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

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21 **Abstract**

22 In the present study, the two fungi *Cladosporium ramotenellum* strain PGP02 and *Phoma* spp. strain
23 PGP03) and the bacterium *Pseudomonas oryzihabitans* PGP01 were isolated from *Pyrus* and *Prunus in*
24 *vitro* rescued embryos, whose plantlets showed a better growth than non-contaminated cultures. Upon
25 identification, concentrated solutions of the three microorganisms were applied to pear (*Pyrus communis*
26 L.) *in vitro* rooted plantlets, increasing in different ways biometric parameters such as plant fresh weight
27 (FW), stem length and root length. Then, these microorganisms were tested in embryos derived from three
28 directed crosses between early ripening nectarine varieties (*Prunus persic*a cv. Nectarina). In a first cross,
29 in vitro cultured embryos were inoculated with both fungi, *C. ramotenellum* PGP02 and *Phoma* spp.
30 PGP03, at $2x10^7$ esp/mL, and the bacterium *P. oryzihabitans* PGP01, at $2x10^8$ CFU/mL. In the following
31 crosses, only the bacterium *P. oryzihabitans* PGP01, at $2x10^8$ CFU/mL, was employed. The effects on
32 number of germinated embryos, development of the subsequent plants, after 24 weeks of *in vitro* culture,
33 as well as their *ex vitro* acclimatization performance were analysed. These microorganisms had no effect
34 on the germination efficiency of nectarine embryos. However, the presence of the bacterium *P.*
35 *oryzihabitans* PGP01 modified root system architecture in the three crosses, increasing root volume and
36 thickness, which in consequence enhanced the acclimatization efficiency to soil in those crosses with poor
37 acclimation efficiencies. These results enforce a breakthrough in the use of microorganisms along the in
38 vitro embryo rescue used in early ripening peaches and nectarines breeding programmes, and the production
39 of plants more resistant to the stressful conditions imposed by the acclimatization to soil.

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

42 **1. Introduction**

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

60 Nowadays, the use of Plant Growth Promoting Microorganisms (PGPM), as biological sources to
61 stimulate plant growth, represents an ecological alternative in the implementation of a sustainable
62 agriculture (Vejan et al., 2016). Plant Growth Promoting Rhizobacteria (PGPR) are non-pathogenic
63 microorganisms, present in soil and rhizosphere, that improve host plant growth through a large number of
64 mechanisms, all of them related with the close contact to the root system. This interaction encourage some
65 adaptations involving the increase of root length or the development of lateral roots (Della Mónica et al.,
66 2018; Zamioudis et al., 2013). Moreover, in *Arabidopsis thaliana,* has been previously reported that
67 changes in root morphology are associated with the production of some plant-growth related
68 phytohormones, including auxins and ethylene (Contesto et al., 2010; Iqbal and Hasnain, 2013). As a
69 consequence, the morphological changes occurred in plants by these microorganisms increase the uptake
70 of nutrients from soils (Calvo et al., 2014; Collavino et al., 2010; Vessey, 2003), improvement of abiotic
71 stress tolerance (Chu et al., 2019; Skz, 2018; Yang et al., 2009) and therefore, crop quality. Within the

72 PGPR cluster, *Pseudomonas* represents one of the most common genus of bacteria widely involved in
73 atmospheric nitrogen (N_2) fixation by legume-rhizobia symbioses (Vessey, 2003). Nevertheless, it has been
74 recently shown that some species of this genus are involved in plant growth promotion by improving root
75 development (Trinh et al., 2018; Zamioudis et al., 2013). Although most of the authors focus their attention
76 on the implication of PGPR in plant growth, a significant group of Plant Growth Promoting Fungi (PGPF)
77 also exists. In this case, the principal mechanisms through which these fungi increase plant growth include
78 the production of hormones such as gibberellins and Volatile Organic Compounds (VOCs) (Hamayun et
79 al., 2010, 2009; Naznin et al., 2013).

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

89 **2. Material and methods**

90

91 *2.1. In vitro plant material*

92 Pear seedlings derived from directed crosses between *Pyrus communis* accessions belonging to the
93 IRTA's pear breeding program, oriented to produce new varieties, were used in the study. In addition, seeds
94 from three different crosses between nectarine (*P. persica* cv Nectarina) varieties, also belonging to IRTA's
95 peach breeding program, were collected in different years and used for this study. Pear and nectarine seeds
96 were extracted from cold stored fruits, harvested two weeks before commercial ripening stage in the IRTA's
97 Experimental Field Station at Gimenells (Lleida). Seeds extracted out of 10 fruits were placed in 50 mL
98 Falcon tubes filled with distilled water, and washed twice before disinfection. This was done immersing
99 and shaking the seeds for 15 minutes in a solution with 1% (w:v) NaOCl, followed by three 5-minute-long
100 rinses in sterile doubled distilled water. Embryos were dissected out of the seed teguments, at naked eye or
101 with the help of a dissecting scope, under the flow hood. Afterwards, embryos were cultured in Woody

102 Plant Medium (WPM) (McCown and Lloyd, 1981) supplemented with different hormonal combinations
103 depending on embryo size and plant species. While nectarine embryos were cold stratified (3-5ºC) under
104 darkness and during 12 weeks, for seed germination, embryo dormancy of pear embryos was broken with
105 culture in GA_3 containing medium.

