the two trials.

### *2.6. Gas content, biometrical measurements and chlorophyll content*

In parallel to the determination of the evolution of microorganisms, the $CO_2$ and $O_2$ curves were monitored in control and PGPM-treated bioreactors for the different trials, by air sampling through one of the two ventilation ducts, protected from external contamination by a 0.22 µm filter unit, the GreenTray® is provided with. Measures were taken every two hours during 12 h using the portable gases analyser Dansensor® CheckPoint3 (AMETEK Instruments, Barcelona, Spain). On the other hand, after 15 days of co-culture, RP-20 plants from control and inoculated bioreactors following the two trials were removed for the measures of plant fresh weight (FW) in g, and the number of roots, which were measured as previously described in Cantabella et al (2020). For chlorophyll determination, 0.04 g of leaves were taken from RP-20 *in vitro* explants cultured 15 days in the absence or presence of the two microorganisms in the case of the first trial, as well as in the presence of *P. oryzihabitans* PGP01 in MS medium at pH 5.7 or 7, in the second trial, and incubated in 10 mL of 80% acetone (v/v) during 24 h under darkness. After 24 h of incubation, absorbance at 645 and 663 nm was measured using the spectrophotometer SP-2000UV (Shangai Spectrum Instruments Co., Ltd, Shangai, China), and the contents of total chlorophyll

(Chlt), chlorophyll a (Chla) and chlorophyll b (Chlb) were determined as previously described (Arnon, 1949).

*2.7. Determination of the phytohormone content in liquid culture medium*

Thoroughly homogenized samples of 3 mL culture medium samples were measured into 15 mL Falcon centrifuge tubes and spiked with 5 ng of each internal standard, homogenized and frozen at -80°C prior to lyophilizing. The lyophilized samples were re-dissolved with 0.5 mL of water/acetonitrile (98+2) 0.1% formic acid and filtered through 0.22 µm hydrophilic PTFE filter before UHPLC-MS/MS analysis. The standards of phytohormones such as abscisic acid (ABA), indole-3-acetic acid (IAA), $N^6$-isopentenyladenine (iP) and dihydrozeatin (DHZ) and their stable isotopically labeled counterparts [$^2H_6$]-ABA, [$^2H_5$]-IAA, [$^2H_6$]-iP and [$^2H_3$]-DHZ used as internal standards were purchased from OlchemIm Ltd. (Olomouc, Czech Republic). Stock solutions (100 µg $mL^{-1}$) and working solutions (10 µg $mL^{-1}$) of phytohormones and internal standards were separately prepared in methanol (Fisher Scientific, Madrid, Spain). All standard solutions were stored at -80°C. Thus, a solution of the mixture of phytohormones and another mixture of internal standards at 1 µg·$mL^{-1}$ in water/acetonitrile (Fisher Scientific, Madrid, Spain) (98+2) 0.1% formic acid (Fisher Scientific, Madrid, Spain) were prepared. A six-point calibration curve was obtained in water/acetonitrile (98+2) 0.1% formic acid to cover 0.5 – 50 ng $mL^{-1}$ range with 10 ng·$mL^{-1}$ of internal standards. Ultrapure Milli-Q water ($H_2O$) was obtained by Synergy UV (Merck KGaA, Darmstadt, Germany). UHPLC-MS/MS (ultra-high performance liquid chromatography tandem mass spectrometry) analysis was performed on Waters Acquity UPLC binary system coupled to a Xevo TQ-S triple-quadrupole mass spectrometer equipped with an ESI source (Waters, Milford, MA, USA). A Waters Acquity UPLC® HSS T3 1.8 µm 2.1 × 100 mm column (Milford, MA, USA) was used for chromatographic separation. A water/acetonitrile (98+2) 0.1% formic acid solutions was used as the mobile phase A and acetonitrile was used as the mobile phase B with gradient elution (Table S1). The flow rate was 0.250mL min-1. The sample injection volume was 2.5µL and the column oven temperature was kept at 40°C. The sample was separated for 10 min. The tandem mass spectrometer (MS/MS) was operated in ESI positive/negative mode and data were acquired in the multiple reaction monitoring (MRM) mode (Table S2). The MS/MS parameters were set as follows: Capillary voltage 3000V/-2.500V, source temperature 150°C, desolvation temperature 350°C, desolvation gas (nitrogen) flow 800 L/h, cone

gas (nitrogen) flow 150L/h and collision gas (argon) flow 0.15 mL/min. Acquired data were processed by MassLynx™ MS Software with TargetLynx™ program version 4.1 (Waters, Milford, MA, USA).

### *2.8. Statistical analysis*

All the experiments were repeated at least three times for confirmation of the results. The experiment was designed considering a completely random design (CRD), and data analysis were carried out by using JMP Pro Software (version 13.1.0, SAS Institute Inc., Cary, NC). Statistical significance was judged at $P < 0.05$, and the Tukey test was used to separate means when the differences were statistically significant.

## 3. Results

### 3.1. Shoot proliferation in the GreenTray® bioreactor versus conventional cultures in flasks

Comparing the multiplication rates of RP-20 in the GreenTray® temporal immersion system bioreactor (Figure 1A) with the culture system in flasks with semisolid agar-containing medium, although differences in the multiplication rates were observed, after the 6-week-long culture process (Figure 2A), no statistically significant differences were found for RP-20. As shown in Figure 1B and C, shoot development after culture in the two culture systems were comparable. Moreover, significant statistical differences were observed on the shoot length and the fresh weight per shoot between both culture systems (Figure 2B, C). RP-20 rootstocks were almost 50% longer when growing in the GreenTray® bioreactor than in flasks with semisolid medium (Figure 2B), which was in concordance with a 28% increase in the shoot fresh weight per new shoot (Figure 2C).

### 3.2. Interaction of RP-20 explants and PGPMs in GreenTray® bioreactor

In this experiment, a TIS bioreactor was used for the first time as a system to evaluate the interaction between *in vitro* micropropagated plantlets and PGPMs.

