Inoculation of in vitro cultures with rhizosphere microorganisms improve plant development and acclimatization during immature embryo rescue in nectarine and pear breeding programs.

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Abstract
In the present study, the two fungi *Cladosporium ramotenellum* strain PGP02 and *Phoma* spp. strain PGP03) and the bacterium *Pseudomonas oryzihabitans* PGP01 were isolated from *Pyrus* and *Prunus in vitro* rescued embryos, whose plantlets showed a better growth than non-contaminated cultures. Upon identification, concentrated solutions of the three microorganisms were applied to pear (*Pyrus communis* L.) *in vitro* rooted plantlets, increasing in different ways biometric parameters such as plant fresh weight (FW), stem length and root length. Then, these microorganisms were tested in embryos derived from three directed crosses between early ripening nectarine varieties (*Prunus persica* cv. Nectarina). In a first cross, in vitro cultured embryos were inoculated with both fungi, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03, at 2x10⁷ esp/mL, and the bacterium *P. oryzihabitans* PGP01, at 2x10⁸ CFU/mL. In the following crosses, only the bacterium *P. oryzihabitans* PGP01, at 2x10⁸ CFU/mL, was employed. The effects on number of germinated embryos, development of the subsequent plants, after 24 weeks of *in vitro* culture, as well as their *ex vitro* acclimatization performance were analysed. These microorganisms had no effect on the germination efficiency of nectarine embryos. However, the presence of the bacterium *P. oryzihabitans* PGP01 modified root system architecture in the three crosses, increasing root volume and thickness, which in consequence enhanced the acclimatization efficiency to soil in those crosses with poor acclimation efficiencies. These results enforce a breakthrough in the use of microorganisms along the in vitro embryo rescue used in early ripening peaches and nectarines breeding programmes, and the production of plants more resistant to the stressful conditions imposed by the acclimatization to soil.

**Keywords:** Acclimatization process; Bacterium; Fungi; *In vitro* embryo rescue; Plant growth promotion; Root morphology.
1. Introduction

Peach (*Prunus persica* L.) and pear (*Pyrus communis* L.) are two of the most popular fruit trees cultured in temperate growing regions (Heidari et al., 2019; Topp et al., 2008). In 2017, the worldwide production of both peach and pear exceeded the twenty millions of tonnes, being Spain one of the main producing countries (FAO 2017). Moreover, Catalonia ranks the first and second region of Spain in terms of surface of production of peach and pears, respectively (MAPAMA 2017). Given the above, the application of *in vitro* culture techniques are useful to provide large number of disease-free and true-to-type plant materials (Ko et al., 2018). In addition, *in vitro* embryo rescue under aseptic environment, is a valuable technique to provide of new genetic variation to modern peach breeding programs aiming to get new early ripening varieties (Batlle et al., 2012; Devi et al., 2017; Sundouri et al., 2014). By using this methodology, the poor germination caused by incomplete embryo and seed development is solved using an aseptic and nutrient-rich environment (Liu et al., 2007; Sinclair and Byrne, 2003). Currently, the culture in Woody Plant Medium (WPM) supplemented with sucrose and plant growth regulators (McCown and Lloyd, 1981), following of an stratification at 4-5 °C in dark conditions (Anderson and Byrne, 2002) is the most common practice to culture both small (5-10-mm-long) and large (>10 mm) embryos. To ensure the normal plant development, *in vitro* plantlets need to be transplanted to *ex vitro* conditions (Ko et al., 2018). Nevertheless, the acclimatization needs to be undertaken in a controlled plant growth environment since there are several external factors that seriously affect plant survival (Chandra et al., 2010; Maleki Asayesh et al., 2017).

