Rhizosphere microorganisms enhance *in vitro* root and plantlet development of *Pyrus* and *Prunus* rootstocks

Daniel Cantabella[1,2]; Neus Teixidó[1]; Guillem Segarra[1]; Rosario Torres[1]; Maria Casanovas[2]; Ramon Dolcet-Sanjuan[2]*

[1]IRTA Postharvest Programme, [2]Plant *In Vitro* Culture Laboratory, Fruticulture Programme; IRTA Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, 25003 Lleida, Catalonia, Spain.

*Corresponding author: ramon.dolcet@irta.cat

Main conclusion: The *in vitro* application of rhizosphere microorganisms led to a higher rooting percentage in *Pyrus* Py12 rootstocks and increased plant growth of *Pyrus* Py170 and *Prunus* RP-20.

Abstract

The rooting of fruit tree rootstocks is the most challenging step of the *in vitro* propagation process. The use of rhizosphere microorganisms to promote *in vitro* rooting and plant growth as an alternative to the addition of chemical hormones to culture media is proposed in the present study. Explants from two *Pyrus* (Py170 and Py12) rootstocks and the *Prunus* RP-20 rootstock were inoculated with *Pseudomonas oryzihabitans* PGP01, *Cladosporium ramotenellum* PGP02 and *Phoma* sp. PGP03 following two different methods to determine their effects on *in vitro* rooting and plantlet growth. The effects of the microorganisms on the growth of fully developed Py170 and RP-20 plantlets were also studied *in vitro*. All experiments were conducted using vermiculite to simulate a soil system *in vitro*. When applied to Py12 shoots, which is a hard-to-root plant material, both *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 fungi were able to increase the rooting percentage from 56.25% to 100% following auxin indole-3-butyric acid (IBA) treatment. Thus, the presence of these microorganisms clearly improved root development, inducing a higher number of roots and causing shorter roots. Better overall growth and improved stem growth of treated plants was observed when auxin treatment was replaced by co-culture with microorganisms. A root growth-promoting effect was observed on RP-20 plantlets after inoculation with *C. ramotenellum* PGP02, while *P. oryzihabitans* PGP01 increased root numbers for both Py170 and RP-20 and increased root growth over stem growth for RP-20. It was also shown that the three microorganisms *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 were able to naturally produce auxin, including indole-3-acetic acid (IAA), at different levels. Overall, our results demonstrate that the microorganisms *P. oryzihabitans*

PGP01 and *C. ramotenellum* PGP02 had beneficial effects on *in vitro* rooting and plantlet growth and could be applied to *in vitro* tissue culture as a substitute for IBA.

Keywords: Auxins, Fruit tree rootstocks, Micropropagation, Plant-growth promoting fungi, Plant-growth promoting rhizobacteria.

Abbreviations

CRD, completely randomized design; NYDA, nutrient yeast dextrose agar; PDA, potato dextrose agar; PDB, potato dextrose broth; PGPMs, plant growth-promoting microorganisms; REM, root elongation media; REM-V, root elongation media with vermiculite; RIM, root induction media; TSB, tryptone soy broth.

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Author contribution statement

DC, NT and RDS contributed to the design of the study. All the experiments, as well as the data collection, were conducted by DC, MC, and GS. The data analysis and interpretation were performed by DC, NT, RT, and RDS. The writing and revision of the manuscript were performed by DC, NT, RT, and RDS. All the authors have read the article and made critical contributions to improve the quality of the manuscript.

Introduction

Of the different steps of *in vitro* plant propagation, the rooting of micropropagated shoots of different fruit tree species belonging to the *Prunus* genus is not easy (Quambusch et al. 2016; Wiszniewska et al. 2016; Arab et al. 2018), and rooting enhancement is crucial to ensure plant production and survival in soil conditions. The main procedures used to stimulate the formation of roots in *in vitro* environments involve a reduction in the concentration of macronutrients, as well as the addition of exogenous auxin (Iglesias et al. 2004; Dobránszki and Teixeira da Silva 2010; Goel et al. 2018; Lucchesini et al. 2019). The effectiveness of the process depends on several factors, including the genotype, the type of auxin and the dose of hormone applied (Magyar-Tábori et al. 2002; Ruzic and Vujovic 2007).

In recent years, restrictions imposed by the European Commission concerning the use of chemicals in plant production (including auxin) have led to the development of new strategies to improve *in vitro* rooting using more ecological sources to avoid the application of exogenous auxin (Pacholczak et al. 2012; Elmongy et al. 2018). It is well known that some plant growth-promoting microorganisms (PGPMs) have an impact on *in vitro* plant development, increasing plant growth or the efficacy of the propagation and rooting of explants (Contesto et al. 2010; Trinh et al. 2018). Bacteria and fungi are able to produce hormones such as auxin or gibberellins (Waqas et al. 2012; Iqbal and Hasnain 2013; Meents et al. 2019), making the use of microorganisms a promising alternative to the use of chemical compounds. In a study conducted by our research group, the plant growth-promoting effects of two fungi (*Cladosporium ramotenellum* PGP02 and *Phoma* sp. PGP03) and one bacterium (*Pseudomonas oryzihabitans* PGP01) isolated from *Pyrus* and *Prunus* endogenously contaminated *in vitro* cultures were reported (Cantabella et al. 2020).

On this basis, the aim of the present study involves the evaluation of the effects of these three microorganisms on root induction and development and on the growth of micropropagated plantlets from different rootstocks belonging to species of the *Pyrus* and *Prunus* genera. This application has generated universal interest in agricultural research as an instrument to increase abiotic stress tolerance or disease resistance (Asín et al. 2011; Elias-Roman et al. 2019; Riaz et al. 2019; Silva et al. 2019), and the use of *in vitro* tissue culture techniques serves as a way to study tolerance to abiotic stresses, such as tolerance to lime-induced chlorosis (Dolcet-Sanjuan et al. 1992, 2004a, 2008; Donnini et al. 2009). To ensure better plant-microbe coexistence, an *in vitro* culture system using media comprising vermiculite instead of the traditional approach in which semisolid agar-containing media are used was used in this study.

Material and methods

Plant materials and *in vitro* culture conditions

Rootstocks for which there is commercial interest for fruit production were used in this study. A *Prunus* rootstock marketed for commercial use named Rootpac® 20 (RP-20) (Agromillora Group, Barcelona, Spain) and two *Pyrus* rootstocks named "Py12" and "Py170", which are under agronomic evaluation by the IRTA, were propagated by axillary branching through *in vitro* shoot tip cultures. RP-20 is a natural hybrid between Myrobalan plum (*Prunus cerasifera* Ehr.) and almond (*Prunus dulcis*) and can be used as a rootstock for Japanese plum, peach, nectarine, almond and several apricot cultivars (Pinochet 2010). Py170 is a hybrid between OH11 (Simard and Michelesi 2002) and *Pyrus amygdaliformis*, while Py12 is derived from the open pollination of *Pyrus communis* cv. Williams. Both clones are in the last selection phase of an IRTA pear rootstock breeding programme that aims to obtain tolerance to both lime-induced chlorosis and reduced vigour.

Shoot-tip cultures of both *Pyrus* rootstocks (Py12 and Py170) and commercial *Prunus* (RP-20) rootstock were the source of shoot explants used to induce rooting and produce full plantlets. The three plant materials were propagated by axillary branching in MS media (Murashige and Skoog 1962) supplemented with 5 μM benzylaminopurine (BAP), as described by Iglesias et al. (2004). For shoot elongation, 50 ml of liquid MS media without hormones was added to each flask after a 4-week-long culture period in 100 ml of semisolid multiplication media. Root induction on *in vitro* elongated shoots was conducted in ½-strength MS media supplemented with 10 μM indole-3-acetic acid (IAA), henceforth referred to as RIM-10IBA, for a 7-day-long period in darkness, followed by a transfer to ½-strength MS auxin-free media, henceforth referred to as root elongation media (REM). The *in vitro* coexistence of shoot explants or full plantlets with the microorganisms took place in ½-strength MS media without hormones but amended with vermiculite (50:40, v/v, vermiculite:medium), as described by Dolcet-Sanjuan et al. (2004b), henceforth referred to as root elongation media with vermiculite (REM-V). The pH of the different liquid media was adjusted to 5.7 using NaOH before the addition of gelling agar (8 g l^{-1} in RIM and REM and 6 g l^{-1} in REM-V). For semisolid agar-containing media, 15-mm-diameter tubes, each with 15 ml of media, were used. When REM-V was used, 38-mm-diameter tubes, each containing 50 ml of vermiculite and 40 ml of semisolid media, were used. The media were autoclaved at 121°C for 20 min, and once they had cooled, they were stored at 14°C. All *in vitro* cultures, including those in co-culture with the

microorganisms, were maintained in a culture chamber set at 24°C and providing 100-120 µmol m^{-2} s^{-1} cool-white fluorescent light under a 16-h-light photoperiod.