#### 3.2.1. Growth of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and endophytic contaminations in GreenTray® bioreactor

The evolution of the PGPMs under study (*P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02) in the first trial is shown in Figure 3A. In the first trial, where both *P. oryzihabitans* PGP01 and *C.*

*ramotenellum* PGP02 were used to inoculate GreenTray® bioreactors containing RP-20 explants, it was found that the bacterium *P. oryzihabitans* PGP01 peaked its growth after 1 dpi, achieving a 5.66 Log CFU $mL^{-1}$ (Figure 3A). After this period, bacterial growth decreased, showing the lowest growth after 5 dpi (2.25 Log CFU $mL^{-1}$). Bacterial cell population remained practically unchanged from 5 dpi until the end of the process, ranging from 2.56 to 2.98 Log CFU $mL^{-1}$ at 7 and 15 dpi, respectively (Figure 3A). Regarding *C. ramotenellum* PGP02, although a 1.86 Log CFU $mL^{-1}$ reduction of growth was observed after 1 dpi, fungal population rapidly recovered and reached a maximum of 3.95 Log CFU $mL^{-1}$ (Figure 3A). *C. ramotenellum* PGP02 stabilized after this period, and at the end of the process, 3.41 Log CFU $mL^{-1}$ was observed (Figure 3A). On the other hand, populations dynamics of endophytes in GreenTray® bioreactors non-treated and treated with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 at $1x10^3$ CFU $mL^{-1}$ and $1x10^5$ esp $mL^{-1}$, respectively, were registered and represented in Figure 3B. The co-culture of RP-20 explants with *P. oryzihabitans* PGP01 in GreenTray® bioreactors did not reduce the endophytes population as significant differences in Log CFU $mL^{-1}$ were not found between both treatments (Figure 3B). However, the inoculation with the fungal isolate *C. ramotenellum* PGP02 drastically reduced contaminant population, and no endophytes were counted after 5 dpi (Figure 3B).

#### 3.2.2. Effects of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 in the content of $CO_2$ and $O_2$ in the vessel of the GreenTray® bioreactor

Along with the population dynamics of PGPMs and endophytes, the evolution of the $CO_2$ and $O_2$ within the plant material vessel of the control and PGPM-treated GreenTray® bioreactors was registered during the whole process. In the first trial, it is noteworthy to mention that while the content of both $CO_2$ and $O_2$ was not altered in the presence of *P. oryzihabitans* PGP01 at $1x10^3$ CFU $mL^{-1}$, a huge production of $CO_2$ in the presence of *C. ramotenellum* PGP02 at $1x10^5$ esp $mL^{-1}$ was registered from 2 dpi to the end of the co-culture process, being the highest level after 6 dpi (Figure 4A). This large production of $CO_2$ was accompanied by a consumption of $O_2$ in the same periods of time (Figure 4B).

#### 3.2.3. Effects of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 in RP-20 *in vitro* plant growth and chlorophyll content

After 15 days of co-culture with *P. oryzihabitans* PGP01 no significant changes in the plantlets FW of RP-20 were observed in the first trial (Figure 5A). In contrast, *C. ramotenellum* PGP02 negatively

affected RP-20 growth, observing a decrease of about a 26% in this parameter (Figure 5A). Although no effects on plant growth were observed in RP-20 plant FW in response to the inoculation with *P. oryzihabitans* PGP01, a 29% decrease in the number of roots was recorded after 15 days of co-culture (Figure 5B), being this decrease even more pronounced in *C. ramotenellum* PGP02-treated RP-20 plants (Figure 5B).

Concerning the chlorophyll content observed in RP-20 leaves cultured in GreenTray® bioreactor treated or not with PGPMs, significant changes in the content of Chlt (Figure 5C), Chla and Chlb (data not shown) were not registered in response to the inoculation with *P. oryzihabitans* PGP01 at $1x10^3$ CFU $mL^{-1}$ when compared to non-treated RP-20 plants (Figure 5C). The negative effects of *C. ramotenellum* PGP02 observed in plant growth were also correlated with an important decrease in the content of Chlt, Chla and Chlb (64, 67 and 53%, respectively) in comparison to control GreenTray® bioreactors after 15 days of co-culture (Figure 5C).

3.2.4. Growth of *P. oryzihabitans* PGP01 and endophytic contaminations in GreenTray® bioreactor using MS medium at different pHs

Effects of inoculating GreenTray® with *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$ depended on the pH of the MS medium. In general, bacterial growth was favored in the medium MS pH 7 as significantly higher values of bacterial growth were observed at several points of the co-culture process (Figure 6A). During the most active bacterial growth period, thus mean, between 0 and 2 dpi, 7.45 and 7.31 Log CFU $mL^{-1}$ were reached at 1 and 2 dpi, respectively, when *P. oryzihabitans* PGP01 was cultured in the medium MS at pH 7 (Figure 6A). It is important to remark that a characteristic curve showing bacterial growth was obtained when *P. oryzihabitans* PGP01 was cultured at pH 7. On the contrary, at the same period, significantly lower values of *P. oryzihabitans* PGP01 (5.92 and 5.69 Log CFU $mL^{-1}$) were observed at pH 5.7 (Figure 6A). Furthermore, endophytic population existing in RP-20 rootstocks cultured in GreenTray® bioreactors in the presence or absence of *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$ also displayed a different response when the pH of the MS medium was adjusted to 5.7 (Figure 6B) or 7 (Figure 6C). In this sense, a very slight reduction of the endophytic population in the presence of *P. oryzihabitans* PGP01 in GreenTray® bioreactor with medium MS 5.7 occurred; however, these reduced values were only significant after one and 7 dpi (Figure 6B). Conversely, the inoculation of GreenTray® bioreactors with the medium MS pH 7 with *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$

somewhat promoted endophytes development in comparison to non-treated GT bioreactors although no statistically significant differences were found at any sampling period (Figure 6C).

### 3.2.5. Effects of *P. oryzihabitans* PGP01 cultured at different pHs in the content of $CO_2$ and $O_2$ in the vessel of the GreenTray® bioreactor

When only *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$ was used for the inoculation of GreenTray® bioreactors, the pH of the MS medium differently affected the content of $CO_2$ and $O_2$.in the vessel atmosphere containing the plantlets (Figure 1A). The inoculation of GreenTray® bioreactors containing RP-20 explants with *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$ did not alter the $CO_2$ and $O_2$ content when the medium pH was 5.7 (Figure 7A, B). Nevertheless, the response was quite different when the inoculation of this bacterium took place using the medium adjusted at pH 7 (Figure 7C, D). In this case, a significant production of $CO_2$ compared to non-treated GT bioreactors was observed during the first 48 h of bacterial growth (Figure 7C). On the other hand, during the same period, a significant reduction on the $O_2$ content was also observed (Figure 7D).

### 3.2.6. Effects of *P. oryzihabitans* PGP01 on plantlet growth and chlorophyll content of RP-20 plantlets cultured at different pHs

It is important to remark that, in the absence of the bacterium *P. oryzihabitans* PGP01, the culture of RP-20 *in vitro* plantlets in the medium MS at pH 7 negatively affected plant growth, leading to significant reductions of several growth parameters such as plant shoot and root FW, number of leaves, and root length (data not shown). When *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$ was co-cultured for 15 days with RP-20 in the medium MS at pH 5.7, no significant changes in plantlet FW occurred (Figure 8A). However, RP-20 plantlets from GreenTray® bioreactors inoculated with *P. oryzihabitans* PGP01 displayed a 53% increase in the number of roots after 15 days of co-culture (Figure 9B). When the co-culture took place in the medium MS at pH 7, the co-culture with *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$ did not promote changes neither in the plant FW nor in the number of roots of RP-20 *in vitro* plantlets (Figure 8C, D).