Nowadays, the use of Plant Growth Promoting Microorganisms (PGPM), as biological sources to stimulate plant growth, represents an ecological alternative in the implementation of a sustainable agriculture (Vejan et al., 2016). Plant Growth Promoting Rhizobacteria (PGPR) are non-pathogenic microorganisms, present in soil and rhizosphere, that improve host plant growth through a large number of mechanisms, all of them related with the close contact to the root system. This interaction encourage some adaptations involving the increase of root length or the development of lateral roots (Della Mónica et al., 2018; Zamioudis et al., 2013). Moreover, in *Arabidopsis thaliana*, has been previously reported that changes in root morphology are associated with the production of some plant-growth related phytohormones, including auxins and ethylene (Contesto et al., 2010; Iqbal and Hasnain, 2013). As a consequence, the morphological changes occurred in plants by these microorganisms increase the uptake of nutrients from soils (Calvo et al., 2014; Collavino et al., 2010; Vessey, 2003), improvement of abiotic stress tolerance (Chu et al., 2019; Skz, 2018; Yang et al., 2009) and therefore, crop quality. Within the
PGPR cluster, *Pseudomonas* represents one of the most common genus of bacteria widely involved in atmospheric nitrogen (N\textsubscript{2}) fixation by legume-rhizobia symbioses (Vessey, 2003). Nevertheless, it has been recently shown that some species of this genus are involved in plant growth promotion by improving root development (Trinh et al., 2018; Zamioudis et al., 2013). Although most of the authors focus their attention on the implication of PGPR in plant growth, a significant group of Plant Growth Promoting Fungi (PGPF) also exists. In this case, the principal mechanisms through which these fungi increase plant growth include the production of hormones such as gibberellins and Volatile Organic Compounds (VOCs) (Hamayun et al., 2010, 2009; Naznin et al., 2013).

The work presented herein represents a first innovative approach that implies the introduction of microorganisms into an aseptic in vitro culture environment to stimulate woody plants growth and development. The aim of this research is to investigate whether three endophytic microorganisms (two fungi and one bacterium) isolated from *Pyrus* or *Prunus* immature embryos are able to improve the growth of in vitro plantlets obtained by in vitro embryo rescue. First, we applied these microorganisms to in vitro rooted pear plantlets to test their plant-growth promoting effect. Then, in vitro rescued nectarine embryos were inoculated with either one of the three microorganisms, and their impact on the percentage of germinated embryos, as well as on in vitro plantlet growth and the subsequent acclimatization of plants to greenhouse conditions were analysed.

2. Material and methods

2.1. *In vitro* plant material

Pear seedlings derived from directed crosses between *Pyrus communis* accessions belonging to the IRTA’s pear breeding program, oriented to produce new varieties, were used in the study. In addition, seeds from three different crosses between nectarine (*P. persica* cv Nectarina) varieties, also belonging to IRTA’s peach breeding program, were collected in different years and used for this study. Pear and nectarine seeds were extracted from cold stored fruits, harvested two weeks before commercial ripening stage in the IRTA’s Experimental Field Station at Gimenells (Lleida). Seeds extracted out of 10 fruits were placed in 50 mL Falcon tubes filled with distilled water, and washed twice before disinfection. This was done immersing and shaking the seeds for 15 minutes in a solution with 1% (w:v) NaOCl, followed by three 5-minute-long rinses in sterile doubled distilled water. Embryos were dissected out of the seed teguments, at naked eye or with the help of a dissecting scope, under the flow hood. Afterwards, embryos were cultured in Woody
Plant Medium (WPM) (McCown and Lloyd, 1981) supplemented with different hormonal combinations depending on embryo size and plant species. While nectarine embryos were cold stratified (3-5°C) under darkness and during 12 weeks, for seed germination, embryo dormancy of pear embryos was broken with culture in GA₃ containing medium.

2.2. In vitro culture media

In this study, pear embryos were germinated in WPM supplemented with 3% sucrose (Duchefa Biochemie, Haarlem, The Netherlands) and 1µM of GA₃, while nectarine embryos were cultured in the same media without hormones, but with vermiculite (50:40 v/v) (Dolcet-Sanjuan et al., 2017). The pH of the medium was adjusted to 5.7 using 1N NaOH prior the addition of 6 g/L gelling agar (Quimivita, Barcelona, Spain). Forty mL of medium was dispensed in each 38-mm-diameter tubes, with or without vermiculite. Media was then autoclaved at 121°C for 20 min, cooled down at room temperature, and stored at 14°C before culturing the embryos. Pear germinated embryos were transferred to tubes with WPM without hormones for plant development.