106 *2.2. In vitro culture media*

107 In this study, pear embryos were germinated in WPM supplemented with 3% sucrose (Duchefa
108 Biochemie, Haarlem, The Netherlands) and 1µM of GA_3, while nectarine embryos were cultured in the
109 same media without hormones, but with vermiculite (50:40 v/v) (Dolcet-Sanjuan et al., 2017). The pH of
110 the medium was adjusted to 5.7 using 1N NaOH prior the addition of 6 g/L gelling agar (Quimivita,
111 Barcelona, Spain). Forty mL of medium was dispensed in each 38–mm-diameter tubes, with or without
112 vermiculite. Media was then autoclaved at 121°C for 20 min, cooled down at room temperature, and stored
113 at 14ºC before culturing the embryos. Pear germinated embryos were transferred to tubes with WPM
114 without hormones for plant development.

115 *2.3. Preparation of microorganisms inocula*

116 The two fungi and the one bacterium inocula used in the present experiment were originally
117 isolated from contaminated *Prunus* and *Pyrus in vitro* cultured embryos. Their potential effect as PGPM
118 was suspected since the resulting plantlets from contaminated in vitro cultured embryos showed a greater
119 shoot, leaf and root growth than the non-contaminated ones (data not shown). Samples of contaminated
120 culture media were taken and cultured in nutrient yeast dextrose agar (NYDA: nutrient broth, 8 g/L; yeast
121 extract, 5 g/L; dextrose, 10 g/L; and agar, 20 g/L) and potato-dextrose agar (PDA: potato, 200 mL; glucose,
122 20 g/L; and agar, 20 g/L) to obtain pure cultures of the three microorganisms. Then, the selected strains,
123 renamed as PGP01, PGP02 and PGP03, were identified as *P. oryzihabitans*, *C. ramotenellum* and *Phoma*
124 spp. by the Instrumental techniques laboratory from University of León (Spain) and they were included in
125 the microorganisms' Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). After
126 isolation, bacterium and fungi were preserved on NYDA and PDA plates, respectively, and stored in 20%
127 glycerol at -80ºC. Both bacterial and fungi inoculants were prepared in solutions of 160 mL which contained
128 the microorganism and sterile water. Plate dilution technique on solid PDA + 25 ppm of the antibiotic
129 gentamycin (for fungi) and NYDA (in case of the bacterium) media was used to determine the real colony
130 forming unit (CFU)/mL. Moreover, conidia were also determined for fungi by haemocytometer. Finally,

131 the concentrations of the inocula were adjusted to $2x10^7$ esp/mL for both fungi and $2x10^8$ CFU/mL in the
132 case of the bacterium.

133 *2.4. Inoculation of in vitro cultured embryos with microorganisms and growth conditions*

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

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

148 *2.5. Embryo germination, plant morphometric and photosynthetic parameters*

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

152 At the final stage, pictures of the seedlings were taken and some morphological and photosynthetic
153 characters were measured, including plant fresh weight (FW) in grams, total number of leaves, root volume
154 (mL), stem length (cm), root length (cm) and photosystem II quantum yield (QY). Afterwards, plantlets
155 were moved to the greenhouse for acclimatization into soil. Root volume was determined by volume of
156 water displaced using a sectioned graded 25 mL cylinder. Root length was measured on the longest root
157 from each plant. The QY parameter was determined using a PAR-FluorPen FP 100-MAX-LM (PSI spol.
158 s.r.o., Drasov, Czech Republic), which reflects the maximum efficiency of photosystem II (PSII) or its

159 quantum efficiency when all the centres are open (Maxwell and Johnson, 2000). Changes in this parameter
160 provide information about the state of plants under stress conditions, being the values considered standard
161 those ranged between 0.64-0.83.

162 *2.6. Microorganisms population dynamics*

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

172 *2.7. Acclimatization of plants to greenhouse conditions*

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

181 *2.8. Statistical analysis*

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

186

187 **3. Results**

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

195 *3.1. Effect of the inoculation with three microorganisms in pear in vitro rooted seedlings*

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

211 *3.2. Effect of the inoculation with three microorganisms in nectarine embryos emergence*

212 The percentage of germinated nectarine embryos (inoculated and non-inoculated) along with the
213 population dynamics of the three microorganisms (*P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and
214 *Phoma* spp. PGP03) at the end of each culture period (12, 16 and 24 weeks after embryos inoculation) are