Conversely, the culture of RP-20 *in vitro* plantlets with *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$ induced a slight but significant increase in the content of Chlt and Chla (11 and 12%, respectively), but not Chlb (Figure 7A) when the liquid MS medium was adjusted at pH 5.7 (Figure 9A). The

inoculation of GT bioreactors with medium MS at pH 7 with *P. oryzihabitans* PGP01 did not significantly change the content of Chlt, Chla and Chlb (Figure 9B).

#### 3.2.7. Effects of *P. oryzihabitans* PGP01 and pH on the hormonal content of the culture media

The inoculation of GreenTray® bioreactors with PGPMs also promoted changes in the content of iP, DHZ, ABA and IAA in the culture medium. In the presence of $1x10^3$ CFU $mL^{-1}$ of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 at pH 5.7, hormones levels in the culture medium were so low that could not be detected (data not shown). A higher inoculum concentration of *P. oryzihabitans* PGP01 in GreenTray® bioreactors did not produce significant differences in the levels of DHZ and ABA at pH 5.7 compared to control (data not shown). In fact, DHZ was not detected for neither control nor *P. oryzihabitans* PGP01-treated bioreactors under these conditions (data not shown). However, the inoculation with *P. oryzihabitans* PGP01 at $1x10^6$ CFU $mL^{-1}$ and pH 5.7 reduced (3-fold decrease) the IAA levels compared to non-treated bioreactors (Figure 10). Furthermore, the behavior of *P. oryzihabitans* PGP01 in GreenTray® bioreactors at pH 7 altered the levels of iP in the culture medium, observing a significant 1.47-fold increase compared to non-treated GT bioreactors (Figure 10). The CK DHZ was not detected in the culture medium inoculated *P. oryzihabitans* PGP01 at pH 7 (data not shown). IAA levels were not significantly changed in the presence of the bacterium *P. oryzihabitans* PGP01 at pH 7 (Figure 10).

## 4. Discussion

The results demonstrated the feasibility of the recently patented GreenTray® bioreactor for the *in vitro* micropropagation and monitoring of the interaction between RP-20 plantlets and the two PGPMs *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02. So far, studies concerning the application of beneficial microorganisms to improve *in vitro* plant performance have been conducted by directly inoculating the base of the explant (Larraburu et al., 2010; Perez-Rosales et al., 2018) or by incubation of *in vitro* shoots in microbial suspensions (Della Mónica et al., 2018; Quambusch et al., 2014). In all the above cases, *in vitro* plantlets as well as beneficial microorganisms were developed in the semisolid agar media commonly used in conventional micropropagation methods. Therefore, this investigation represents a breakthrough in the field of plant-microbe interactions exploiting a plant propagation system

based on liquid culture principles as a system to host microorganisms with potential effects on plant development. Nowadays, there are available different TIS bioreactors conformations used in commercial and research propagation including RITA® (Teisson et al., 1996), SETIS® (Bello-Bello et al., 2019), TIB® (Escalona et al., 1999) and MATIS® (Etienne et al., 2013), being SETIS® and RITA® the most widely implemented. In the case of our study, the new TIS bioreactor GreenTray®, developed by Dolcet-Sanjuan and Mendoza (2018), was used to analyze the evolution of the interaction between RP-20 *in vitro* plantlets and both PGPMs, *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02. This bioreactor has proven to be effective in improving the performance of *in vitro* fruit tree RP-20 rootstocks micropropagation as longer shoots were obtained, which would also explain the increase in shoot FW observed after the culture on this system. The multiplication rate, although slightly higher, was not significantly enhanced by the cultivation of RP-20 shoots in GreenTray® bioreactors. In line with this approach, a similar response was reported in three ornamental species (*Digitalis*, *Echinacea* and *Rubus*) in a TIS bioreactor, observing a higher FW of *Echinacea* and *Rubus* shoots cultured in TIS bioreactor compared to those cultured in agar medium (Welander et al., 2014). The proven effectiveness of the TIS bioreactors in increasing the throughput of the *in vitro* micropropagation process had led to many authors focusing on *in vitro* woody plant species to develop profitable micropropagation protocols. For instance, it has been reported that the culture of *in vitro* banana (*Musa* spp.) explants in TIS bioreactors led to an increased multiplication rate and higher survival in acclimatization conditions in comparison to the culture in semi-solid agar medium (Bello-Bello et al., 2019). In spite of the multiple advantages of the TIS bioreactors in *in vitro* micropropagation, the number of studies concerning the use of TIS bioreactors to monitor the interaction between in vitro plantlets and beneficial microorganisms are scarce in literature. For that reason, a complete experiment where the interaction between individual *in vitro* RP-20 shoots, prior rooting elongation, and the two PGPMs *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 was studied using GreenTray® bioreactors. In this study, the three major objectives explored were (1) the performance of the two PGPMs and RP-20 plantlets in a TIS bioreactor, (2) the ability of these two PGPMs to control the growth of endophytic contaminations naturally habiting RP-20 explants, and (3) the study of the effect of the application of the two PGPMs in the hormonal status of the culture medium. Two independent experiments were conducted, modifying the culture conditions or the microbial inoculum in order to meet all these objectives.

Regarding the performance of PGPMs and RP-20 *in vitro* plantlets in the presence of *C. ramotenellum* PGP02 and *P. oryzihabitans* PGP01, it was observed that, in general, *P. oryzihabitans* PGP01 did not importantly altered RP-20 growth, possibly attributed to the low inoculum concentration. In contrast, the conditions given in the plant culture vessel of the GreenTray® bioreactors inoculated with *C. ramotenellum* PGP02 negatively affected *in vitro* RP-20 plant development as a considerably reduced plant FW was registered after 15 days of co-culture. In these bioreactors, a high production of $CO_2$ was observed in the plant culture vessel in response to the inoculation with *C. ramotenellum* PGP02 after 2 dpi together with a concomitant decrease in the $O_2$ content. These conditions were associated with a *C. ramotenellum* PGP02 overgrowth that disrupted *in vitro* plantlet development. However, these changes in the content of these two gases may be caused by the use of the sucrose present in the culture medium as a substrate for fungal metabolism as previously reported by many authors (Brannon, 1923; Mason and Righelato, 1976). It is widely known that $CO_2$ has an important role in both *in vivo* and *in vitro* culture, increasing the rate of photosynthetic carbon fixation rates and improving the performance of plants on the acclimatization stage (Pérez-Jiménez et al., 2015). In fact, the enrichment of the environment with $CO_2$ constitutes the basis for the establishment of a photoautotrophic culture leading to the removal of sugars from the culture medium (Xiao et al., 2011). However, $CO_2$ concentration present in the culture vessel must be strictly controlled as high levels of $CO_2$ lead to growth disturbances in some plant genotypes (Tisarum et al., 2018). In this study, this high production of $CO_2$ within the culture vessel could also explain the reduced values of the chlorophyll content observed in the leaves of RP-20 plantlets at the end of the culture process. Long term exposure to high concentrations of $CO_2$ may seriously affect the photosynthesis process by a negative adaptation of the enzyme RUBISCO, resulting in a reduction in the chlorophyll content (Arigita et al., 2002). On the other hand, endophytic contaminant population was determined in GreenTray® bioreactors together with the growth of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02. Endophytic contaminations in *in vitro* cultures are often responsible of high losses of plant material due to that bacterial growth may overrun the cultures reducing micropropagation rates (Leifert and Cassells, 2001). Most of the studies published in literature have reported the efficiency of antibiotics such as gentamicin, tetracycline or tobramycin in the successful elimination of endophytes growth (Fang and Hsu, 2012; Khan et al., 2018). Moreover, other authors have proposed the application of the Plant Preservative Mixture™ (PPM) in the suppression of the growth of some bacterial endophytes species belonging to *Sphingomonas* genus (Lotfi et al., 2020; Miyazaki et al., 2010). In this study, the