2.3. Preparation of microorganisms inocula

The two fungi and the one bacterium inocula used in the present experiment were originally isolated from contaminated Prunus and Pyrus in vitro cultured embryos. Their potential effect as PGPM was suspected since the resulting plantlets from contaminated in vitro cultured embryos showed a greater shoot, leaf and root growth than the non-contaminated ones (data not shown). Samples of contaminated culture media were taken and cultured in nutrient yeast dextrose agar (NYDA: nutrient broth, 8 g/L; yeast extract, 5 g/L; dextrose, 10 g/L; and agar, 20 g/L) and potato-dextrose agar (PDA: potato, 200 mL; glucose, 20 g/L; and agar, 20 g/L) to obtain pure cultures of the three microorganisms. Then, the selected strains, renamed as PGP01, PGP02 and PGP03, were identified as P. oryzihabitans, C. ramotenellum and Phoma spp. by the Instrumental techniques laboratory from University of León (Spain) and they were included in the microorganisms’ Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). After isolation, bacterium and fungi were preserved on NYDA and PDA plates, respectively, and stored in 20% glycerol at -80°C. Both bacterial and fungi inoculants were prepared in solutions of 160 mL which contained the microorganism and sterile water. Plate dilution technique on solid PDA + 25 ppm of the antibiotic gentamycin (for fungi) and NYDA (in case of the bacterium) media was used to determine the real colony forming unit (CFU)/mL. Moreover, conidia were also determined for fungi by haemocytometer. Finally,
the concentrations of the inocula were adjusted to 2x10^7 esp/mL for both fungi and 2x10^8 CFU/mL in the case of the bacterium.

2.4. Inoculation of in vitro cultured embryos with microorganisms and growth conditions

In the case of the first nectarine cross, four hundred in vitro rescued embryos were used, grouped in four treatments: (i) control non inoculated embryos, (ii) *P. oryzihabitans* strain PGP01, (iii) *C. ramotenellum* strain PGP02 and (iv) *Phoma* spp. strain PGP03-inoculated embryos. Each in vitro culture vessel containing one embryo was inoculated with 1 mL of the microorganism suspension, adding 1 mL of sterile water to those non-treated embryos (control). After inoculation, all embryos were submitted to a 12-week-long cold stratification period, in the dark and at 3 to 5°C (First stage). Seedlings starting germination were transferred to in vitro growth chambers, in which they were cultured for a 4-week-long period at 14°C and a 12h photoperiod (12h light / 12h dark), of cool white fluorescent lightening at 100-120 μEm^-2s^{-1} photoactive radiation (PAR) (Second stage). At the final in vitro plant growth phase (Third stage), which was 8-week-long, the culture temperature was increased to 24°C, and the photoperiod to 16h light, of the same intensity as in the previous stage.

For the two following crosses, considering the results observed in the first trial, the inoculation with only the bacteria *P. oryzihabitans* PGP01 was taken into account. As a control treatment 100 embryos were used, and 100 embryos were inoculated with bacteria, following the same procedure described above.

2.5. Embryo germination, plant morphometric and photosynthetic parameters

At the end of the second embryo germination stage, the percentage of germinated seeds, accounting for those with open cotyledons showing the embryo axis, was calculated. At the end of the third stage, the percentage of germinated seeds, with a growing shoot apex, was calculated.

At the final stage, pictures of the seedlings were taken and some morphological and photosynthetic characters were measured, including plant fresh weight (FW) in grams, total number of leaves, root volume (mL), stem length (cm), root length (cm) and photosystem II quantum yield (QY). Afterwards, plantlets were moved to the greenhouse for acclimatization into soil. Root volume was determined by volume of water displaced using a sectioned graded 25 mL cylinder. Root length was measured on the longest root from each plant. The QY parameter was determined using a PAR-FluorPen FP 100-MAX-LM (PSI spol. s.r.o., Drasov, Czech Republic), which reflects the maximum efficiency of photosystem II (PSII) or its
quantum efficiency when all the centres are open (Maxwell and Johnson, 2000). Changes in this parameter provide information about the state of plants under stress conditions, being the values considered standard those ranged between 0.64-0.83.