215 shown in Fig. 2. None of the three microorganisms induced an improvement in the germination of nectarine
216 embryos, since for the crosses used, after the 24-week-long *in vitro* embryo rescue protocol, almost 100%
217 of the control embryos were germinated (Fig. 2B). Indeed, both fungi (*C. ramotenellum* PGP02 and *Phoma*
218 spp. PGP03) reduced the percentage of germinated embryos (Fig. 2B). After 12 weeks of culture, at the end
219 of the first stage at 4ºC, while control and *P. oryzihabitans* PGP01 inoculated embryos had exceeded the
220 50% of germination, similar to the control, none of the embryos treated with *C. ramotenellum* PGP02 had
221 germinated, and those inoculated with *Phoma* spp. PGP03 had reached only 27% of germination. Related
222 to the growth of the microorganisms in the culture media, it is worthy to mention that the stratification
223 phase (12 weeks at 4ºC and darkness) induced a decrease on the *P. oryzihabitans* PGP01 population,
224 reaching values of almost 3.5 Log (CFU/mL) (Fig. 2A). However, the growth of this bacterium was highly
225 recovered at the end of the second phase (14ºC, 16-h photoperiod), obtaining values of 7.20 Log (CFU/mL),
226 which remains unchanged until the end of the *in vitro* culture process. Conversely, a time-depending
227 increase in the populations of the two fungi was observed at 12 and 16 weeks. However, after 24 weeks of
228 the in vitro culture process, *Phoma* spp. PGP03 population increased 0.86 Log (CFU/mL) while *C.*
229 *ramotenellum* PGP02 population showed a 0.44 Log (CFU/mL) decrease with regard to the previous period.

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

233 *3.3. Effect of the inoculation of embryos with three microorganisms on growth and development*
234 *of resulting nectarine seedlings*

235 Morphometric (FW, number of leaves, stem and root length and root volume), and photosynthetic
236 (QY) parameters were measured in nectarine seedlings derived from immature embryos rescued in different
237 years through a 24-week-long *in vitro* culture process (Fig. 3). Compared with the control, no inoculated
238 embryos, inoculation with the bacterium *P. oryzihabitans* PGP01 did not affect nectarine seedlings growth
239 since effects on FW were not detected (Fig. 3A). By contrast, plantlets from embryos inoculated with both
240 fungi, *Phoma* spp. and *C. ramotenellum* PGP02*,* were drastically affected, detecting reductions of almost
241 70% in both treatments (Fig. 3A). The treatment with the three microorganisms resulted in plants with
242 minor number of leaves than non-inoculated (control) seedlings (Fig. 3B). In this case, *Phoma* PGP03-
243 treated plantlets showed the lowest values, detecting a decrease of 64% in comparison to control (Fig. 3B).

244 Regarding stem length, inoculation with the three microorganisms caused a decrease in this parameter,
245 being less affected by the bacterium *P. oryzihabitans* PGP01 inoculated plants (22% lower than control)
246 than for fungi inoculated plants (56% less than control plants for both fungi) (Fig. 3C). The most prominent
247 effect of the inoculation of embryos with the bacterium *P. oryzihabitans* PGP01, was that nectarine plantlets
248 exhibited an increase of 61% in root volume when compared to control; while, the presence of fungi, *Phoma*
249 spp. PGP03 or *C. ramotenellum* PGP02, resulted in seedlings with significant lower root volume (Fig. 3D).
250 All the inoculated seedlings displayed a statistically significant shorter and compact root system as shown
251 by the measures of maximum root length (Fig. 3E). The shortest roots were detected in *C. ramotenellum*
252 PGP02-treated plantlets, which exhibited a decrease up to 60% compared to the control (Fig. 3E).
253 Significant morphological differences were observed on the produced nectarine plantlets, those plantlets
254 derived from culture media inoculated with *P. oryzihabitans* PGP01 showed thicker roots than non-
255 inoculated plantlets (Fig. 4), a similar effect to that observed in pear plantlets (Fig. 1B). While no
256 statistically significant differences in QY were found in *C. ramotenellum* PGP02 and *Phoma* spp. PGP03-
257 inoculated seedlings, compared with the control, a slight significant decrease in this parameter was
258 registered in *P. oryzihabitans* PGP01-treated plants (6% than control, approximately) (Fig. 3F). These
259 reduced values are in concordance with the fact that these plants had not as green leaves as control and *C.*
260 *ramotenellum* PGP02-plants (Fig. 4).

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

268 *3.4. Effect of the in vitro inoculation of nectarine embryos on the acclimatization of seedlings*

269 In the first experiment using nectarine embryos, the inoculation with the three microorganisms
270 resulted in plants more resistant to acclimatization in the greenhouse, displaying survival rates of 88, 85
271 and 87% for *C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01, respectively,
272 against the 71% recorded for the control plants (Fig. 6A). These percentages were moderately significantly

273 better in the case of *Phoma* spp. PGP03 (*p* – value=0.020) and *C. ramotenellum* PGP02 (*p* – value=0.013),
274 and highly significantly for the *P. oryzihabitans* PGP01-inoculated plants (*p* – value=0.005). Moreover,
275 this improved survival rate did correlate with an increase of 92% on the new shoot length in the *P.*
276 *oryzihabitans* PGP01 acclimated plants when compared with control (Fig. 6B). An inhibition of the shoot
277 growth under acclimatization conditions occurred in those plants from embryos inoculated with *C.*
278 *ramotenellum* PGP02 and *Phoma* spp. PGP03, observing decreases of 55 and 70% regarding to control,
279 respectively (Fig. 6B). In the following trials with two different nectarine genotypes, the survival rates
280 after the 4-week-long acclimatization process were higher, but not statistically significant, for plantlets
281 inoculated with *P. oryzihabitans* PGP01 than the control ones (data not shown). This could be explained
282 by a plant genotype effect on the acclimatization survival rates.