446 results obtained in this research may suggest the role of the pH of the culture medium in the modulation
447 of the biological control activity of the two tested PGPMs. In the presence of *C. ramotenellum* PGP02
448 drastically suppressed the growth of endophytic contaminants after 5 dpi, but associated to the detriment
449 of plantlet growth. After 7 days of co-culture, *C. ramotenellum* PGP02 decreased the pH of the MS
450 medium up to approximately 2.6, making difficult the growth of bacterial contaminants. In contrast, the
451 low inoculum concentration of *P. oryzihabitans* PGP01 used in this first trial did not promote important
452 changes in the endophytes populations as no important changes in the pH of the MS medium were
453 recorded regarding non-treated bioreactors.

454 As the initial pH of the culture medium has been shown to be a key factor to ensure bacterial
455 growth, in a subsequent experiment, GreenTray® bioreactors were inoculated solely with
456 *P. oryzihabitans* PGP01 at pH 5.7 and 7, which is the optimum for bacteria growth, to analyse if a better
457 bacterial growth induced the effects in roots reported in previous studies (Cantabella et al., 2021, 2020).
458 As expected, a better growth of *P. oryzihabitans* PGP01 was registered during the whole process when
459 co-culturing with RP-20 plants at pH 7. These results are also supported by the decrease of $O_2$ and the
460 increase of $CO_2$ observed during the most active growth of this bacterium, revealing a higher activity at
461 pH 7. Nevertheless, the greater performance of *P. oryzihabitans* PGP01 did not result in an improvement
462 of RP-20 growth explants after 15 days of co-culture, mostly due to the negative effect of pH 7 on plantlet
463 growth. This factor is crucial not only for bacterial growth but also for plantlet development as it is
464 known that the optimum pH for *in vivo* plants ranges between 4.2 and 6.5 (Leifert et al., 1992).
465 Surprisingly, the best results in terms of plant development were obtained when RP-20 explants and *P.*
466 *oryzihabitans* PGP01 coexist in the medium MS at 5.7 as a stimulation of root development occurred to
467 by increasing the number of roots as previously reported (Cantabella et al., 2021). In the scientific
468 literature, there are recent evidences available that reports the effect of PGPMs in the chlorophyll
469 pigments (Khanghahi et al., 2019), most of them using this parameter as an indicator of both biotic and
470 abiotic stress alleviation (Arkhipova et al., 2020; Jain et al., 2020; Kour et al., 2020). In this process,
471 chlorophyll do not act alone in this photosynthesis process as $CO_2$ is also involved through its fixation by
472 the RUBISCO enzyme (Parry et al., 2013). In the present study, the inoculation with *P. oryzihabitans*
473 PGP01 induced positive changes in the photosynthetic pigments content, observing an increase in the
474 amount of Chl t mainly provoked by an increase in the Chl *a* content. Thus, the results herein presented
475 might suggest that *P. oryzihabitans* PGP01, as a PGPM, could stimulate the photosynthesis process.

However, in the GreenTray® bioreactors inoculated with this bacterium, similar evolution of the percentage of $CO_2$ was recorded in comparison to those non-treated bioreactors. Therefore, if the higher chlorophyll content observed in RP-20 leaves after 15 days of co-culture in the presence of *P. oryzihabitans* PGP01 is related to a better photosynthetic performance remains unclear, requiring further investigations to fully understand this relationship. For instance, the inoculation with a mixture of PGPMs lead to a higher photosynthetic rate in plants by the improvement of leaf anatomical attributes (Paradiso et al., 2017). In this study, authors did not report differences in the levels of leaf chlorophyll in the presence of this PGPMs mixture. Higher inoculum concentration of *P. oryzihabitans* PGP01 at the same pH 5.7 either produced an evident control of endophytes growth. In addition, the increase of the initial pH of the MS medium up to 7, more suitable for *P. oryzihabitans* PGP01 growth, also favoured endophytes performance, slightly surpassing *P. oryzihabitans* PGP01 growth. Considering these results, endophytic bacteria population existing in RP-20 *in vitro* displayed a dynamic behaviour, being the pH the key factor modulating their population. Endophytes in micropropagated plants have been shown as dynamic microorganisms, adapted to *in vitro* conditions and displaying fluctuations in populations over the years of culture (Quambusch et al., 2016). In the case of our study, future experiments will be needed to corroborate if these two PGPMs are able to control the growth of endophytic contaminations in RP-20 *in vitro* cultures.

It is widely reported that root colonization and the subsequent plant growth promotion mediated by PGPMs is commonly associated to changes in the hormonal homeostasis (Tsukanova et al., 2017). For that reason, in the present study, it was decided to analyse how the inoculation of GreenTray® bioreactors with the PGPMs *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 may modify the hormonal status of the culture medium. In this case, it has been demonstrated that the inoculation of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 co-cultured *in vitro* with RP-20 plantlets under different conditions affected the pattern of the different hormones analysed. In general, in the first trial, the application of either the two microorganisms did not promote plant growth of RP-20 *in vitro* plantlets after 15 days of co-culture. Furthermore, the results obtained in terms of plant FW and number of roots in RP-20 plantlets in GreenTray® bioreactors treated with both microorganisms in comparison to those non-inoculated might suggest an induction of a senescence process, as it was revealed by the increase on ABA content. This hormone has been widely associated with the stimulation of senescence signalling (Xie et al., 2004). In contrast, in the second trial, it was found that the inoculation with *P. oryzihabitans* PGP01 at