2.6. Microorganisms population dynamics

At the end of the second and third embryo germination stages, cell population of the three inoculated microorganisms was determined in order to assess population dynamics in parallel to the growth of the embryos. Samples of in vitro culture media along with the contained cultured embryo were removed from the culture tubes and were placed together into sterile plastic filter bags (BagPage 400 ml, Interscience BagSystem, St Nom la Brêtech, France) with 100 mL of water + Tween. Each bag was homogenized in a stomacher blender (Masticator Basic 400 mL, IUL SA, Torrent de l’Estadella, Barcelona, Catalonia, Spain) for 90 s at high speed. Serial ten-fold dilutions of the washings were made and plated on PDA + 25 ppm gentamycin plates for fungi and NYDA plates for bacterium. Plates were incubated at 25ºC and population dynamics were collected as CFU/mL.

2.7. Acclimatization of plants to greenhouse conditions

Nectarine rooted plantlets derived from the 24-week-long in vitro culture process, were transferred to 200 mL pots filled with peat and vermiculite (2:1, v:v). Plants were acclimated in plastic tunnels, within a conventional greenhouse, designed to gradually and automatically decrease the relative humidity (RH) from 100% to 60%, in a 4-week-long period. Acclimatization tunnels had a soil temperature above 22/18 ºC (day/night) and a photoperiod of 16h light, supplemented with LED lights (SUP12100DC, AlternativaLED, Spain) to extend the day light hours, with 230 µEm-2s-1 PAR at leaf level. At the end of this period, plantlet survival rate and growth, measuring its new shoot length (cm), were used to determine the plantlet performance during the acclimatization process.

2.8. Statistical analysis

The experiment was set up as a completely randomized design (CRD), and the data was analysed by a one-way ANOVA. Statistical significance was judged at the level \( P < 0.05 \). When the analysis was statistically significant, Student’s t-test was used for separation of means. Data analysis was performed using JMP Pro software (version 13.1.0, SAS Institute Inc., Cary, NC).
3. Results

In this study, three microorganisms (P. oryzihabitans PGP01, C. ramotenellum PGP02, and Phoma spp. PGP03) were tested under in vitro culture conditions, using in vitro fully developed pear seedlings in order to test their potential plant-growth promoting effect. Afterwards, three independent directed crosses of in vitro cultured nectarine embryos, collected in different years, were inoculated. The effects of the microorganism presence in the culture media on the in vitro embryo rescue efficiency, in terms of germination percentage and resulting seedlings development were studied. Moreover, the growth of the acclimated plants was also monitored after 4 weeks in acclimatization tunnels.

3.1. Effect of the inoculation with three microorganisms in pear in vitro rooted seedlings

Pear in vitro-rooted plantlets inoculated with each of the three microorganisms showed some preliminary plant-growth promoting effect (Fig. 1A and B). All of the microorganisms tested in the study induced a significant increase in plant FW when compared to control (199, 173 and 207% for P. oryzihabitans PGP01, Phoma spp. PGP03 and C. ramotenellum PGP02, respectively) (Fig. 1A). Moreover, a significant 125% rise in comparison to non-inoculated plantlets was recorded in stem length in those plantlets inoculated with P. oryzihabitans PGP01 (Fig. 1A). Although an important increase in this parameter was also observed in C. ramotenellum PGP02-inoculated plantlets (50% regarding to control), the differences found were not statistically significant from the control (Fig. 1A). On the other hand, whereas the application of P. oryzihabitans PGP01 and Phoma spp. PGP03 did not produce significant changes in root length, the inoculation of pear plantlets with C. ramotenellum PGP02 displayed a strong increase in root length (259% when compared with control plantlets) (Fig. 1A). It is noteworthy to mention the thicker and lignified aspect of the roots in those plantlets inoculated with P. oryzihabitans PGP01 compared with the non-inoculated plantlets or those inoculated with either of the other two microorganisms (Fig. 1B). Moreover, these roots had a darker colour as well, probably due to the close contact between root and bacterium.