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

287 **4. Discussion**

288 The three microorganisms (two fungi and a bacterium) tested in the present study were obtained
289 from immature *Pyrus* and *Prunus* embryos, rescued under *in vitro* conditions, following the described
290 sterilization and culture process. Some of those in vitro contaminated embryos resulted in plantlets with a
291 better growth than not contaminated embryos. After their posterior isolation and identification, the impact
292 of these endophytic microorganisms on *in vitro* rooted pear plantlets was analysed, and the three
293 microorganisms showed an important plant-growth promoting effect on this plant material improving one
294 or two of the three parameters analysed (fresh weight, stem and root length). The use of microorganisms
295 with an endophytic origin to improve plant growth have been previously described in plants under
296 greenhouse conditions (Dias et al., 2009). However, their application in an aseptic *in vitro* environment is
297 being recently implemented (Perez-Rosales et al., 2018; Regalado et al., 2018), and the information found
298 in the literature is scarce, even more among woody plants grown *in vitro*. For instance, Kavino and
299 Manoranjitham (2018) and Rajamanickam et al. (2018) used endophytic bacterial strains isolated from
300 different parts of banana field plants to induce resistance against pathogens in banana *in vitro* manipulated
301 plantlets.

302 Given the potential effect on plant growth seen in the preliminary experiment with *Pyrus* rooted
303 plants, the effects of these microorganisms on the *in vitro* embryo germination and plantlet growth from
304 three different crosses of nectarine were studied. The *in vitro* rescue of embryos is being increasingly
305 applied in peach breeding programmes to widen the diversity of commercially productive early ripening
306 varieties with a higher commercial interest (Mancuso et al., 2002; Batlle et al., 2012). Herein, almost 100%
307 of the rescued nectarine embryos, in the three crosses, sprouted at the end of the *in vitro* culture process, in
308 contrast with the 70% of germination observed in interspecific hybrid embryos of peach and plum (Liu et
309 al., 2007). This procedure allows to increase the germination of these immature embryos, that otherwise
310 would degenerate, by culturing them in an aseptic and nutritionally enriched environment (Sinclair and
311 Byrne, 2003). On the other hand, although there is much evidence that the *in vitro* application of
312 microorganisms could influence the propagation or *in vitro* rooting (Kavino and Manoranjitham, 2018;
313 Quambusch et al., 2014), the knowledge regarding the inoculation with beneficial microorganisms to
314 enhance the in vitro germination of rescued embryos and posterior seedling development is currently rare.
315 In our case, none of the three microorganisms applied in the first experiment improved nectarine embryos
316 sprouting, mostly due to the high effectiveness of the in vitro rescue procedure mentioned above.
317 Nevertheless, the most notable finding is that while *C. ramotenellum* PGP02 and *Phoma* spp. PGP03-
318 inoculated embryos showed a negative effect on germination, no detrimental effect was reported in the case
319 of *P. oryzihabitans*-inoculated embryos. The key difference of this behaviour lies in the growth of these
320 microorganisms in the plant in vitro culture medium. Whereas the growth of the *P. oryzihabitans* PGP01
321 was highly recovered after 16 and 24 weeks of *in vitro* culture, the low temperatures (4ºC) of the
322 stratification phase dramatically affected the growth of this microorganism, which is quite logical because
323 most of bacteria grow better at high temperatures (Dobrić and Bååth, 2018). By contrast, these conditions
324 along with the important concentration of sugar present in the culture medium favoured the development
325 of both fungi, negatively affecting in most cases the embryo germination. This fact explained the results
326 obtained at the end of the *in vitro* rescue in terms of morphological parameters, and helped us to discard
327 the inoculation with fungi for the experiments with the following nectarine crosses. In these experiments,
328 the same behaviour of *P. oryzihabitans* PGP01 in the culture medium was observed, with an important
329 decrease of its growth after the stratification phase at 4ºC, and a rapid recovery when the embryos were
330 transferred to 14ºC. Moreover, *P. oryzihabitans* PGP01 in the culture medium did not affect embryo

331 germination percentage, suggesting that there is no interference of the microorganism with the embryo
332 physiology during the stratification at 4ºC.

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

351 Regarding the acclimatization of seedlings from control and inoculated nectarine embryos to
352 greenhouse conditions, the application of microorganisms produced more resistant plants with higher
353 survival rate values than control plants. These results were most remarkable for the seedlings from embryos
354 inoculated with *P. oryzihabitans* PGP01 since a strong stimulation of the new shoot growth after 4 weeks
355 of acclimatization was registered. This better performance of plants derived from *P. oryzihabitans* PGP01-
356 inoculated embryos was not observed in two other nectarine genotypes, for which almost 100% of control
357 plants survived to acclimatization in greenhouse conditions. This suggests that the genotype of the nectarine
358 embryos interacts with *P. oryzihabitans* PGP01 on the survival and growth response during acclimatization,
359 since this is a physiologically complex process, highly influenced by several environmental factors
360 including humidity, temperature, light, CO_2 or nutrient levels (Chen, 2004; Hazarika, 2006; Tisarum et al.,