pH 5.7 only produced a significant decrease in the IAA content in MS medium after 15 days of co-culture with RP-20. Lower auxin levels in the culture medium might help root elongation and root number, and consequently plantlets cultured under these conditions showed higher number of roots than non-treated plantlets. For unravelling the mechanisms underlying *in vitro* plant growth promotion, many authors have paid particular attention in auxins as the main hormones involved in root development (Asghar et al., 2002; Dias et al., 2009; Iqbal and Hasnain, 2013). In a previous study, it has been suggested the IAA-producing ability of *P. oryzihabitans* PGP01 as the responsible of root modifications occurred in RP-20 *in vitro* micropropagated explants after 8 weeks of co-culture (Cantabella et al., 2021). On the other hand, some bacteria belonging to *Pseudomonas* spp. are able to produce auxin-like compounds that triggers auxin signalling stimulating root development (Ortiz-Castro et al., 2020; Zamioudis et al., 2013). In this study, auxins produced during the most active growth period of *P. oryzihabitans* PGP01 could have promoted root induction, explaining the increase in the number of roots observed in RP-20 *in vitro* plantlets. As a result, the higher root surface in RP-20 likely increased auxins uptake throughout the rest of the co-culture process, explaining the lower levels of IAA present in MS medium of *P. oryzihabitans* PGP01-treated bioreactors after 15 days of co-culture. Moreover a lower IAA content in the culture medium could have facilitated root elongation. The negative effects of the increase on the initial medium pH on plant FW could in the absence of *P. oryzihabitans* PGP01 be corroborated by the higher contents of ABA observed in this GreenTray® bioreactors. Under these conditions, RP-20 senescence could be promoted by the presence of *P. oryzihabitans* PGP01 as higher values of this hormone were found in MS medium after 15 days of co-culture.

## 5. Conclusions

To conclude, this study demonstrated the feasibility of the GreenTray® bioreactor for an efficient *in vitro* micropropagation of the *Prunus* rootstock RP-20, increasing the FW and length of the produced explants. In addition, this system has also been effective for the study of the interaction between RP-20 plantlets and PGPMs, especially *P. oryzihabitans* PGP01, inducing a stimulation of root development by an increase in the number of roots. Both PGPMs tested in this study were not able to control endophytes growth; however, the absence of endophytic contaminations observed in the presence of the microorganism *C. ramotenellum* PGP02 suggested the role of low pHs (acidic) in the removal of endophytes in the culture medium. Further experiments will be conducted to study in depth how the pH

might modify endophytes population without affecting in vitro micropropagation in the absence of microorganisms.

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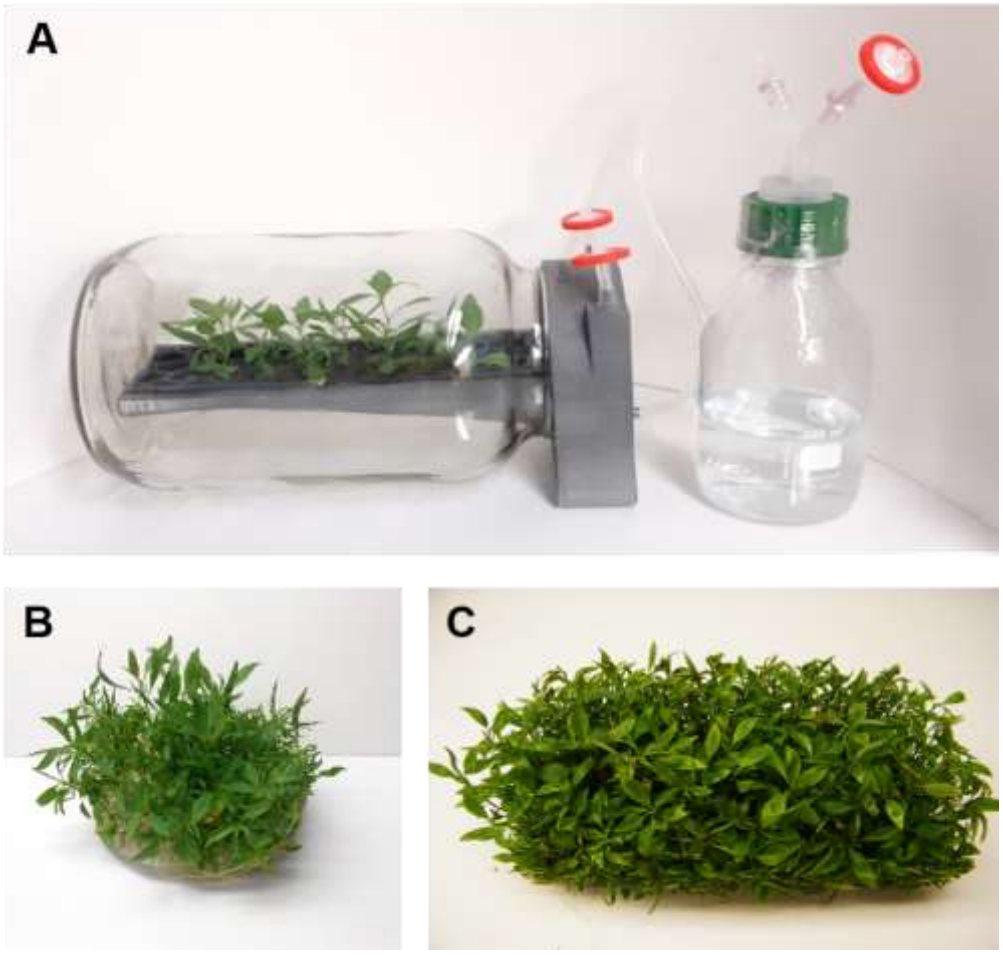
745

746 **FIGURE LEGENDS**




747

748 **Figure 1. Use of GreenTray® TIS bioreactor for *in vitro* micropropagation RP-20 (A), and explants**

749 **appearance in comparison to conventional culture using semi-solid medium with agar (B, C).**