3.2. Effect of the inoculation with three microorganisms in nectarine embryos emergence

The percentage of germinated nectarine embryos (inoculated and non-inoculated) along with the population dynamics of the three microorganisms (P. oryzihabitans PGP01, C. ramotenellum PGP02 and Phoma spp. PGP03) at the end of each culture period (12, 16 and 24 weeks after embryos inoculation) are
shown in Fig. 2. None of the three microorganisms induced an improvement in the germination of nectarine embryos, since for the crosses used, after the 24-week-long in vitro embryo rescue protocol, almost 100% of the control embryos were germinated (Fig. 2B). Indeed, both fungi (C. ramotenellum PGP02 and Phoma spp. PGP03) reduced the percentage of germinated embryos (Fig. 2B). After 12 weeks of culture, at the end of the first stage at 4°C, while control and P. oryzihabitans PGP01 inoculated embryos had exceeded the 50% of germination, similar to the control, none of the embryos treated with C. ramotenellum PGP02 had germinated, and those inoculated with Phoma spp. PGP03 had reached only 27% of germination. Related to the growth of the microorganisms in the culture media, it is worthy to mention that the stratification phase (12 weeks at 4°C and darkness) induced a decrease on the P. oryzihabitans PGP01 population, reaching values of almost 3.5 Log (CFU/mL) (Fig. 2A). However, the growth of this bacterium was highly recovered at the end of the second phase (14°C, 16-h photoperiod), obtaining values of 7.20 Log (CFU/mL), which remains unchanged until the end of the in vitro culture process. Conversely, a time-depending increase in the populations of the two fungi was observed at 12 and 16 weeks. However, after 24 weeks of the in vitro culture process, Phoma spp. PGP03 population increased 0.86 Log (CFU/mL) while C. ramotenellum PGP02 population showed a 0.44 Log (CFU/mL) decrease with regard to the previous period.

Considering the results obtained in this first trial, only the bacterium P. oryzihabitans PGP01 was used in the next assays to inoculate the nectarine in vitro rescued embryos, and the same response was reported for both germination percentage and dynamics population (data not shown).

3.3. Effect of the inoculation of embryos with three microorganisms on growth and development of resulting nectarine seedlings

Morphometric (FW, number of leaves, stem and root length and root volume), and photosynthetic (QY) parameters were measured in nectarine seedlings derived from immature embryos rescued in different years through a 24-week-long in vitro culture process (Fig. 3). Compared with the control, no inoculated embryos, inoculation with the bacterium P. oryzihabitans PGP01 did not affect nectarine seedlings growth since effects on FW were not detected (Fig. 3A). By contrast, plantlets from embryos inoculated with both fungi, Phoma spp. and C. ramotenellum PGP02, were drastically affected, detecting reductions of almost 70% in both treatments (Fig. 3A). The treatment with the three microorganisms resulted in plants with minor number of leaves than non-inoculated (control) seedlings (Fig. 3B). In this case, Phoma PGP03-treated plantlets showed the lowest values, detecting a decrease of 64% in comparison to control (Fig. 3B).
Regarding stem length, inoculation with the three microorganisms caused a decrease in this parameter, being less affected by the bacterium *P. oryzihabitans* PGP01 inoculated plants (22% lower than control) than for fungi inoculated plants (56% less than control plants for both fungi) (Fig. 3C). The most prominent effect of the inoculation of embryos with the bacterium *P. oryzihabitans* PGP01, was that nectarine plantlets exhibited an increase of 61% in root volume when compared to control; while, the presence of fungi, *Phoma* spp. PGP03 or *C. ramotenellum* PGP02, resulted in seedlings with significant lower root volume (Fig. 3D). All the inoculated seedlings displayed a statistically significant shorter and compact root system as shown by the measures of maximum root length (Fig. 3E). The shortest roots were detected in *C. ramotenellum* PGP02-treated plantlets, which exhibited a decrease up to 60% compared to the control (Fig. 3E). Significant morphological differences were observed on the produced nectarine plantlets, those plantlets derived from culture media inoculated with *P. oryzihabitans* PGP01 showed thicker roots than non-inoculated plantlets (Fig. 4), a similar effect to that observed in pear plantlets (Fig. 1B). While no statistically significant differences in QY were found in *C. ramotenellum* PGP02 and *Phoma* spp. PGP03-inoculated seedlings, compared with the control, a slight significant decrease in this parameter was registered in *P. oryzihabitans* PGP01-treated plants (6% than control, approximately) (Fig. 3F). These reduced values are in concordance with the fact that these plants had not as green leaves as control and *C. ramotenellum* PGP02-plants (Fig. 4).