In vitro root induction and development in the presence of microorganisms

The effects of co-culture with microorganisms during root induction and development were evaluated using 3-cm-long elongated shoots of RP-20, Py12 and Py170. Two different protocols were followed to study the impact of the three microorganisms on *in vitro* rooting. In the first protocol (method 1), elongated shoots were transferred to RIM-10IBA for 7 days in dark conditions. Afterward, the shoots were transferred to REM-V and immediately inoculated with 1 ml of the microorganism suspension. In the second protocol (method 2), elongated shoots were immersed for 5 min in 1 ml of microorganism suspension and then placed in a sterile 2-cm-diameter well, which enabled immersion of 3 mm of the shoot base. Afterward, the shoots were cultured in tubes with REM for one week to ensure the microorganisms grew in direct contact with the shoot base. After this period, the shoots were transferred to REM-V to favour root development, as was done for method 1. For both methods, a total of fifteen shoots per treatment were used. Four treatments per experiment were used, namely, inoculation with PGP01 (*P. oryzihabitans*), inoculation with PGP02 (*C. ramotenellum*), inoculation with PGP03 (*Phoma* spp.) and inoculation with a control. The control treatment involved the addition of 1 ml of sterile water for method 1 or immersing the shoot base in 1 ml of sterile water for method 2. After 8 weeks of co-culture, the rooting percentage was calculated by dividing the number of rooted shoots by the total number of shoots. Morphometric and photosynthetic parameters, including the number of leaves, root number, stem length (cm), root length (cm), total plant fresh weight (FW, in g), root fresh weight (g) and stem fresh weight (g) were measured for each plantlet, as described by Cantabella et al. (2020).

In vitro plant development in the presence of microorganisms

To test whether *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 had plant growth-promoting effects, *in vitro* fully developed RP-20 and Py170 plantlets were used. In previous experiments (Cantabella et al. 2020), the three microorganisms were able to increase the FW, stem length and root length of rooted pear seedlings derived from *in vitro* embryo rescue.

For these assays, a total of ten 3-cm-long micropropagated shoots per treatment were induced for rooting in RIM-10IBA for 7 days, followed by a 1- to 2-week-long culture period in REM to favour root elongation and development. After this period, the shoots that displayed visible roots were removed from

the agar-containing semisolid media and transplanted into REM-V, followed by inoculation with 1 ml of each microorganism suspension or the same volume of sterile double distilled water for the control treatment. After 8 weeks of co-culture, morphometric and photosynthetic parameters, including the number of leaves, root number, stem length (cm), root length (cm), total plant fresh weight (FW, in g), root fresh weight (g) and stem fresh weight (g) were measured for each plantlet. The root fresh weight (g)/stem fresh weight (g) ratio was calculated for each plantlet and used as an indicator of differential plant growth distribution.

Preparation of microorganism inocula

In the present study, the bacterium *P. oryzihabitans* PGP01 and fungi *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 belonging to the IRTA postharvest programme PGPMs collection, were used to test their potential effects on the *in vitro* rooting and growth of *Prunus* and *Pyrus* rootstocks. These microorganisms were isolated from *Pyrus* and *Prunus* embryos germinated in an aseptic environment and preliminarily tested in *in vitro* rooted pear plantlets, the results of which showed beneficial effects (Cantabella et al. 2020). *P. oryzihabitans* PGP01 cultures preserved at -80°C were 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}) plates at 25°C, while *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 cultures were grown in potato dextrose agar (PDA: potato tissue, 200 ml; glucose, 20 g l^{-1}; and agar, 20 g l^{-1}) plates at the same temperature. Forty-eight-hour-old plates of bacteria and 14-d-old plates of both fungi were used to prepare the microorganism suspensions. *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) and washed by centrifugation at 6164 × *g*. After suspension in phosphate buffer, the bacterial concentration was adjusted with sterile distilled water at $2x10^8$ CFU ml^{-1} by measuring the absorbance at 420 nm with a spectrophotometer (SP-2000 UV, Shanghai Spectrum Instruments Co., Ltd., Shanghai, China). The conidia of both *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 were measured using a haemocytometer, and the concentration was ultimately set to $2x10^6$ spores ml^{-1} with sterile distilled water (sp ml^{-1}). In both cases, the plate dilution technique on solid PDA and NYDA media was applied to calculate the true colony forming units (CFU ml^{-1}).

Determination of auxin production via spectrophotometry

The indole-3-acetic (IAA) production ability of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 was tested spectrophotometrically according to the methods described by Gordon

and Weber (1951). *P. oryzihabitans* PGP01 bacteria and *C. ramotenellum* PGP02 and Phoma sp. PGP03 fungi were grown in flasks containing 50 ml of tryptone soy broth (TSB) and potato dextrose broth (PDB) supplemented or not supplemented with 0.5 and 1 g l^{-1} tryptophan for 24 h and 168 h, respectively, and incubated at 25°C and 150 rpm. Bacterial and fungal cultures were pelleted by centrifugation for 10 min at 6164 × *g*, and 1 ml of the supernatants was tested for the presence of indole compounds using 2 ml of Salkowski reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$). After 25 min of incubation in darkness, the absorbance at 530 nm was measured with an SP-2000 UV spectrophotometer (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China). The content of auxin was determined via a standard curve of synthetic IAA (Duchefa Biochemie, Haarlem, The Netherlands) at different concentrations (from 0 to 20 µg ml^{-1}) and treated in the same way as were the bacterial and fungal supernatants.

Data analysis

The different experiments were designed in accordance with a completely randomized design (CRD), and the data were analysed by one- or two-way factorial ANOVA using JMP Pro Software (version 13.1.0, SAS Institute, Cary, NC, USA). Different letters were used to denote significant differences according to Student's t-test ($p \leq 0.05$). Statistical significance was judged at the level $P < 0.05$, and Tukey's test was used to separate the means within one factor when the differences were statistically significant. Significant differences in *in vitro* rooting percentages between treatments and the control were analysed via Fisher's exact test ($P \leq 0.05$).

Results

Effects of the applications of three microorganisms on *in vitro* root induction, development and biometric parameters

To study the effect of the three microbes *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 on *in vitro* root induction and plantlet development, microorganisms were applied following two different methods: inoculation after root induction in the medium supplemented with 10 µM IBA (RIM-10IBA, Method 1), and immersion of the shoot basis in microbe suspensions without root induction with IBA (REM, Method 2). Using these methods, the effects of these microorganisms were tested in three rootstock genotypes: the easy-to-root *Prunus* RP-20, the easy-to-root *Pyrus* Py170 and the hard to root *Pyrus* Py12. To ensure the coexistence of plants and microorganisms, all the experiments were conducted in REM supplemented with vermiculite (REM-V). After 8 weeks of co-culture, the *in vitro*

rooting percentage, as well as several parameters regarding root induction and development (number of roots and root length) and plantlet development (plant, shoot and root FW) were measured.

As expected, the plant material had an important effect on the rooting response. The control treatment of the shoots, with neither inoculation with microorganisms (method 1) nor immersion into suspensions of microorganisms (method 2), induced to root in RIM-10IBA (method 1) or by a 7-day-long culture in REM (method 2), followed by an 8-week-long culture in REM-V, induced 100% rooting on RP-20 and Py170, while it was only 56.3% for Py12, a hard-to-root *Pyrus* clone (Table 1).

Inoculation with *P. oryzihabitans* PGP01 or *C. ramotenellum* PGP02 through either methodology had no detrimental influence on rooting percentage, which remained at 100%, for the easy-rooting clones RP-20 or Py170 (Table 1). For Py12 in co-culture with *C. ramotenellum* PGP02 and *Phoma* sp. PGP03, the rooting percentage doubled, increasing to 100% when root elongation (method 1) or root induction and elongation (method 2) were performed in co-culture with the tested microorganisms (Table 1).