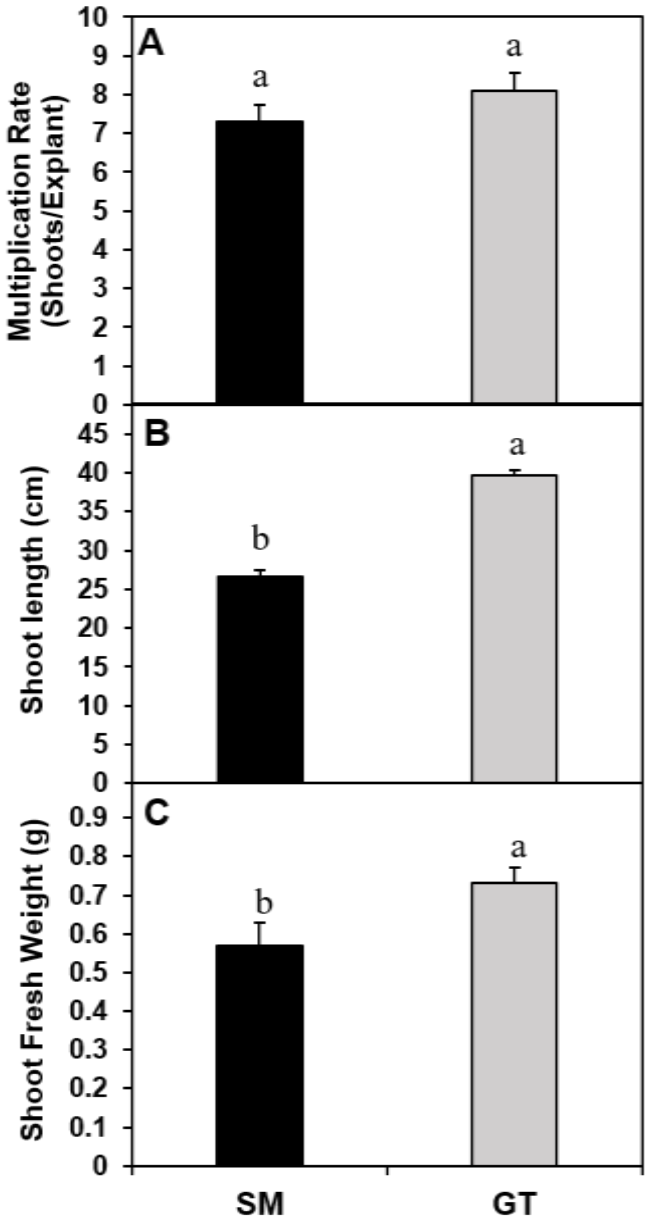



**Figure 2. Effects in the multiplication rate (A), shoot length (B), and shoot FW (C) of RP-20 *in vitro* explants in GreenTray® TIS bioreactor compared to the conventional culture in semi-solid medium (SM) with agar.** Data represents the mean ± SE of the shoots obtained from the 25 and 10 explants initially cultured in the GreenTray® bioreactor and flasks, respectively. Different letters denote significant differences according to Student t test ($P < 0.05$).

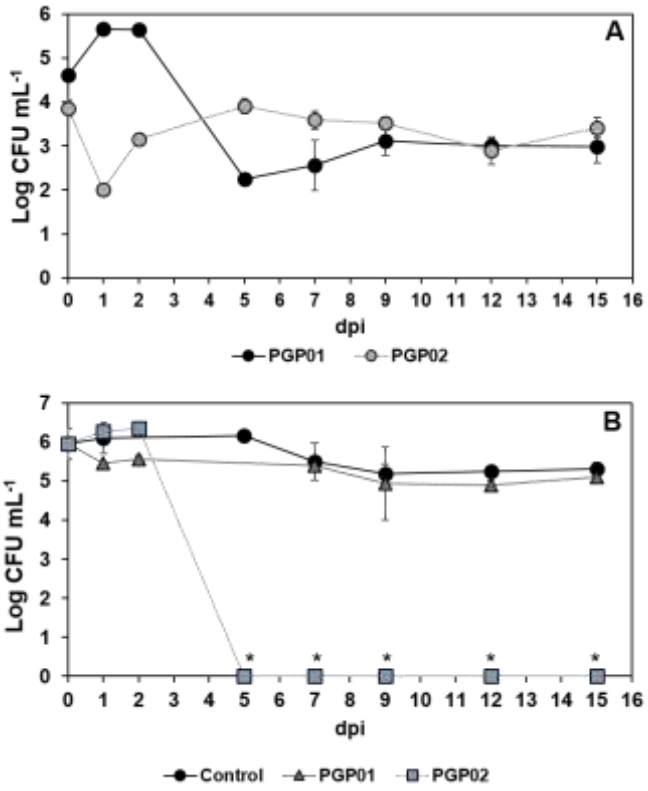
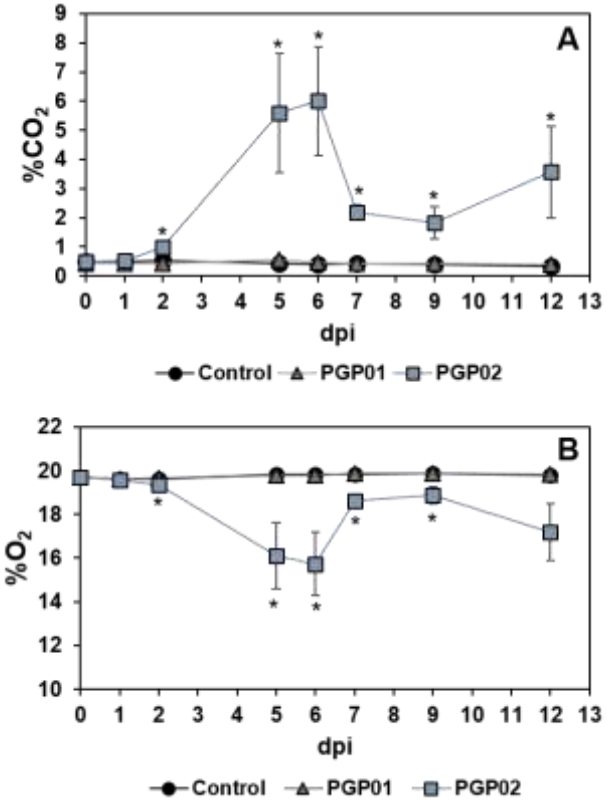
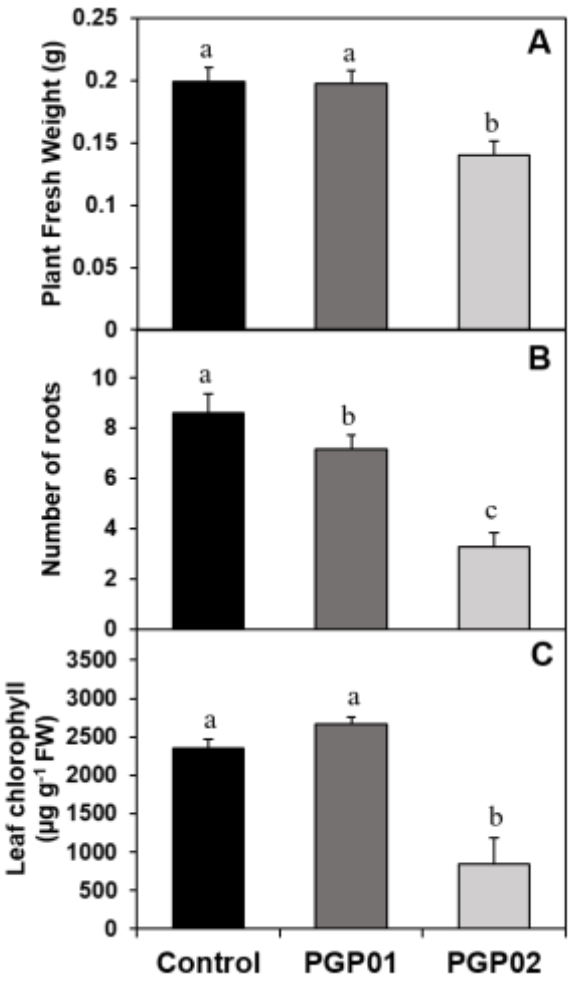



**Figure 3. Dynamics population of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 (A) and bacterial endophytes population (B) in GreenTray® TIS bioreactors inoculated with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 with MS medium at pH 5.7.** Data represents the mean ± SE of at least three replicates. Asterisks (*) symbol indicate significant differences between control and treatments according to Student t test ($P < 0.05$).