361 2018). The awareness of using beneficial microorganisms on *in vitro* plants lies in overcoming the great
362 losses of plant material recorded at the acclimatization stage (Orlikowska et al., 2017). Trivedi and Pandey
363 (2007) demonstrated that the inoculation *Picrorhiza kurrooa* plantlets with three PGPR increased plant
364 survival as well as growth parameters in a greenhouse environment. Similarly, banana hardened plants
365 bacterized with two strains of *Bacillus subtilis* displayed a 100% of survival plants in comparison to 89%
366 recorded for control treatment (Rajamanickam et al., 2018). Most studies using the term "biohardening"
367 involve the application of microorganisms on acclimated plantlets to ensure plant establishment under
368 acclimatization conditions (Harish et al., 2008; Yadav et al., 2013). In consideration with the above, the
369 results presented in this work should be treated as an outcome of the *in vitro* inoculation and co-culture of
370 embryos with this microorganism, *P. oryzihabitans* PGP01, and not as an independent effect of the
371 inoculation with this bacterium on the acclimatization process. Moreover, plant defence mechanisms
372 induced by PGPR could contribute to a success in plant endurance (Chandra et al., 2010). Nevertheless, in
373 this study, the mechanisms of action of these microorganisms have not been studied, being this point the
374 focus of a future research.

375 **6. Conclusions**

376 To sum up, among the three microorganisms (*P. oryzihabitans* PGP01, *C. ramotenellum* PGP02
377 and *Phoma* spp. PGP03) with a plant growth promoting effect in pear *in vitro* rooted plantlets, *P.*
378 *oryzihabitans* PGP01 had no detrimental effects on the *in vitro* embryo rescue efficiency of three early
379 ripening nectarine crosses. Even so, the subsequent seedlings from the embryos inoculated with *P.*
380 *oryzihabitans* PGP01displayed highly significant modifications in root morphology that improved the
381 acclimatization to *ex vitro* conditions of those genotypes with difficult adaptability to the acclimation
382 process. This project could mean the first step of a significant change in woody plants breeding programmes
383 favouring the adaptation of plants whose endurance under greenhouse conditions resulted complex.
384 However, further investigations in this regard will be required in order to understand the mechanisms
385 underlying the plant growth promotion induced by the three microorganisms tested as well as the strong
386 root development and better survival observed in plants inoculated with *P. oryzihabitans* PGP01.

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395

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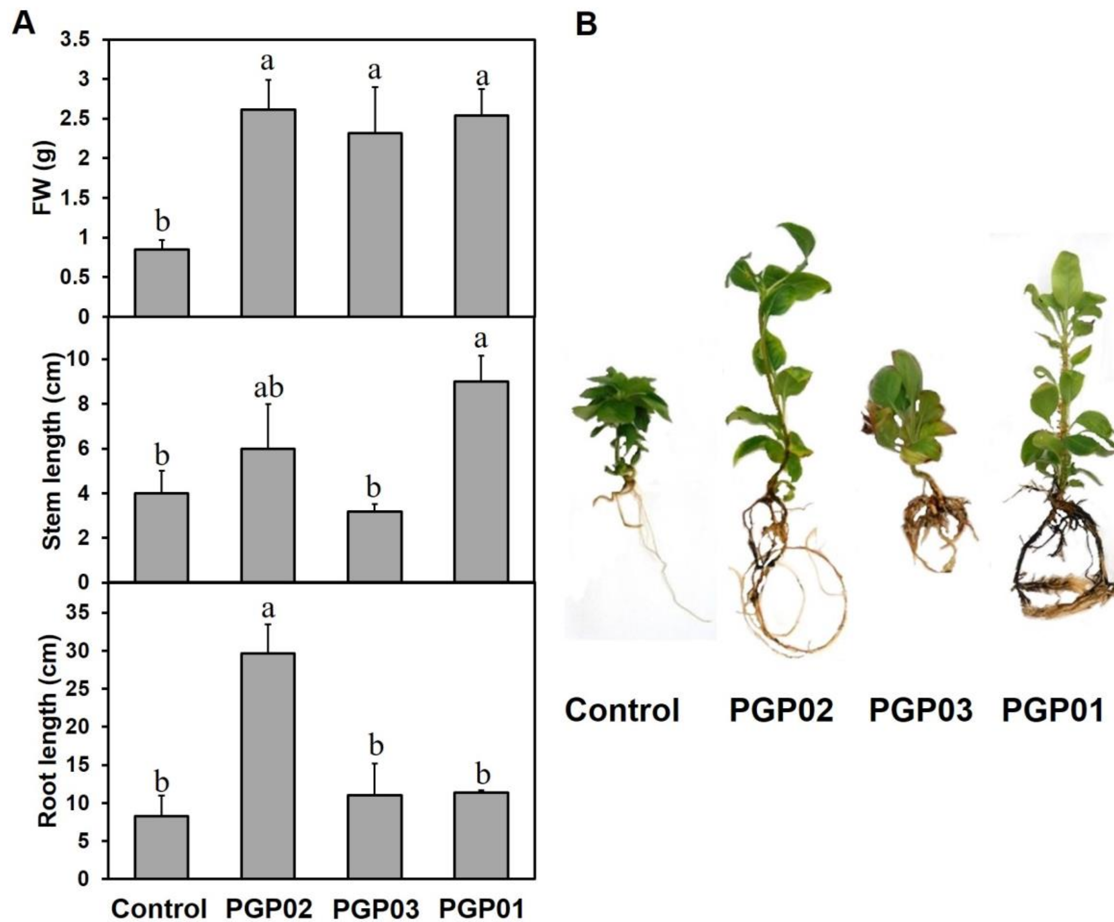
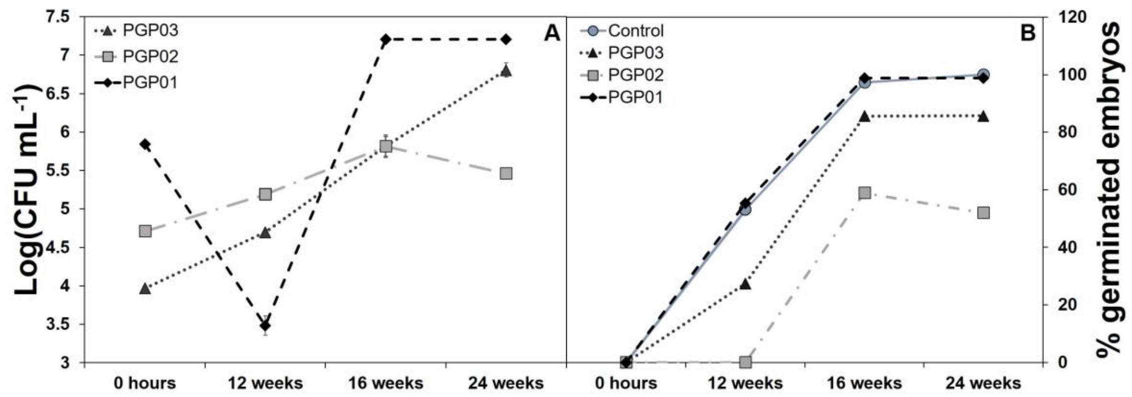
565

