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1 **Acidification of the culture medium as a strategy to control endophytic contaminations in *Prunus***
2 **spp. rootstocks cultured in GreenTray® TIS bioreactor**

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8

9

Abstract

Overgrowth of endophytes in some *in vitro* cultures may disrupt the normal shoot tip growth and proliferation, being necessary to obtain endophytes-free cultures to achieve a normal plant micropropagation process. To remove these contaminations from the culture medium, antibiotics are commonly added to the culture medium. However, its use in plant production must be urgently reduced because of the current restrictions imposed by the European Union. For that purpose, the effect of acidic low (pH 3) and neutral (pH 7) pH was tested in the GreenTray® TIS bioreactor as an alternative to control endophytes growth without affecting the micropropagation of the *Prunus* rootstock RP-20 explants. The results demonstrated that culture at pH 3 did not affect the number of shoots, shoot FW, shoot length and the amount of chlorophyll pigments, but significantly reduced endophytes population. The identification also revealed that *Roseomonas mucosa*, *Microbacterium oxydans*, *Bacillus subtilis* and *Luteibacter yeojuensis* were the bacterial isolates responsible of those contaminations. These results might suppose a real breakthrough in the *in vitro* tissue culture field, although more research is required to meet the pH requirements for the different plant species and other endophytic microorganisms.

Keywords: *In vitro* micropropagation; GreenTray®, *Prunus* rootstock; Inhibition of endophytes growth; Low pH; Chlorophyll content.

27 **Introduction**

28 Endophytes have been defined as microorganisms with the ability to colonize inner plant tissues
29 without expressing disease symptoms (Petrini, 1991; Wilson, 1995). In *in vitro* cultures, most are bacteria
30 belonging to *Methylobacterium* and *Curtobacterium* genus (Panicker et al., 2007; Pohjanen et al., 2014).
31 Although, in natural environment they do not induce harmful effects in plants, it is known that their
32 presence in *in vitro* cultures might somehow modify explants behaviour. In some cases, endophytes in *in*
33 *vitro* cultured plants led to a plant growth promotion and the improvement of *in vitro* processes such as
34 multiplication or rooting of recalcitrant genotypes (Cantabella et al., 2021; Quambusch et al., 2014;
35 Zawadzka et al., 2014). Nevertheless, in other plant species, endophytes may disturb *in vitro* explant
36 performance, seriously affecting shoot micropropagation and leading to high losses of plant material
37 (Cheong et al., 2020) in commercial plant micropropagation. In those cases, it is of crucial importance to
38 establish a protocol for their removal from the culture medium. In the last years, the use of antibiotics or
39 the Plant Preservative Mixture (PPM™) to obtain endophytes-free cultures has been reported as an
40 effective procedure (Khan et al., 2018; Lotfi et al., 2020). However, these approaches should be
41 abandoned due to the restrictions imposed by the European Commission concerning the addition of
42 chemicals to the culture medium for plant production (Elmongy et al., 2018; Wiszniewska et al., 2016). In
43 this context, more sustainable alternatives to achieve this goal are required. In the previous research, the
44 ability of the two plant growth-promoting microorganisms (PGPMs) *Pseudomonas oryzihabitans* PGP01
45 and *Cladosporium ramotenellum* PGP02 to control endophytic contaminants was evaluated using the
46 GreenTray® TIS bioreactor (Cantabella et al., unpublished data). In this study, although an effective
47 biological control of these contaminations in the presence of both microorganisms did not occur, it was
48 suggested that the effect of the pH might represent a crucial factor for endophytes control. For this reason,
49 the present study has been designed to evaluate whether culture media adjusted to more acidic (pH 3) or
50 more basic (pH 7) pH values, compared with the optimal pH 5.7 used in plant growth are able to control
51 endophytes without affecting *in vitro* micropropagation of the commercial *Prunus* rootstock Rootpac®
52 20.

53 **Material and methods**

54 *In vitro plant material*

55 Explants of the *Prunus* commercial rootstock Rootpac® 20 (RP-20) (Agromillora, Barcelona,
56 Spain) were used for the study. Twenty 2-cm-long shoots were transferred from glass flasks to each

57 GreenTray® bioreactor (Dolcet-Sanjuan and Mendoza, 2018) after 3 weeks of culture. Flasks for
58 micropropagation in semi-solid media contained Murashige and Skoog medium (MS) (Murashige and
59 Skoog, 1962) supplemented with 3% sucrose and 5 µM 6-Benzylaminopurine (BAP), pH to 5.7, agar (7 g
60 L^{-1}) and autoclaved at 121 °C for 20 minutes. Shoot explants were apparently clean, with no endophytic
61 appearance in the culture media at the shoot clump base.