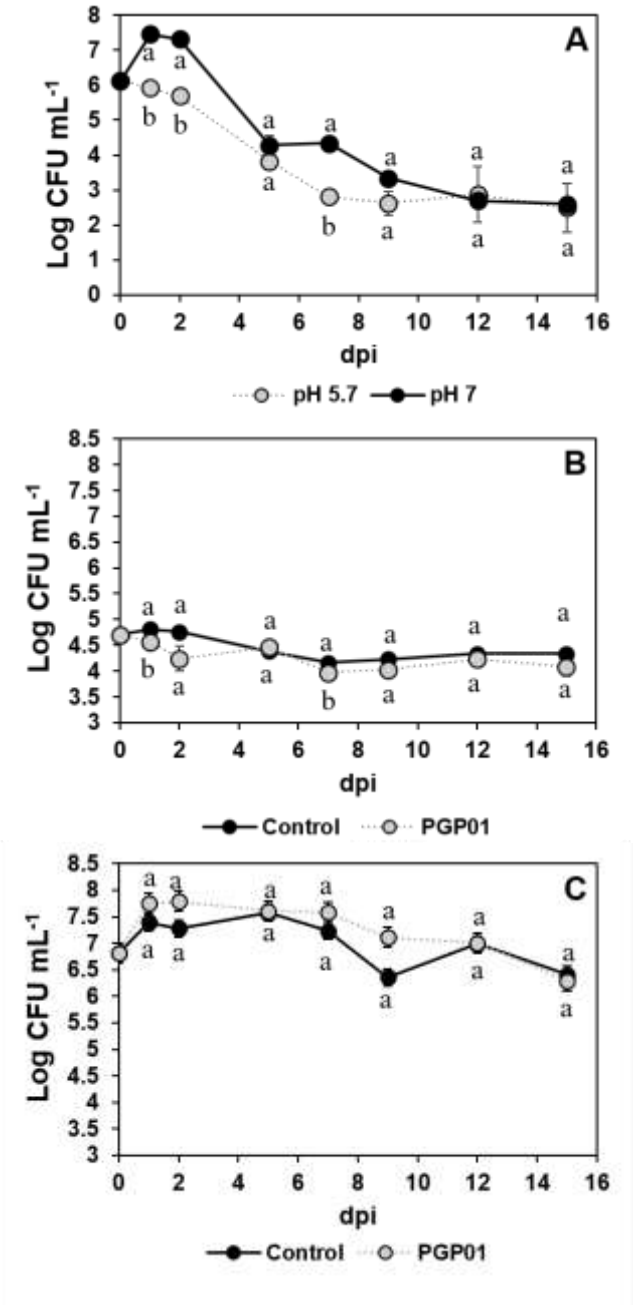

762

763 **Figure 4. $CO_2$ (A) and $O_2$ (B) evolution in the plant culture vessel of GreenTray® TIS bioreactors**
764 **inoculated with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 during 15 days.** Data represents
765 the mean ± SE of at least three replicates. Asterisks (*) symbol indicate significant differences between
766 control and treatments according to Student t test ($P < 0.05$).



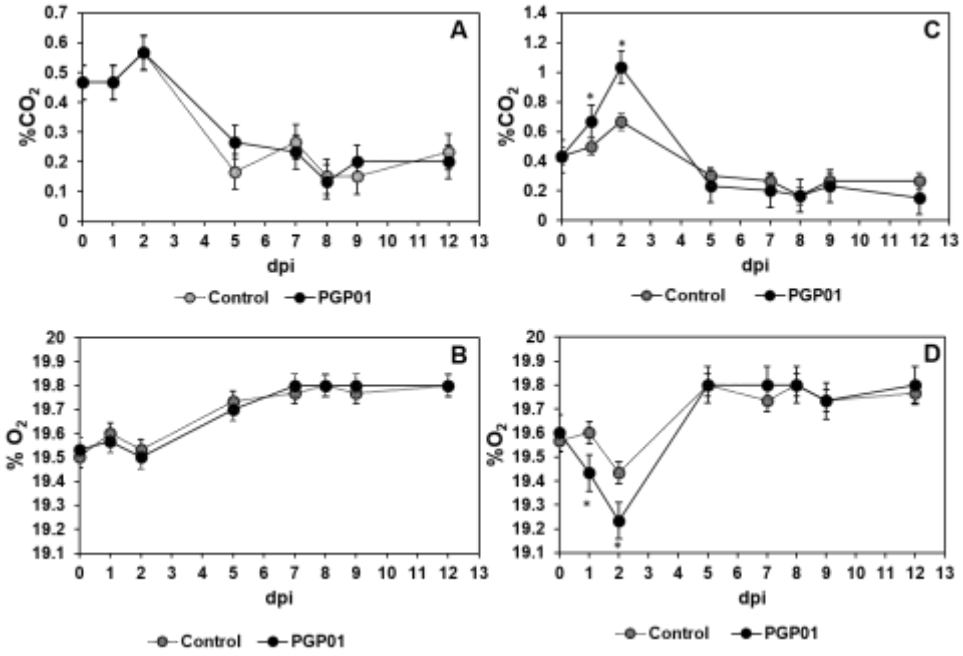

767

768 **Figure 5. Effects on *in vitro* plant FW (A), number of roots (B) and chlorophyll content in leaves (C)**
769 **of RP-20 explants after 15 days of co-culture in GreenTray® TIS bioreactor.** Data of plant FW and
770 number of roots represents the mean ± SE of at least ten plants, and chlorophyll content data represents
771 the mean ± SE of three independent replicates. In all cases, different letters denote significant differences
772 between control and treatments according to Student t test ($P < 0.05$).