The effect of the microorganisms on the number of developed roots ($P < 0.001$) and the length of rooted shoots of RP-20, Py170 and Py12 (Fig. 1a) were highly influenced ($P < 0.001$) by the method of inoculation. After the first protocol, we observed that *P. oryzihabitans* PGP01 was able to induce a 35% significant increase in the number of roots in *Prunus* RP-20 shoots but not in either the *Pyrus* Py170 or Py12 rootstocks (Figs. 1a and 2). However, this increase in the number of roots was not accompanied by a greater root length for either of the three tested plant genotypes compared with non-treated plantlets (Figs. 1b and 2). Inoculation with *C. ramotenellum* PGP02 led to a higher number of roots for the three plant genotypes tested compared with their respective controls, with increases of 57, 54 and 312% for RP-20, Py170 and Py12, respectively (Figs. 1a and 2). Together with the positive effect of inoculation with this microorganisms on the number of roots, a significant (135%) increase in root length was observed only in *Pyrus* rootstock Py170 after 8 weeks of co-culture (Figs. 1b and 2). *Phoma* sp. PGP03 drastically affected this parameter in the hard-to-root Py12 rootstock compared with the non-treated plantlets; the root length increased 225% after 8 weeks of co-culture (Figs. 1a and 2). However, a significant inhibition in root elongation was reported for the RP-20 and Py12 genotypes after inoculation with *Phoma* sp. PGP03 in comparison to that of the control plants (Figs. 1b and 2).

Overall, the number of roots observed on RP-20 and Py170 shoots was similar or lower than that on the control plants when they were immersed in the microorganism suspensions (method 2) with no

previous exogenous auxin treatment (Fig. 1a). However, with the Py12 clone, inoculation with *C. ramotenellum* PGP02 significantly increased the number of roots (almost 50% higher than those of the control) (Figs. 1a and 2). Regarding root length, inoculation by root immersion in the suspension of *P. oryzihabitans* PGP01 induced significantly longer roots (110% increase) from shoots of Py170 (Figs. 1b and 2). As observed with method 1, *Phoma* sp. PGP03 inhibited root elongation in RP-20 and Py12, and this reduction was significant only in RP-20 (58% lower than that of the control) (Figs. 1b and 2).

Regardless of the genotype, the effects of the three microorganisms on the total plant ($P < 0.001$), shoot ($P < 0.001$) and root FW ($P = 0.003$, $P = 0.004$ and $P < 0.001$ for RP-20, Py170 and Py12, respectively) were affected by the method used for their application (Fig. 3a, b, c). After inoculation, during root elongation (method 1) of RP-20 and Py170 with the suspension of *P. oryzihabitans* PGP01, while plant FW was not affected, an important reduction in this parameter was reported in the hard-to-root Py12 genotype (Fig. 3a). The reduction in shoot FW occurred in the RP-20 and Py12 plantlets in response to inoculation with *P. oryzihabitans* PGP01 (48 and 67% lower than that of the control, respectively). Inoculation with *P. oryzihabitans* PGP01 following this method induced a nearly 70% increase in RP-20 root FW (Fig. 3c). However, the plant, shoot and root FWs remained unchanged or were lower than those of the control plants after 8 weeks of co-culture with both *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 inoculated according to method 1 (Fig. 3a, b, c).

In method 2, inoculation with *P. oryzihabitans* PGP01 significantly increased the plant and shoot FW of *Pyrus* Py170 rootstock (225% higher than that of the control) but not in RP-20 or Py12 rootstock (Fig. 3a). When this bacterium was applied via this method, we reported an increase in root FW for all three genotypes: RP-20, Py170 and Py12 (68, 120 and 80% more than that of the non-treated plantlets) (Fig. 3c). In this sense, it is important to highlight that this increase in root FW observed for RP-20 in response to inoculation with *P. oryzihabitans* PGP01 was quite similar to that obtained via method 1. Surprisingly, *C. ramotenellum* PGP02 significantly promoted plant, shoot and root FW of *Pyrus* rootstocks Py170 and Py12, whereas no effects on the *Prunus* RP-20 rootstock occurred after 8 weeks of co-culture with this microbe (Fig. 3a, b, c). Finally, while a negative effect of the application of *Phoma* sp. PGP03 was observed on RP-20 shoots, the plant, shoot and root FW decreased (74, 86 and 171% lower than those of control, respectively), but no significant changes in these parameters were found in Py170 or Py12 (Fig. 3a, b, c).

In general, inoculation with the three microorganisms following method 1 stimulated root development, mainly increasing the number of roots of RP-20, Py170 and Py12 explants. On the other hand, increased growth of plantlets was observed when the inoculation was carried out by immersing the shoots in *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 suspensions, since better values of plant, shoot and root FW were observed in response to these microbes in the three genotypes tested in the study.

***In vitro* plantlet development in the presence of microorganisms**

In this experiment, we focused our efforts in elucidating the effect of these three microorganisms *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 on *in vitro* plant development of the two rootstock genotypes *Prunus* RP-20 and *Pyrus* Py170. For that purpose, *in vitro* micropropagated explants were inoculated with the three microorganisms in REM-V after root induction and development in RIM-10IBA and REM, respectively. Again, several biometrical parameters including the number of roots, root and shoot FW and root/shoot FW ratio were measured after 8 weeks of co-culture with microbes.

Plant material had a significant effect on root and plantlet development after inoculation with microorganisms, and this interaction was significant for the number of roots ($P < 0.001$), shoot FW ($P = 0.002$), root FW ($P = 0.001$) and root/shoot FW ratio ($P = 0.002$). In this sense, we observed that *P. oryzihabitans* PGP01 positively affected all the parameters concerning root growth for the RP-20 plants, such as the number of roots, root FW and the root/shoot FW ratio compared with those of the non-treated plantlets (Fig. 4a, b and d). This root-localized effect was also observed when the bacterium was applied to Py170 *in vitro* explants, since a significant increase in the number of roots was recorded after 8 weeks of co-culture (Fig. 4a and 5). On the other hand, both plant genotypes behaved differently after inoculation with *C. ramotenellum* PGP02. The application of the fungus favoured the root growth of RP-20 *in vitro* plantlets, increasing their root FW (129% compared with that of the control plants). This fungus also increased shoot length compared with that of the non-treated plantlets (data not shown). Nevertheless, only shoot growth increased when the application was carried out on the *Pyrus* Py170 explants (Fig. 4b, c and 5). In this sense, the shoot FW significantly increased by only 32% compared with that of the control plantlets. Again, no significant plant growth promotion was observed in response to inoculation with *Phoma* sp. PGP03, except for the higher number of roots observed for Py170 plantlets after 8 weeks of co-culture with this fungus (Fig. 4a and 5). Other parameters, including root length and number of leaves, were not significantly affected by either of the three microbes (data not shown).

Determination of IAA in *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 cultures.

The content of IAA was determined in *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 cultures. The ability to produce auxins was suggested in the three microorganisms, as significant increases in the IAA content were detected when the different culture media were supplemented with Trp. In this context, it is noteworthy to mention that the concentration of IAA detected in P. oryzihabitans PGP01 cultures was higher than that observed for *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 In the case of *P. oryzihabitans* PGP01, an increase in the IAA content was observed when the tryptophan was added to the medium in a dose-dependent manner, registering a 4.35- and a 7.07-fold increase at 0.5 and 1 g l^{-1}, respectively (Fig. 6). On the other hand, IAA was also found in *C. ramotenellum* PGP02 cultures when fungal cells were grown in PDB without Trp (Fig. 6), and these levels remained unchanged when the lowest concentration of tryptophan was tested. However, the addition of 1 g l^{-1} induced a 3.57-fold increase in IAA levels in comparison to those recorded in PDB without tryptophan (Fig. 6). Regarding PGP03, the addition of tryptophan at 0.5 or 1 g l^{-1} to the PDB medium induced significant increases on IAA content in comparison to non-supplemented medium (3.2 and 3.7-fold), but, in that case, no differences between both doses of tryptophan were registered (Fig. 6).

Discussion

Rootstocks have important agronomic value in fruit tree crops since they are used for grafting commercial varieties, providing desirable fruit production and quality and tree tolerance to edaphic factors associated with abiotic and biotic stresses (Prodhomme et al. 2019). Some clones selected for their desirable agronomic characteristics, especially those clones that result in low vigour, have poor rooting efficiencies and root development, such as the following: apple rootstock M9 (*Malus* x *domestica* Borkh) (Sun and Bassuk 1991; Amiri and Elahinia 2011); pear rootstocks OHxF 333, Brossier P.2274 and P.227 (Mielke and Turner 2008; Necas and Kosina 2008); and peach rootstocks Lovel and *Prunus americana* (Mayer et al. 2015). As a consequence, *in vitro* propagation is not feasible. Herein, owing to its tolerance to lime-induced chlorosis and reduced vigour (Asín et al. 2011), Py12, a pear rootstock clone selected in an IRTA breeding programme, also has low rooting efficiency compared to that of another *Pyrus* rootstock, Py170, or the commercial *Prunus* rootstock Rootpac® 20 (RP-20). When *in vitro* rooting is the main obstacle to obtaining complete viable plants in woody plant species (Wiszniewska et al. 2016), research to improve the

efficiency of this step frequently emphasizes determining the hormone type and level (Dobránszki and Teixeira da Silva 2010) to be added during *in vitro* growth, though the results are highly dependent on genotype. In three apple rootstocks (M.26, MM.106 and JTE-H) in which three different levels of IBA were used, different responses in terms of rooting percentage and root length were observed for the different genotypes (Magyar-Tábori et al. 2002).