566 **LEGENDS OF FIGURES.**



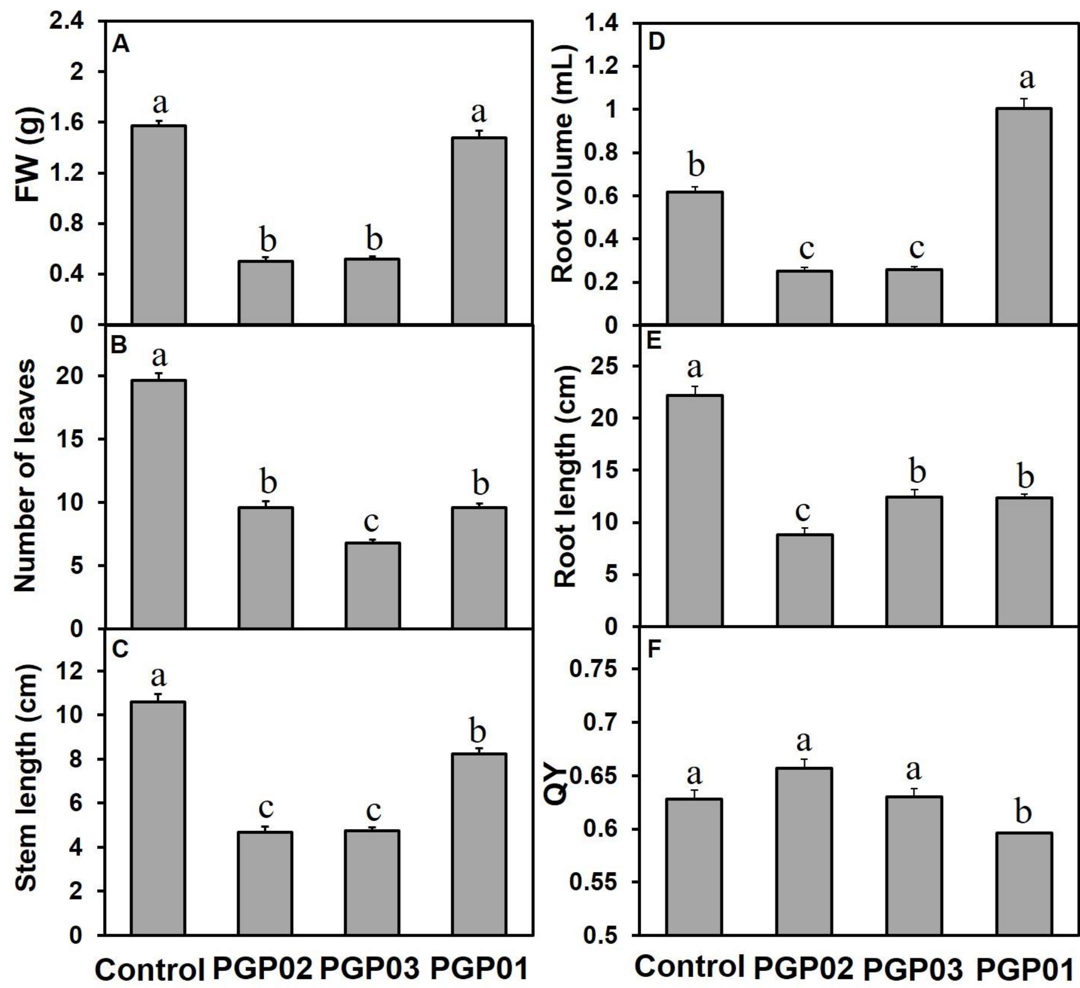
567

568 **Figure 1.- Effects of the application of three microorganisms (*C. ramotenellum*
569 PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01) on growth parameters (A)
570 and morphology (B) of pear rooted plantlets growing *in vitro*.** Data represents the
571 mean ± SE of at least three measurements. Different letters on bars of the same chard
572 indicate significant differences according to the Student's t-Test ($p \leq 0.05$).