When inoculation of in vitro cultured embryos was done in two additional nectarine crosses, seedlings from embryos inoculated with *P. oryzihabitans* PGP01 displayed reduced values of all biometrical and photosynthetic parameters analysed except for root volume at the end of the in vitro embryo rescue process. Higher root volume values were observed in both crosses, being statistically significant in one of them (Fig. 5B). Nevertheless, the *P. oryzihabitans*-inoculated plantlets of the two nectarine crosses displayed thicker and shorted roots than non-inoculated seedlings (Fig. 5C and D), as previously observed (Fig. 4), most likely due to the activity of the bacterium during the seedling root development.

### 3.4. Effect of the in vitro inoculation of nectarine embryos on the acclimatization of seedlings

In the first experiment using nectarine embryos, the inoculation with the three microorganisms resulted in plants more resistant to acclimatization in the greenhouse, displaying survival rates of 88, 85 and 87% for *C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01, respectively, against the 71% recorded for the control plants (Fig. 6A). These percentages were moderately significantly
better in the case of *Phoma* spp. PGP03 (p – value=0.020) and *C. ramotenellum* PGP02 (p – value=0.013), and highly significantly for the *P. oryzihabitans* PGP01-inoculated plants (p – value=0.005). Moreover, this improved survival rate did correlate with an increase of 92% on the new shoot length in the *P. oryzihabitans* PGP01 acclimated plants when compared with control (Fig. 6B). An inhibition of the shoot growth under acclimatization conditions occurred in those plants from embryos inoculated with *C. ramotenellum* PGP02 and *Phoma* spp. PGP03, observing decreases of 55 and 70% regarding to control, respectively (Fig. 6B). In the following trials with two different nectarine genotypes, the survival rates after the 4-week-long acclimatization process were higher, but not statistically significant, for plantlets inoculated with *P. oryzihabitans* PGP01 than the control ones (data not shown). This could be explained by a plant genotype effect on the acclimatization survival rates.

The differential response among microorganisms might be explained by the morphological changes occurred in plantlets from embryos inoculated with *P. oryzihabitans* PGP01 that provided a thicker root system related with a larger capacity to deal with *ex vitro* conditions, and therefore, a better resilience under stressful conditions.

4. Discussion

The three microorganisms (two fungi and a bacterium) tested in the present study were obtained from immature *Pyrus* and *Prunus* embryos, rescued under *in vitro* conditions, following the described sterilization and culture process. Some of those in vitro contaminated embryos resulted in plantlets with a better growth than not contaminated embryos. After their posterior isolation and identification, the impact of these endophytic microorganisms on *in vitro* rooted pear plantlets was analysed, and the three microorganisms showed an important plant-growth promoting effect on this plant material improving one or two of the three parameters analysed (fresh weight, stem and root length). The use of microorganisms with an endophytic origin to improve plant growth have been previously described in plants under greenhouse conditions (Dias et al., 2009). However, their application in an aseptic *in vitro* environment is being recently implemented (Perez-Rosales et al., 2018; Regalado et al., 2018), and the information found in the literature is scarce, even more among woody plants grown *in vitro*. For instance, Kavino and Manoranjitham (2018) and Rajamanickam et al. (2018) used endophytic bacterial strains isolated from different parts of banana field plants to induce resistance against pathogens in banana *in vitro* manipulated plantlets.
Given the potential effect on plant growth seen in the preliminary experiment with *Pyrus* rooted plants, the effects of these microorganisms on the *in vitro* embryo germination and plantlet growth from three different crosses of nectarine were studied. The *in vitro* rescue of embryos is being increasingly applied in peach breeding programmes to widen the diversity of commercially productive early ripening varieties with a higher commercial interest (Mancuso et al., 2002; Batlle et al., 2012). Herein, almost 100% of the rescued nectarine embryos, in the three crosses, sprouted at the end of the *in vitro* culture process, in contrast with the 70% of germination observed in interspecific hybrid embryos of peach and plum (Liu et al., 2007). This procedure allows to increase the germination of these immature embryos, that otherwise would degenerate, by culturing them in an aseptic and nutritionally enriched environment (Sinclair and Byrne, 2003). On the other hand, although there is much evidence that the *in vitro* application of microorganisms could influence the propagation or *in vitro* rooting (Kavino and Manoranjitham, 2018; Quambusch et al., 2014), the knowledge regarding the inoculation with beneficial microorganisms to enhance the *in vitro* germination of rescued embryos and posterior seedling development is currently rare.