Improvement in root development in peach and pear embryo-derived plantlets inoculated with the microorganisms used in the present work was reported in a previous work (Cantabella et al. 2020). Therefore, an *in vitro* model in which these three microorganisms (*P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03) were applied to *in vitro* clonally propagated *Prunus* RP-20 and *Pyrus* Py12 and Py170 rootstocks at different stages of *in vitro* development was constructed to determine their effects on root induction, root development, and whole-plantlet *in vitro* growth. Two independent experiments were conducted: first, to study the effects of the microorganisms on the *in vitro* rooting efficiency of shoots, with or without an IBA root induction treatment, and second, to study the microorganisms' effects on *in vitro* whole-plantlet growth. Inoculation of microorganisms following the two different methods has been previously reported in semiwoody olive microcuttings under nursery conditions (Montero-Calasanz et al. 2013). Herein, to improve the coexistence between shoots or plantlets and microorganisms, vermiculite was added to the culture media under *in vitro* conditions. In the first method, in which IBA was used to induce rooting, which is the standard protocol (Murashige and Skoog 1962; Iglesias et al. 2004), *Pyrus* Py12 shoots showed 56.25% rooting, while in the presence of *C. ramotenellum* PGP02 and *Phoma* sp. PGP03, the *in vitro*-rooted shoots completely rooted (100%). In accordance with our results, other endophytic bacterial isolates demonstrated that inoculation of *in vitro* microshoots of two *Prunus avium* genotypes resulted in increased rooting percentage (Quambusch et al. 2014). Additionally, the results obtained with method 2, without IBA treatment of the shoots, confirmed that the *P. oryzihabitans* PGP01 bacterium and both *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 fungi could act as a natural root-promoting source, likely due to their ability to produce IAA. In this context, it has been proven that the bacterium *P. oryzihabitans* PGP01 was able to produce a higher amount of IAA than both fungi. All inoculated and control shoots, including those of Py12, rooted after 8 weeks of culture, except those inoculated with *Phoma* sp. PGP03, which showed a decrease in this parameter. On the other hand, in the IBA-induced shoots (method 1), invariably reduced growth was observed in all plantlets compared with those derived from method 2. Nonetheless, the three microorganisms increased the number

342 of roots per plantlet in the three different rootstocks in a genotype-dependent way, and these increases were
343 statistically significant for *C. ramotenellum* PGP02 in all three tested rootstocks, *P. oryzihabitans* PGP01
344 in RP-20 and *Phoma* sp. PGP03 in RP-20 and Py12.

345 In the present work, the ability of both *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 to
346 produce IAA could explain the increase in the number of roots. *P. oryzihabitans* PGP01 and *C.*
347 *ramotenellum* PGP02, in the absence of IBA (Method 2), led to better root elongation and shoot growth –
348 more significantly in the Py170- and Py12-treated shoots. Regarding *Phoma* sp. PGP03, no important
349 effects on root length were observed in this method in spite that the ability to produce auxins was proven.
350 Studies revealing the use of microorganisms in microshoots to induce rooting while avoiding the application
351 of exogenous auxin are scarce, and some authors have reported positive effects of other natural compounds
352 such as humic acid on improving the *in vitro* rooting efficiency (Elmongy et al. 2018). Herein, though an
353 improvement in rooting percentage was proven only for Py12 shoots, better growth of the three rootstocks
354 (Py170, Py12 and RP-20) was observed after exposure to *P. oryzihabitans* PGP01 and *C. ramotenellum*
355 PGP02, and this technique is a promising alternative to the use of chemical hormones in plant production.
356 Further assays employing clonal rootstocks with a compromised rooting ability under *in vitro* conditions,
357 such as M9, OHxF 333, Brossier P.2274 and P.2276, Lovell or *P. americana*, will need to be carried out to
358 corroborate and verify this effect. The performance of rhizobacteria in the *in vitro* rooting process is highly
359 documented (Quambusch et al. 2014; Kavino and Manoranjitham 2018; Perez-Rosales et al. 2018; Burygin
360 et al. 2019); however, very little is known about the role of fungi in plant root development. It has been
361 previously established that some beneficial fungi are able to produce and accumulate auxin in their mycelia,
362 which could increase the levels of auxin as well as auxin-responsive plant gene expression in *Arabidopsis*
363 *thaliana* (Meents et al. 2019). The present research constitutes the first work showing the auxin production
364 ability of *C. ramotenellum*. In the case of *P. oryzihabitans*, Belimov et al. (2015) already reported the ability
365 of this bacterium to produce different indole compounds. In the same study, potato (*Solanum tuberosum*)
366 plants showed increased root biomass when *P. oryzihabitans* was present in the rhizosphere compared to
367 that of non-inoculated plants.

368 A different approach was conducted to analyse whether *P. oryzihabitans* PGP01 bacteria and the
369 two *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 fungi are microorganisms with potential plant growth-
370 promoting effects under *in vitro* conditions. For this purpose, fully developed Py170 and RP-20 plantlets
371 were used to evaluate whether they were able to improve root and shoot growth. The effects in response to

inoculation with *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 were somehow different depending on the genotype. In *Prunus* RP-20 rootstocks, a greater root FW was observed in response to *C. ramotenellum* PGP02, and a significantly higher number of roots was registered in Py170 after the inoculation with *Phoma* sp. PGP03. These results might be explained by the ability of both fungi to produce auxins. However, the clear effect of *C. ramotenellum* PGP02 on the growth of the aerial parts of Py170 and RP-20 suggested the role of other compounds produced by the fungus instead of auxin. Some fungi play a crucial role in plant growth and development, mostly due to the endophytic production of plant hormones such as gibberellins (GAs), which are involved in important physiological processes, including stem elongation (Calvo et al. 2014). Previous studies have demonstrated the ability of different isolates from rhizosphere fungi belonging to the *Cladosporium* and *Phoma* genera to produce GAs, which resulted in increased growth of soybean and cucumber seedlings (Hamayun et al. 2009, 2010). Plant growth promotion in *Atriplex gmelinii* by a gibberellin-producing strain of *Gliomastix murorum* was reported (Khan et al. 2009). Thus, considering the above reference, our results could suggest that GAs play a role in the enhancement of shoot growth induced by *C. ramotenellum* PGP02 in *Pyrus* Py170 rootstock. On the other hand, the bacterium *P. oryzihabitans* PGP01 tended to act more specifically in the roots, increasing the number of roots for both Py170 and RP-20 rootstocks, as well as the root FW and root/shoot FW ratio solely in RP-20. Reprogramming root system architecture in response to rhizobacteria belonging to the *Pseudomonas* and *Bacillus* genera is mediated by auxin signalling in *A. thaliana* (López-Bucio et al. 2007; Zamioudis et al. 2013). Pourjasem et al. (2020) recently reported that bacteria belonging to the *Pseudomonas* genus are able to release some elements into the culture media as a result of the decomposition of minerals present in vermiculite that might also favour root development. These results represent a step forward compared with those of a previous publication where the effects of the three microorganisms were described in *Prunus in vitro* rescued embryos for the first time (Cantabella et al. 2020). However, more comprehensive studies are being undertaken to corroborate that this mechanism occurs in Py170 and RP-20 plants with the studied microorganisms.

Conclusions

In summary, the two different procedures concerning inoculation of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 did not reduce the *in vitro* rooting percentage of the efficient-to-root *Pyrus* and *Prunus* rootstocks Py170 and RP-20. On the hard-to-root *Pyrus* Py12 rootstock, both fungi increased the rooting percentage from 56.25% to 100%. As a consequence of the auxin production ability by some of the microorganisms, a higher number of roots was observed with these microorganisms

when co-cultured after IBA induction, and increased plantlet growth was observed when no synthetic auxin was used. Due to the strong potential of rootstocks in fruit tree production, this study constitutes a worthwhile approach to improve the rooting efficiency of difficult-to-root genotypes such as Py12. Furthermore, while *P. oryzihabitans* PGP01 primarily showed a root growth-promoting effect on both RP-20 and Py170 fully developed *in vitro* plantlets, a location-dependent effect in response to inoculation with *C. ramotenellum* PGP02, which positively affected the roots of RP-20 and shoots of Py170 rootstocks, was observed. The present results could suggest that auxin and gibberellins play a role, and the mechanisms underlying this plant growth promotion will be studied in the future.