573

574 **Figure 2.- Microorganism population dynamics (A) and germination percentage of**
575 **in vitro rescued nectarine embryos (B), following the three culture phases (12, 16**
576 **and 24 weeks) of in vitro culture.** Inoculation was done at day 0 with three
577 microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03, *P. oryzihabitans*
578 PGP01). Data of population dynamics represent the mean ± SE from at least three
579 measurements.



580

581 **Figure 3.- Effects of the in vitro co-culture of nectarine embryos with three**
582 **microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans***
583 **PGP01) on (A) total plantlet fresh weight (FW), (B) total number of leaves, (C) stem**
584 **length, (D) total root volume, (E) maximum root length and (F) photosynthetic**
585 **activity or QY, at the end of the 24-week-long *in vitro* culture process.** Different letters
586 on bars of the same chard denote significant differences according to the Student's t-Test
587 ($p \leq 0.05$).

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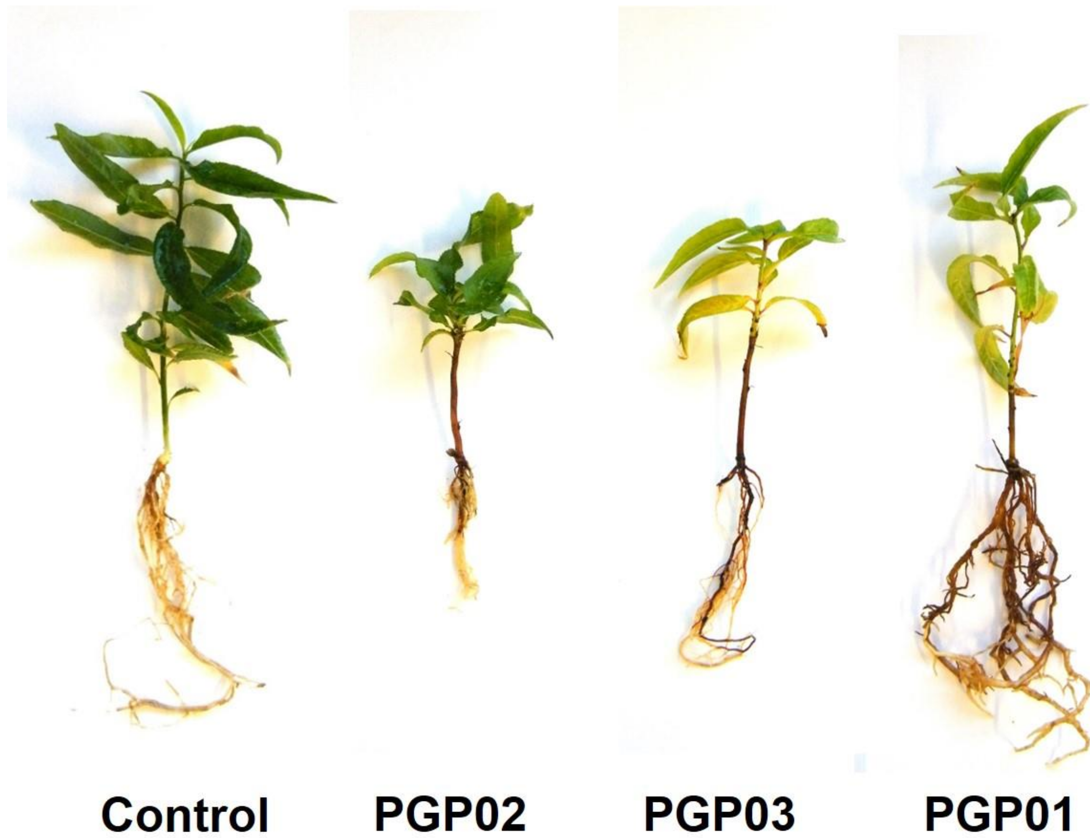
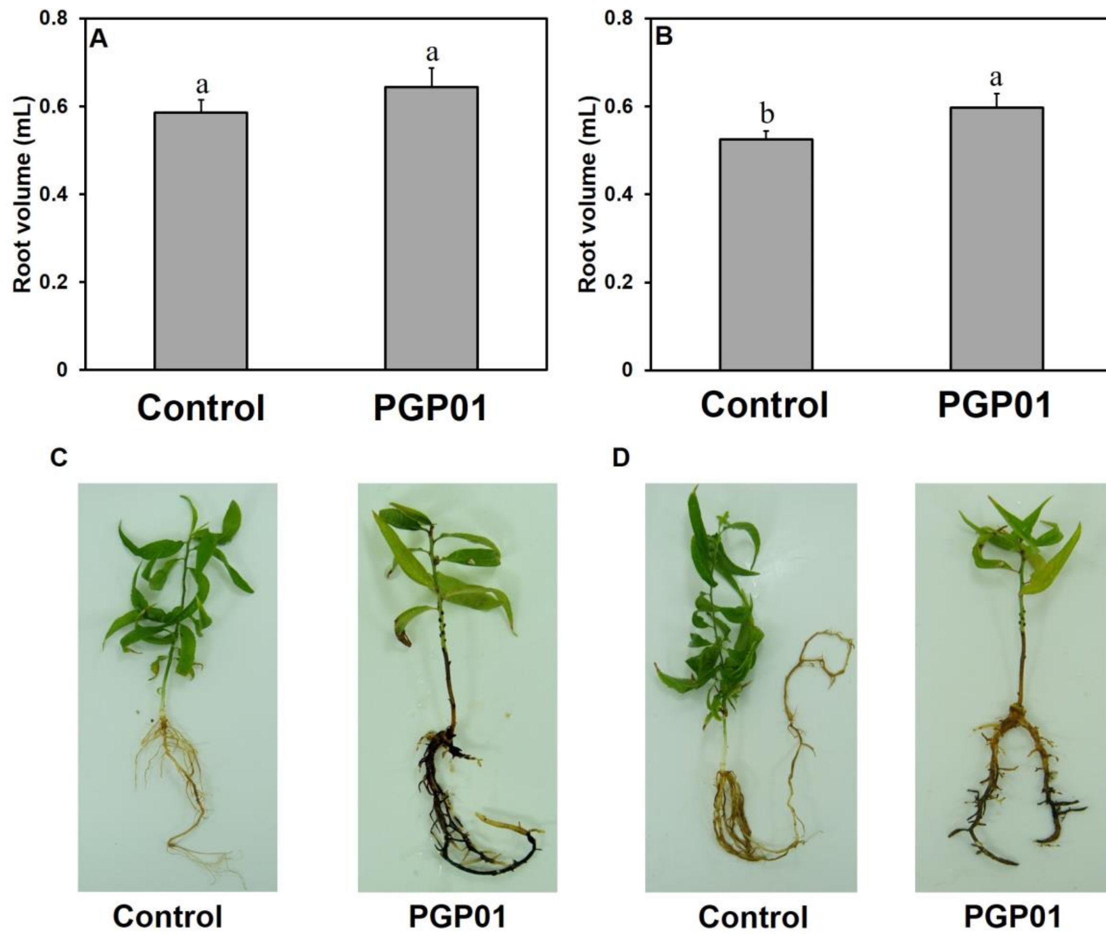