In our case, none of the three microorganisms applied in the first experiment improved nectarine embryos sprouting, mostly due to the high effectiveness of the *in vitro* rescue procedure mentioned above. Nevertheless, the most notable finding is that while *C. ramotenellum* PGP02 and *Phoma* spp. PGP03-inoculated embryos showed a negative effect on germination, no detrimental effect was reported in the case of *P. oryzihabitans*-inoculated embryos. The key difference of this behaviour lies in the growth of these microorganisms in the plant *in vitro* culture medium. Whereas the growth of the *P. oryzihabitans* PGP01 was highly recovered after 16 and 24 weeks of *in vitro* culture, the low temperatures (4ºC) of the stratification phase dramatically affected the growth of this microorganism, which is quite logical because most of bacteria grow better at high temperatures (Dobrić and Bååth, 2018). By contrast, these conditions along with the important concentration of sugar present in the culture medium favoured the development of both fungi, negatively affecting in most cases the embryo germination. This fact explained the results obtained at the end of the *in vitro* rescue in terms of morphological parameters, and helped us to discard the inoculation with fungi for the experiments with the following nectarine crosses. In these experiments, the same behaviour of *P. oryzihabitans* PGP01 in the culture medium was observed, with an important decrease of its growth after the stratification phase at 4ºC, and a rapid recovery when the embryos were transferred to 14ºC. Moreover, *P. oryzihabitans* PGP01 in the culture medium did not affect embryo
germination percentage, suggesting that there is no interference of the microorganism with the embryo physiology during the stratification at 4°C.

At the end of the *in vitro* embryo rescue process, we found that the inoculation of nectarine embryos with *P. oryzihabitans* PGP01 produced shorter, thicker and more vigorous roots in all three crosses studied. Modifications in the root architecture system in response to the inoculation with PGPR represents one of the main mechanisms to induce plant growth (Mantelin et al., 2005; Wang et al., 2016). In other studies using soybean and tomato as plant material, the inoculation with *P. oryzihabitans* led to a higher root biomass that non-inoculated plants (Belimov et al., 2015; Kuzmicheva et al., 2017). However, most of the studies related to the effect of the application of bacteria on root development were conducted using the model *A. thaliana* plants cultured *in vivo*. The inoculation with *Bacillus megaterium* and some strains of *Pseudomonas* inhibited the elongation of primary roots, but stimulate the formation of lateral roots in wild-type plants (López-Bucio et al., 2007; Zamioudis et al., 2013). In contrast, the application of *Aeromonas punctata* stimulated the growth of primary root and lateral root density of Arabidopsis plants (Iqbal and Hasnain, 2013). Furthermore, in the present study, the plantlets inoculated with *P. oryzihabitans* PGP01 also showed reduced values of QY. The chlorophyll fluorescence parameters are a useful approach to predict changes in the status of the plant photosynthetic apparatus under different stress situations (Cantabella et al., 2017; Clemente-Moreno et al., 2015). The activity of the photosystem II (PSII) is strongly associated with root formation (Ślesak et al., 2017). Considering this, the alteration observed in QY for *P. oryzihabitans* PGP01-inoculated nectarine seedlings could be likely linked to the higher root development registered after 24-week-long *in vitro* culture process.