References

Amiri ME, Elahinia A (2011) Influence of medium compositions on growth of apple rootstocks ("M9", "M27", 'MM106') in *in vitro* condition. Acta Hortic 923:139–146

Arab MM, Yadollahi A, Eftekhari M, et al (2018) Modeling and optimizing a new culture medium for *in vitro* rooting of G×N15 *Prunus* rootstock using artificial neural network-genetic algorithm. Sci Rep 8:9977. https://doi.org/10.1038/s41598-018-27858-4

Asín L, Iglesias I, Vilardell P, et al (2011) INRA-IRTA pear rootstock breeding program: Aiming for tolerance to iron chlorosis. Acta Hortic 903:207–213. https://doi.org/10.17660/ActaHortic.2011.903.25

Belimov AA, Dodd IC, Safronova VI, et al (2015) Rhizobacteria that produce auxins and contain 1-amino-cyclopropane-1-carboxylic acid deaminase decrease amino acid concentrations in the rhizosphere and improve growth and yield of well-watered and water-limited potato (*Solanum tuberosum*). Ann Appl Biol 167:11–25. https://doi.org/10.1111/aab.12203

Burygin GL, Kargapolova KY, Kryuchkova Y V., et al (2019) *Ochrobactrum cytisi* IPA7.2 promotes growth of potato microplants and is resistant to abiotic stress. World J Microbiol Biotechnol 35:1–12. https://doi.org/10.1007/s11274-019-2633-x

Calvo P, Nelson L, Kloepper JW (2014) Agricultural uses of plant biostimulants. Plant Soil 383:3–41. https://doi.org/10.1007/s11104-014-2131-8

Cantabella D, Dolcet-Sanjuan R, Casanovas M, et al (2020) Inoculation of *in vitro* cultures with rhizosphere microorganisms improve plant development and acclimatization during immature

embryo rescue in nectarine and pear breeding programs. Sci Hortic (Amsterdam) 273:109643. https://doi.org/10.1016/j.scienta.2020.109643

Contesto C, Milesi S, Mantelin S, et al (2010) The auxin-signaling pathway is required for the lateral root response of arabidopsis to the rhizobacterium *phyllobacterium brassicacearum* . Planta 232:1455–1470. https://doi.org/10.1007/s00425-010-1264-0

Dobránszki J, Teixeira da Silva JA (2010) Micropropagation of apple - A review. Biotechnol Adv 28:462–488. https://doi.org/10.1016/j.biotechadv.2010.02.008

Dolcet-Sanjuan R, Claveria E, Asín L, et al (2008) Towards the selection of a new pear rootstock: *In vitro* and field evaluation for tolerance to iron chlorosis, low vigor and micropropagation of selected clones. Acta Hortic 800 PART 2:683–690

Dolcet-Sanjuan R, Claveria E, Bonany J, et al (2004a) Selection for new pear rootstocks: *In vitro* screening and field evaluation for tolerance to iron chlorosis. Acta Hortic 658:463–468. https://doi.org/10.17660/ActaHortic.2004.658.68

Dolcet-Sanjuan R, Claveria E, Gruselle R, et al (2004b) Practical factors controlling *in vitro* adventitious root formation from walnut shoot microcuttings. J Am Soc Hortic Sci 129:198–203. https://doi.org/10.21273/jashs.129.2.0198

Dolcet-Sanjuan R, Mok DWS, Mok MC (1992) Characterization and *in vitro* selection for iron efficiency in *Pyrus* and *Cydonia*. Vitr Cell Dev Biol - Plant 28:25–29. https://doi.org/10.1007/BF02632188

Donnini S, Castagna A, Ranieri A, Zocchi G (2009) Differential responses in pear and quince genotypes induced by Fe deficiency and bicarbonate. J Plant Physiol 166:1181–1193. https://doi.org/10.1016/j.jplph.2009.01.007

Elias-Roman RD, Calderon-Zavala G, Guzman-Mendoza R, et al (2019) 'Mondragon': A clonal plum rootstock to enhance management of Armillaria root disease in peach orchards of Mexico. Crop Prot 121:89–95. https://doi.org/10.1016/j.cropro.2019.03.011

Elmongy MS, Zhou H, Cao Y, et al (2018) The effect of humic acid on endogenous hormone levels and antioxidant enzyme activity during *in vitro* rooting of evergreen azalea. Sci Hortic (Amsterdam) 227:234–243. https://doi.org/10.1016/j.scienta.2017.09.027

Goel A, Kaur A, Kumar A (2018) Biochemical and histological changes during *in vitro* rooting of microcuttings of *Bacopa monnieri* (L.) Wettst. Acta Physiol Plant 40:1–12. https://doi.org/10.1007/s11738-018-2641-8

Gordon SA, Weber RP (1951) Colorimetric estimation of indoleacetic acid. Plant Physiol 26:192–195. https://doi.org/10.1104/pp.26.1.192

Hamayun M, Khan S, Ahmad N, et al (2009) *Cladosporium sphaerospermum* as a new plant growth-promoting endophyte from the roots of *Glycine max* (L.) Merr. World J Microbiol Biotechnol 25:627–632. https://doi.org/10.1007/s11274-009-9982-9

Hamayun M, Khan SA, Khan AL, et al (2010) Growth promotion of cucumber by pure cultures of gibberellin-producing *Phoma* sp. GAH7. World J Microbiol Biotechnol 26:889–894. https://doi.org/10.1007/s11274-009-0248-3

Iglesias I, Vilardell P, Bonany J, et al (2004) Micropropagation and field evaluation of the pear (*Pyrus communis* L.) "IGE 2002", a new selection of the cultivar Dr. Jules Guyot. J Am Soc Hortic Sci 129:389–393. https://doi.org/10.21273/jashs.129.3.0389

Iqbal A, Hasnain S (2013) *Aeromonas punctata* PNS-1: A promising candidate to change the root morphogenesis of *Arabidopsis thaliana* in MS and sand system. Acta Physiol Plant 35:657–665. https://doi.org/10.1007/s11738-012-1106-8

Kavino M, Manoranjitham SK (2018) *In vitro* bacterization of banana (*Musa* spp.) with native endophytic and rhizospheric bacterial isolates: Novel ways to combat *Fusarium* wilt. Eur J Plant Pathol 151:371–387. https://doi.org/10.1007/s10658-017-1379-2

Khan SA, Hamayun M, Kim HY, et al (2009) Gibberellin production and plant growth promotion by a newly isolated strain of *Gliomastix murorum*. World J Microbiol Biotechnol 25:829–833. https://doi.org/10.1007/s11274-009-9981-x

López-Bucio J, Campos-Cuevas JC, Hernández-Calderón E, et al (2007) *Bacillus megaterium* rhizobacteria promote growth and alter root-system architecture through an auxin- and ethylene-independent signaling mechanism in *Arabidopsis thaliana*. Mol Plant-Microbe Interact 20:207–217. https://doi.org/10.1094/mpmi-20-2-0207

Lucchesini M, Pacifici S, Maggini R, et al (2019) A novel microfloating culture system for the *in vitro* rooting of *Echinacea angustifolia* D.C.: photosynthetic performance and production of caffeic acid derivatives. Plant Cell Tissue Organ Cult 136:123–132. https://doi.org/10.1007/s11240-018-1498-2

Magyar-Tábori K, Dobránszki J, Jambor-Benczúr E, et al (2002) Effects of indole-3-butyric acid levels and activated charcoal on rooting of *in vitro* shoots of apple rootstocks. J Hortic Sci 8:25–28

Mayer NA, Reighard GL, Bridges W (2015) Peach rootstock propagation under intermittent mist system. Acta Hortic 1084:53–61

Meents AK, Furch ACU, Almeida-Trapp M, et al (2019) Beneficial and pathogenic Arabidopsis root-interacting fungi differently affect auxin levels and responsive genes during early infection. Front Microbiol 10:1–14. https://doi.org/10.3389/fmicb.2019.00380

Mielke EA, Turner J (2008) Difficult to propagate pear rootstocks: Will they work as interstems? Acta Hortic 800 PART 2:653–657

Montero-Calasanz MC, Santamaría C, Albareda M, et al (2013) Alternative rooting induction of semi-hardwood olive cuttings by several auxin-producing bacteria for organic agriculture systems. Spanish J Agric Res 11:146–154. https://doi.org/10.5424/sjar/2013111-2686

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473–497

Necas T, Kosina J (2008) Vegetative propagation of pear and quince rootstocks using hardwood cuttings. Acta Hortic 800:701–706. https://doi.org/10.17660/ActaHortic.2008.800.95