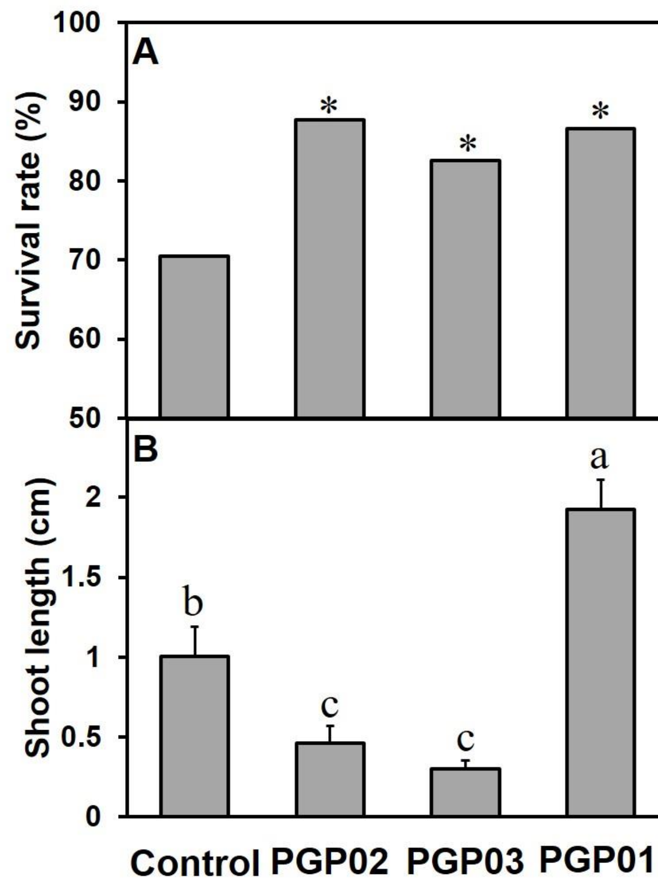
591

592 **Figure 4.- Morphological differences among nectarine plantlets derived from *in vitro***
593 **immature embryo rescue, after a 24-week-long in vitro co-culture process with three**
594 **microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans***
595 **PGP01).**



596

597 **Figure 5.- Root volume values (A,B) and morphological differences (C,D) of**
598 **nectarine plantlets from two independent crosses, after a 24-week-long *in vitro***
599 **embryo rescue process, inoculated or not with the bacteria *P. oryzihabitans* PGP01.**



600

601 **Figure 6.- Percentage of survival rate and length of the new shoot formed of the**
602 **resulting plants obtained from nectarine embryos inoculated with the three**
603 **microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans***
604 **PGP01) after 4 weeks on acclimation tunnels.** Asterisk symbol (*) means significant
605 differences between treatments and control according to the Fisher's Exact Test ($p \leq$
606 0.05). Different letters denote significant differences according to Student's t-Test ($p \leq$
607 0.05).