Regarding the acclimatization of seedlings from control and inoculated nectarine embryos to greenhouse conditions, the application of microorganisms produced more resistant plants with higher survival rate values than control plants. These results were most remarkable for the seedlings from embryos inoculated with *P. oryzihabitans* PGP01 since a strong stimulation of the new shoot growth after 4 weeks of acclimatization was registered. This better performance of plants derived from *P. oryzihabitans* PGP01-inoculated embryos was not observed in two other nectarine genotypes, for which almost 100% of control plants survived to acclimatization in greenhouse conditions. This suggests that the genotype of the nectarine embryos interacts with *P. oryzihabitans* PGP01 on the survival and growth response during acclimatization, since this is a physiologically complex process, highly influenced by several environmental factors including humidity, temperature, light, CO₂ or nutrient levels (Chen, 2004; Hazarika, 2006; Tisarum et al.,
The awareness of using beneficial microorganisms on *in vitro* plants lies in overcoming the great losses of plant material recorded at the acclimatization stage (Orlikowska et al., 2017). Trivedi and Pandey (2007) demonstrated that the inoculation *Picrorhiza kurrooa* plantlets with three PGPR increased plant survival as well as growth parameters in a greenhouse environment. Similarly, banana hardened plants bacterized with two strains of *Bacillus subtilis* displayed a 100% of survival plants in comparison to 89% recorded for control treatment (Rajamanickam et al., 2018). Most studies using the term “biohardening” involve the application of microorganisms on acclimated plantlets to ensure plant establishment under acclimatization conditions (Harish et al., 2008; Yadav et al., 2013). In consideration with the above, the results presented in this work should be treated as an outcome of the *in vitro* inoculation and co-culture of embryos with this microorganism, *P. oryzihabitans* PGP01, and not as an independent effect of the inoculation with this bacterium on the acclimatization process. Moreover, plant defence mechanisms induced by PGPR could contribute to a success in plant endurance (Chandra et al., 2010). Nevertheless, in this study, the mechanisms of action of these microorganisms have not been studied, being this point the focus of a future research.

6. Conclusions

To sum up, among the three microorganisms (*P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03) with a plant growth promoting effect in pear *in vitro* rooted plantlets, *P. oryzihabitans* PGP01 had no detrimental effects on the *in vitro* embryo rescue efficiency of three early ripening nectarine crosses. Even so, the subsequent seedlings from the embryos inoculated with *P. oryzihabitans* PGP01 displayed highly significant modifications in root morphology that improved the acclimatization to *ex vitro* conditions of those genotypes with difficult adaptability to the acclimation process. This project could mean the first step of a significant change in woody plants breeding programmes favouring the adaptation of plants whose endurance under greenhouse conditions resulted complex. However, further investigations in this regard will be required in order to understand the mechanisms underlying the plant growth promotion induced by the three microorganisms tested as well as the strong root development and better survival observed in plants inoculated with *P. oryzihabitans* PGP01.

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Figure 1.- Effects of the application of three microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01) on growth parameters (A) and morphology (B) of pear rooted plantlets growing *in vitro*. Data represents the mean ± SE of at least three measurements. Different letters on bars of the same chard indicate significant differences according to the Student’s t-Test (*p* ≤ 0.05).
Figure 2.- Microorganism population dynamics (A) and germination percentage of in vitro rescued nectarine embryos (B), following the three culture phases (12, 16 and 24 weeks) of in vitro culture. Inoculation was done at day 0 with three microorganisms (C. ramotenellum PGP02, Phoma spp. PGP03, P. oryzihabitans PGP01). Data of population dynamics represent the mean ± SE from at least three measurements.
Figure 3.- Effects of the in vitro co-culture of nectarine embryos with three microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01) on (A) total plantlet fresh weight (FW), (B) total number of leaves, (C) stem length, (D) total root volume, (E) maximum root length and (F) photosynthetic activity or QY, at the end of the 24-week-long *in vitro* culture process. Different letters on bars of the same chard denote significant differences according to the Student’s t-Test (p ≤ 0.05).
Figure 4.- Morphological differences among nectarine plantlets derived from in vitro immature embryo rescue, after a 24-week-long in vitro co-culture process with three microorganisms (C. ramotenellum PGP02, Phoma spp. PGP03 and P. oryzihabitans PGP01).
Figure 5.- Root volume values (A,B) and morphological differences (C,D) of nectarine plantlets from two independent crosses, after a 24-week-long *in vitro* embryo rescue process, inoculated or not with the bacteria *P. oryzihabitans* PGP01.
Figure 6.- Percentage of survival rate and length of the new shoot formed of the resulting plants obtained from nectarine embryos inoculated with the three microorganisms (*C. ramotenellum PGP02, Phoma spp. PGP03 and P. oryzihabitans PGP01*) after 4 weeks on acclimation tunnels. Asterisk symbol (*) means significant differences between treatments and control according to the Fisher’s Exact Test (p ≤ 0.05). Different letters denote significant differences according to Student’s t-Test (p ≤ 0.05).