Pacholczak A, Szydáo W, Jacygrad E, Federowicz M (2012) Effect of auxins and the biostimulator algaminoplant on rhizogenesis in stem cuttings of two dogwood cultivars (*Cornus alba* 'Aurea' and 'Elegantissima'). Sci, Acta Cultus, Hortorum 11:93–103

Perez-Rosales E, Alcaraz-Meléndez L, Puente ME, et al (2018) Endophytic bacteria isolated from wild jojoba [*Simmondsia chinensis* L. (Schneider)] roots improve *in vitro* propagation. Plant Cell Tissue Organ Cult 135:515–522. https://doi.org/10.1007/s11240-018-1483-9

Pinochet J (2010) "Replantpac" (Rootpac R), a plum-almond hybrid rootstock for replant situations. HortScience 45:299–301. https://doi.org/10.21273/HORTSCI.45.2.299

Pourjasem L, Landi A, Enayatizamir N, Hojati S (2020) The release of some elements from vermiculite during the short periods of incubation by heterotrophic bacteria. Eurasian Soil Sci 53:223–229. https://doi.org/10.1134/S106422932002009X

Prodhomme D, Valls Fonayet J, Hévin C, et al (2019) Metabolite profiling during graft union formation reveals the reprogramming of primary metabolism and the induction of stilbene synthesis at the graft interface in grapevine. BMC Plant Biol 19:1–12. https://doi.org/10.1186/s12870-019-2055-9

Quambusch M, Brümmer J, Haller K, et al (2016) Dynamics of endophytic bacteria in plant *in vitro* culture: quantification of three bacterial strains in *Prunus avium* in different plant organs and *in vitro* culture phases. Plant Cell Tissue Organ Cult 126:305–317. https://doi.org/10.1007/s11240-016-0999-0

Quambusch M, Pirttila AM, Tejesvi M V., et al (2014) Endophytic bacteria in plant tissue culture: Differences between easy-and difficult-to-propagate Prunus avium genotypes. Tree Physiol 34:524–533. https://doi.org/10.1093/treephys/tpu027

Riaz S, Pap D, Uretsky J, et al (2019) Genetic diversity and parentage analysis of grape rootstocks. Theor Appl Genet 132:1847–1860. https://doi.org/10.1007/s00122-019-03320-5

Ruzic D, Vujovic T (2007) The protocol for rapid propagation of Plum (*Prunus domestica* L.) by *in vitro* micropropagation. J Pomol 41:79–85

Silva MC, Sousa ARO, Cruz ES, et al (2019) Phenotyping of new hybrid citrus rootstocks under water deficit reveals conserved and novel physiological attributes of drought tolerance. Acta Physiol Plant 41:105. https://doi.org/10.1007/s11738-019-2883-0

Simard MH, Michelesi JC (2002) ’□Pyriam’□: A new pear rootstock. Acta Hortic 351–355. https://doi.org/10.17660/actahortic.2002.596.54

Sun W-Q, Bassuk NL (1991) Stem banding enhances rooting and subsequent growth of M.9 and MM.106 apple rootstock cuttings. HortScience 26:1368–1370. https://doi.org/10.21273/hortsci.26.11.1368

Trinh CS, Lee H, Lee WJ, et al (2018) Evaluation of the plant growth-promoting activity of *Pseudomonas nitroreducens* in *Arabidopsis thaliana* and *Lactuca sativa*. Plant Cell Rep 37:873–885. https://doi.org/10.1007/s00299-018-2275-8

538 Waqas M, Khan AL, Kamran M, et al (2012) Endophytic fungi produce gibberellins and indoleacetic acid
539 and promotes host-plant growth during stress. Molecules 17:10754–10773.
540 https://doi.org/10.3390/molecules170910754

541 Wiszniewska A, Nowak B, Kołton A, et al (2016) Rooting response of *Prunus domestica* L. microshoots
542 in the presence of phytoactive medium supplements. Plant Cell Tissue Organ Cult 125:163–176.
543 https://doi.org/10.1007/s11240-015-0937-6

544 Zamioudis C, Mastranesti P, Dhonukshe P, et al (2013) Unraveling root developmental programs initiated
545 by beneficial *Pseudomonas* spp. bacteria. Plant Physiol 162:304–318.
546 https://doi.org/10.1104/pp.112.212597

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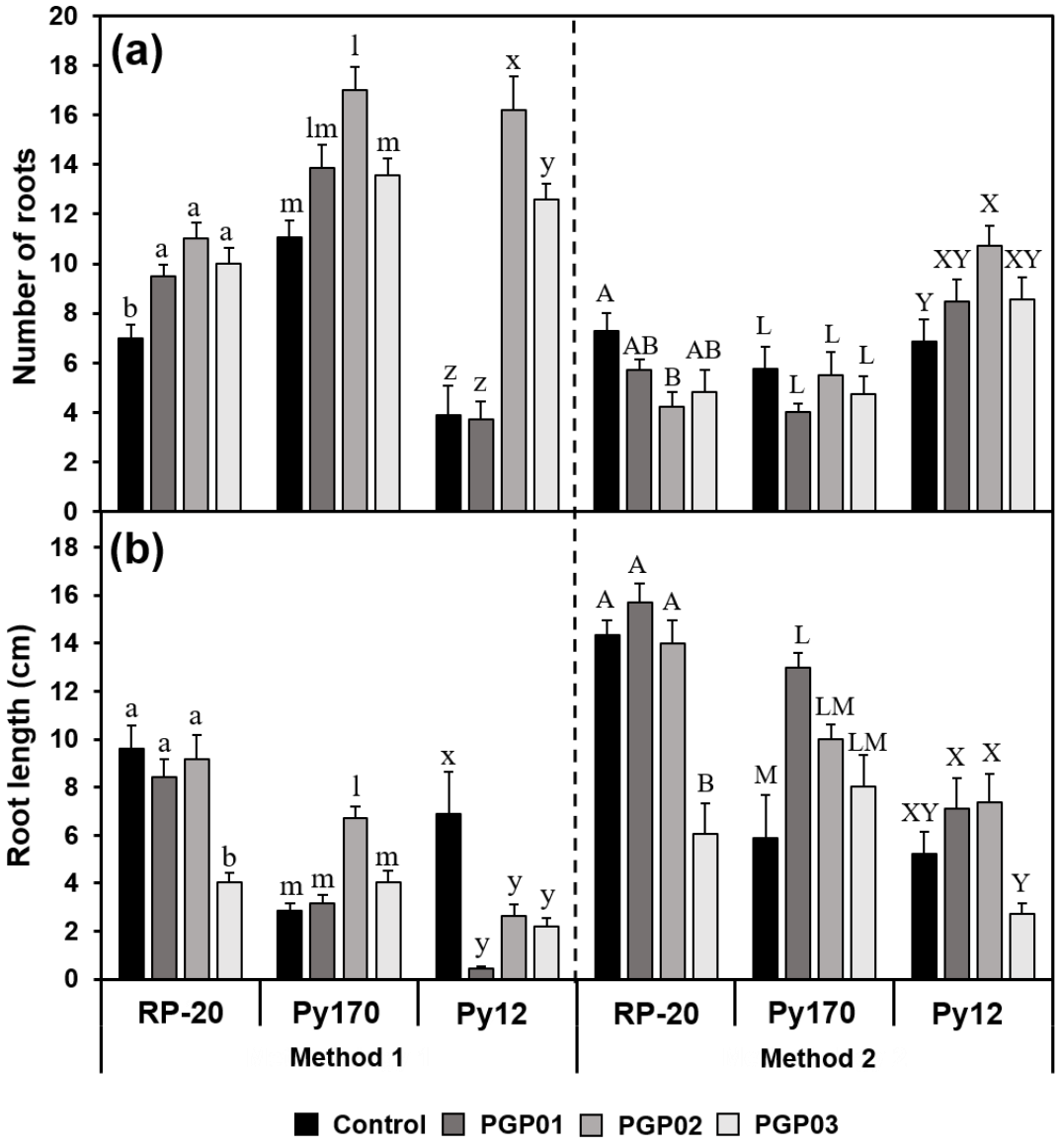


Fig. 1 Effects of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 on the number of roots (**a**) and root length (**b**) of *Prunus* RP-20 and *Pyrus* Py170 and Py12 plantlets after 8 weeks of co-culture and inoculation by two different methods. In method 1, the shoots were inoculated with microorganisms after root induction in 10 m*M* IBA, while in method 2, the shoots were immersed in microorganism suspensions without previous root induction with IBA. In all cases, the data represent the means ± SEs (standard errors) of at least fifteen shoots each. The means with different letters within each genotype are significantly different according to Tukey's test ($P < 0.05$).