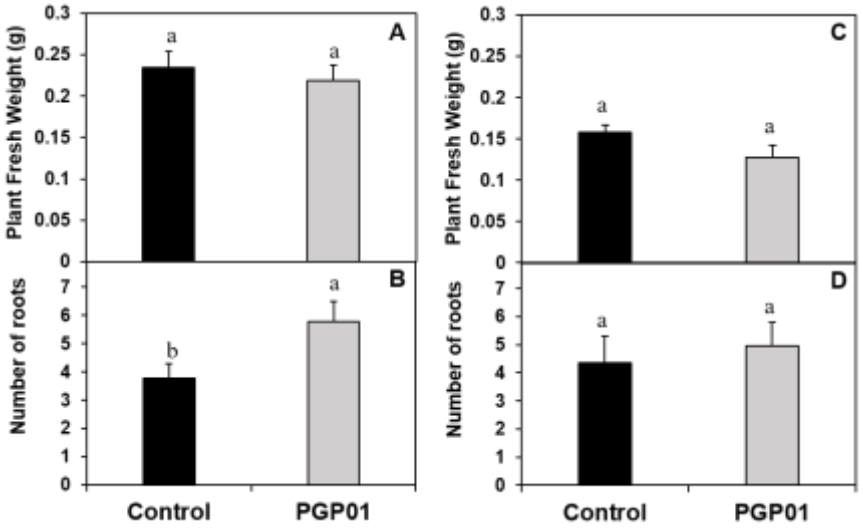

773

774 **Figure 6. Dynamics population of *P. oryzihabitans* PGP01 in GreenTray® TIS bioreactors with MS**
775 **medium at pH 5.7 and 7 (A), and bacterial endophytes population in GreenTray® TIS bioreactors**
776 **at pH 5.7 (B) and 7 (C).** Data represents the mean ± SE of at least three replicates. Different letters
777 represents significant differences between control and treatments according to Student t test ($P < 0.05$).



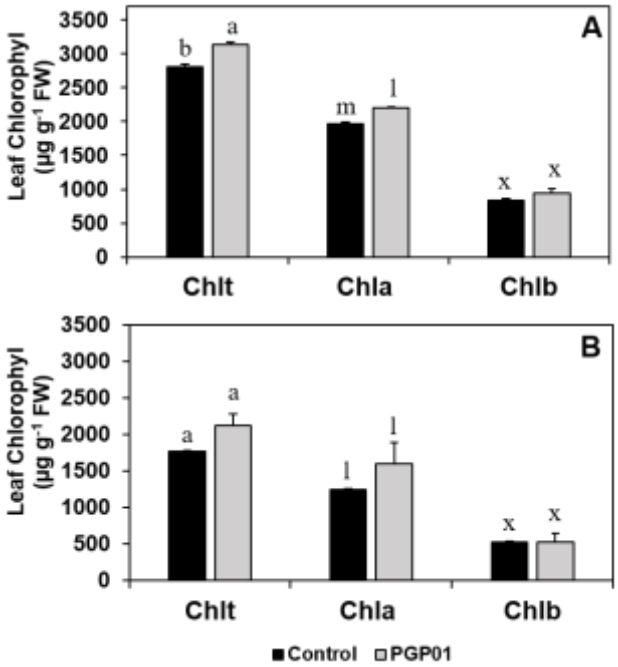

778

779 **Figure 7. Effects on the evolution of the %$CO_2$ and %$O_2$ in GreenTray® TIS bioreactors after the**
780 **inoculation with *P. oryzihabitans* PGP01 in the GreenTray® TIS bioreactor with MS medium at pH**
781 **5.7 (A, B) and 7 (C, D).** Data represents the mean ± SE of at least three replicates. Asterisks (*) symbol
782 represents indicate significant differences between control and treatments according to Student t test ($P <$
783 0.05).




784

785 **Figure 8. Effects on *in vitro* plant FW and number of roots of RP-20 plants after 15 days of co-**
786 **culture in GreenTray® TIS bioreactor with *P. oryzihabitans* PGP01 in MS medium at pH 5.7 (A, B)**
787 **and pH 7 (C, D).** Data represents the mean ± SE of at least ten plants. Different letters denote significant
788 differences between control and treatments according to Student t test ($P < 0.05$).




789

790 **Figure 9. Effects on the content of total chlorophyll (Chl t), chlorophyll *a* (Chl *a*) and chlorophyll *b***
791 **(Chl *b*) in leaves of *in vitro* RP-20 after 15 days of co-culture with *P. oryzihabitans* PGP01 in the**
792 **GreenTray® TIS bioreactor at pH 5.7 (A) and 7 (B).** Data represents the mean ± SE of at least three
793 replicates. Different letters denote significant differences between control and treatments according to
794 Student t test ($P < 0.05$).

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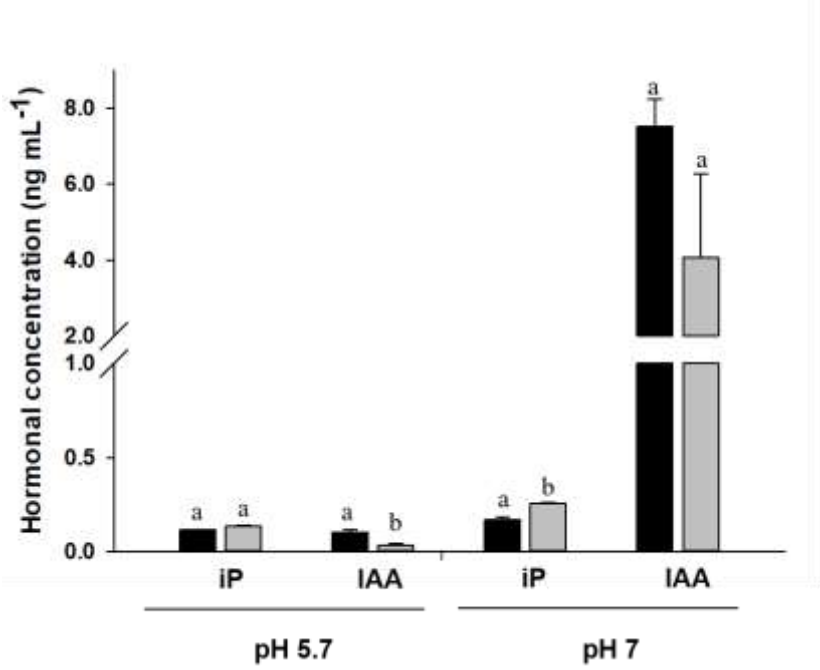


796

797 **Figure 10. Effects on the content of indole-3-acetic acid (IAA) in the culture medium of**
798 **GreenTray® TIS bioreactors in the presence of *P. oryzihabitans* PGP01 inoculated in the MS**
799 **medium at pH 5.7 and 7.** Data represents the mean ± SE of at least three replicates. Different letters
800 denote significant differences between control and treatments according to Student t test ($P < 0.05$).

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803