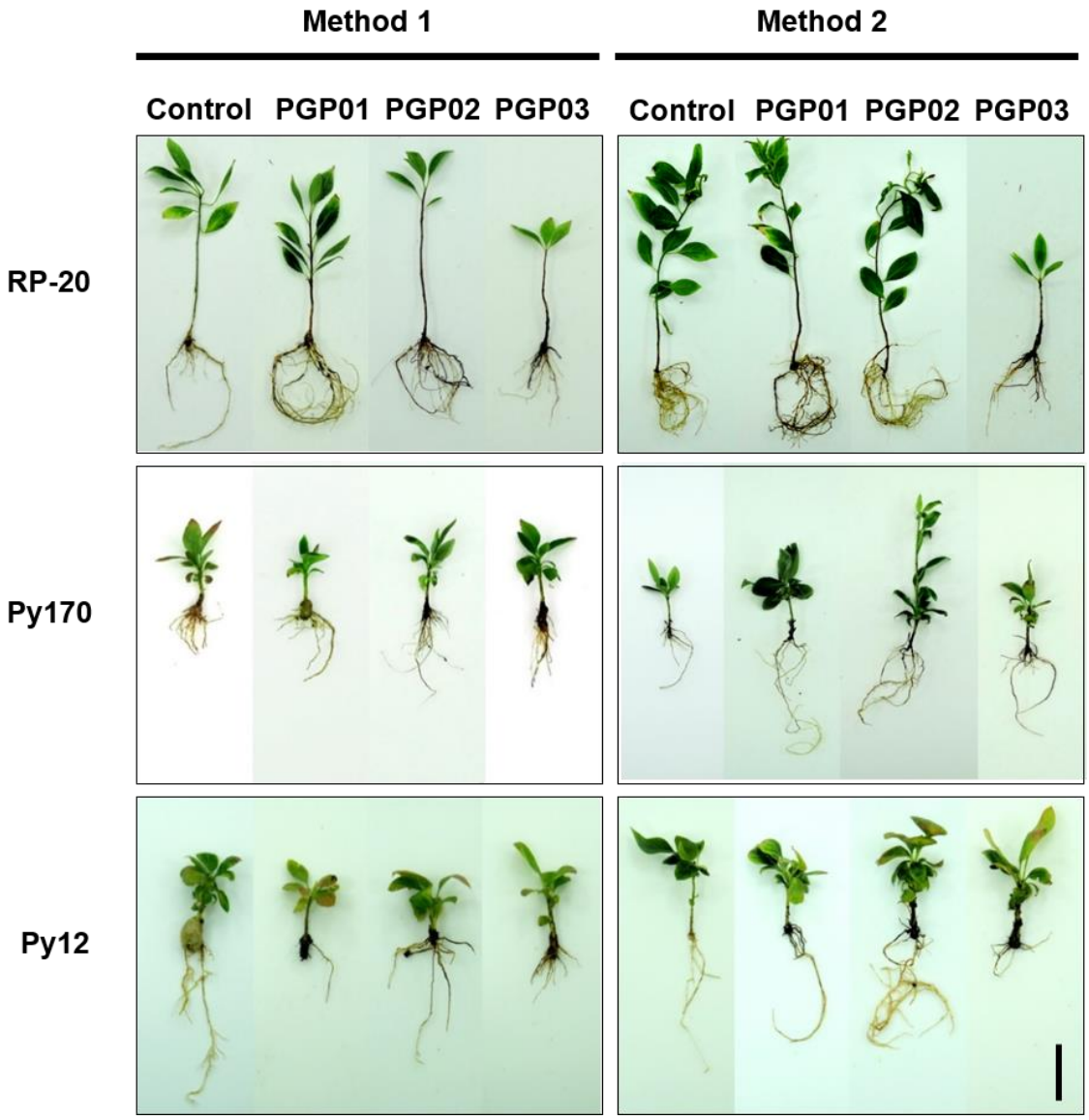


Fig. 2 Plantlets of *Prunus* RP-20 and *Pyrus* Py170 and Py12 after an 8-week-long co-culture with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03. The shoots were inoculated with microorganisms after root induction with 10 m*M* IBA (method 1) or immersed in microorganism suspensions without previous IBA root induction (method 2). The black bar is equivalent to 2 cm of length.

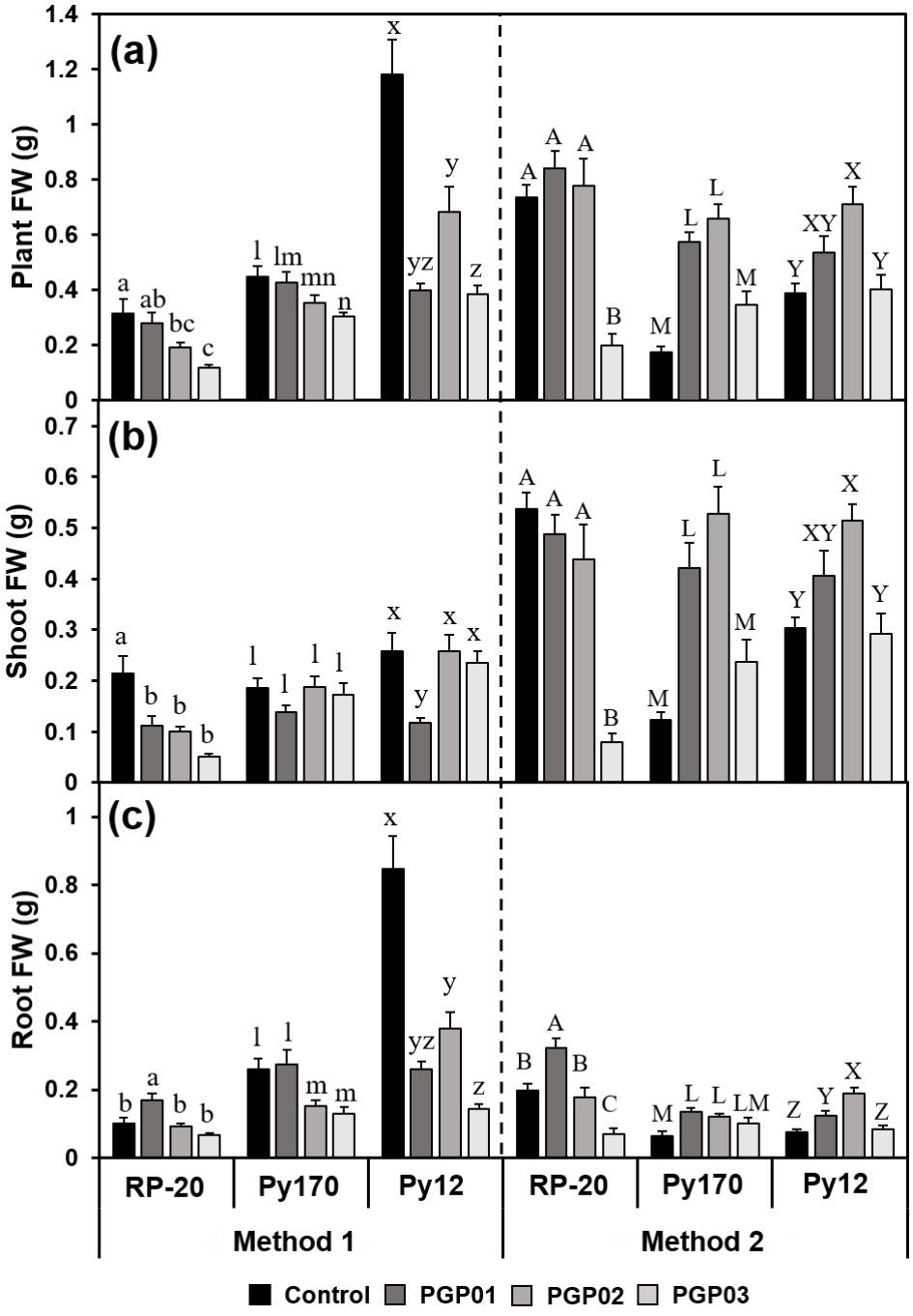
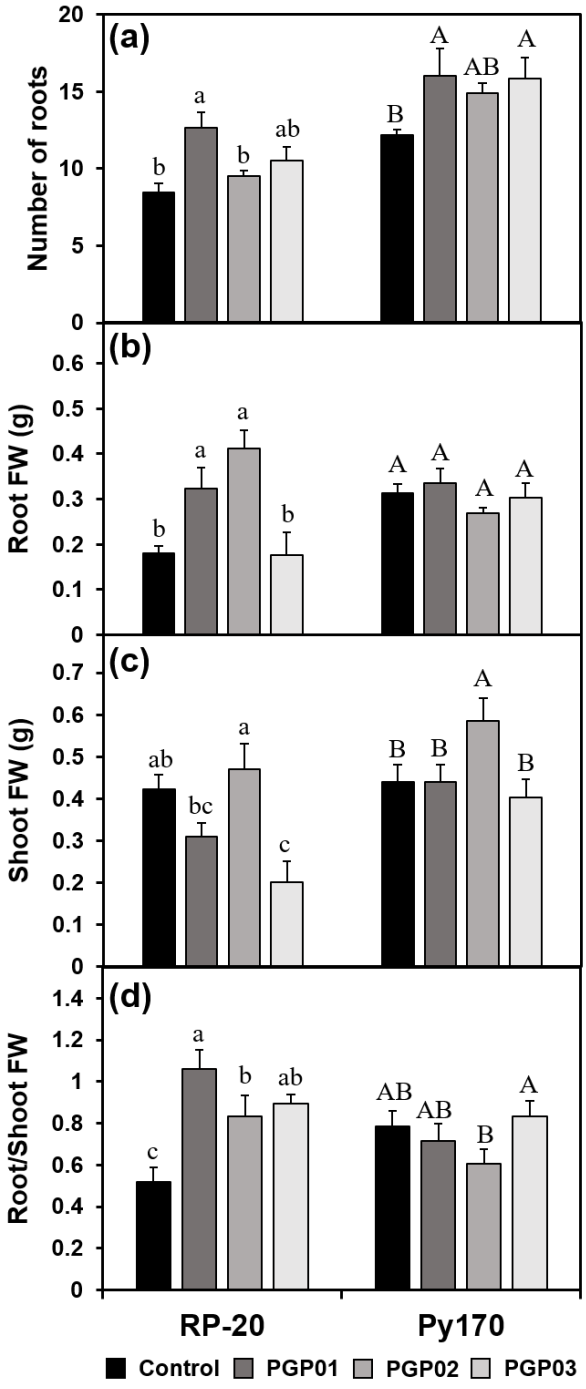


Fig. 3 Effects of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 on plant (**a**), stem (**b**) and root (**c**) FW of *Prunus* RP-20 and *Pyrus* Py170 and Py12 plantlets after 8 weeks of co-culture after inoculation through two different methods. In method 1, the shoots were inoculated with microorganisms after root induction in 10 m*M* IBA, while in method 2, the shoots were immersed in microorganism suspensions without previous root induction with IBA. In all cases, the data represent the means ± SEs of at least fifteen plants. The means with different letters within each genotype are significantly different according to Tukey's test ($P < 0.05$).



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578 **Fig. 4** Effects of inoculation with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03
579 on the number of roots (**a**), root FW (**b**), shoot FW (**c**) and root/stem FW ratio of *Prunus* RP-20 and *Pyrus*
580 Py170 fully developed plantlets. The different lowercase and uppercase letters refer to significant
581 differences between the control and treatments for RP-20 and Py170, respectively, according to Tukey's
582 test ($P < 0.05$).

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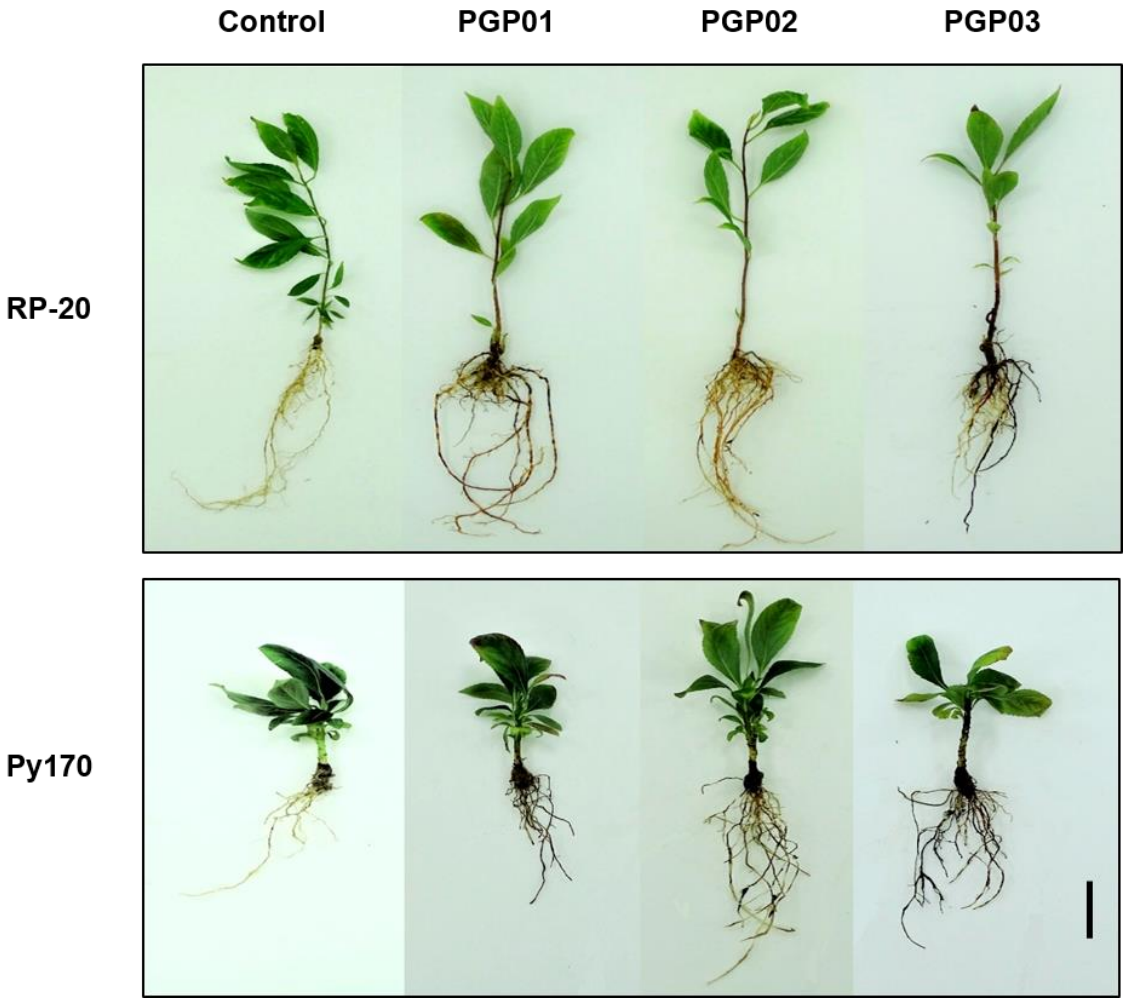

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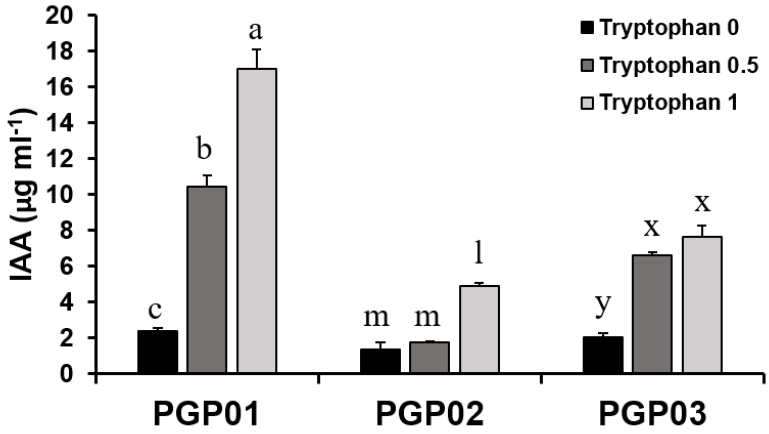
585 **Fig. 5** *Prunus* RP-20 and *Pyrus* Py170 plantlets after 8-week-long co-culture with *P. oryzihabitans* PGP01,

586 *C. ramotenellum* PGP02 and *Phoma* sp. PGP03. The black bar is equivalent to 2 cm of length.



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588 **Fig. 6** IAA production in *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* sp. PGP03 cultures.

589 Bacteria and fungi were grown in TSB and PDB media, respectively, supplemented with 0.5 (dark grey

bars) and 1 g l^{-1} tryptophan (light grey). Supernatants were collected after 24 and 168 h in the case of bacteria and fungi, respectively, and the absorbance at 530 was measured after 25 min of incubation with 2 ml of Salkowski reagent. The bars represent the means ± SEs of three replicates per treatment. The different lowercase and uppercase letters refer to significant differences between tryptophan-treated and non-treated media according to Tukey's test ($P < 0.05$).