62 *Experimental conditions*

63 RP-20 micropropagated explants in GreenTray® bioreactors were cultured using MS medium
64 (Murashige and Skoog, 1962) at pH 3 and pH 7 using a buffer solution based on different proportions of
65 citric acid 0.1 M and Na_2HPO_4 0.2 M following the indications of Buffer Reference Center (Sigma
66 Aldrich). As standard, MS medium was adjusted to pH 5.7 with 0.1 N of NaOH. Media at pH 3 and 7
67 were sterilised by filtration using a 0.22 µm filter, while media at pH 5.7 was sterilized by autoclaving at
68 120ºC for 20 min, following the standard protocol. Three GreenTray® bioreactors for each treatment
69 were set up.

70 *Evaluation of in vitro micropropagation and dynamics of endophytes population*

71 After 8 days of culture, the effects of pHs in RP-20 *in vitro* micropropagation was determined by
72 measuring the number of shoots, shoot length, shoot fresh weight (Shoot FW), as well as the content of
73 total chlorophyll (Chl t), chlorophyll a (Chl *a*) and chlorophyll b (Chl *b*). In addition, a representative
74 number of colonies with different morphological aspect were isolated and identified by sequencing of the
75 16S rDNA and MALDI-TOF by the Laboratory of Instrumental Techniques, University of León (Spain).
76 During the *in vitro* culture process, culture media were sampled to monitor the population dynamics of
77 total endophytes.

78 *Statistical analysis*

79 The experiment was design considering a completely random design (CRD), and data analysis
80 was carried out by using JMP Pro Software (version 13.1.0, SAS Institute Inc., Cary, NC). Statistical
81 significance was judged at $P < 0.05$, and the Tukey test was used to separate the means when the
82 differences were statistically significant.

83 **Results**

84 After 8 days of culture in GreenTray® bioreactors, the micropropagation of RP-20 explants was
85 not negatively affected by pH 3, since no differences were found in the number of shoots produced, shoot
86 FW and shoot length in comparison to when the micropropagation is carried out at optimal pH 5.7 (Figure
87 1A). In contrast, when the pH was adjusted to 7 in RP-20 micropropagation, after 8 weeks of culture, the
88 number of shoots and shoot FW drastically decreased and were 86.5% and 83.9% when compared to
89 those in pH 5.7 (Figure 1A). Regarding endophytes population, it was clearly shown that the
90 micropropagation at pH 3 controlled the growth of bacterial endophytes in RP-20 shoot cultures,
91 observing reductions of 3.01, 2.23 and 2.43 log CFU mL^{-1} after 1, 3 and 6 days of culture, respectively
92 (Figure 1B). Under this acidic pH, endophytes drastically decreased and were not detected in the culture
93 medium after 8 days of *in vitro* culture. Conversely, endophytes in RP-20 cultured in MS medium at pH 7
94 displayed significant increases on their population of 1.5, 2.66, 3.73 and 3.38 log CFU mL^{-1} after 1, 3, 6
95 and 8 days of culture (Figure 1B).

96 After 8 days, the culture of RP-20 at pH 3 did not negatively affect the content of Chl t, Chl *a*
97 and Chl *b* compared to the culture at pH 5.7 (Figure 2A). In addition, RP-20 plantlets cultured at both pHs
98 showed green and fully expanded leaves after 8 days of culture (Figure 2B, C). Nevertheless, a 90 and
99 96% significant decrease in the amount of Chl t and Chl *a* was recorded in RP-20 leaves cultured in MS
100 medium at pH 7 (Figure 2A). Although not significant, it was also registered an almost 72% decrease in
101 the amount of Chl *b* in RP-20 leaves cultured under this pH compared to the culture at pH 5.7 (Figure
102 2A). Under pH 7, RP-20 *in vitro* shoots displayed a stressed appearance with shrunken, yellowish or
103 brownish leaves (Figure 2D) than those cultured at pH 5.7 or 3 (Figure 2C).

104 After identification, it was revealed that four different microbial species were responsible of
105 these endophytic contaminations of RP-20 cultured in the GreenTray® bioreactor (Table 1). The bacterial
106 species *Bacillus subtilis*, *Roseomonas mucosa* and *Microbacterium oxydans* were identified by MALDI-
107 TOF, and the species *Luteibacter yeojensis* was detected by the sequencing of nucleotides of the 16S
108 rDNA (Table 1). The high scores (2.22, 2.45 and 2.22 for *B. subtilis, R. mucosa* and *M. oxydans*,
109 respectively) as well as the high percentage of similarity (>99%) obtained by both techniques revealed
110 high confidence identifications of microbial species (Table 1).

111 **Discussion**

112 In this study, it has been demonstrated that the pH of the culture medium has an important effect
113 in the growth of endophytes in *in vitro* cultures. This experiment has been possible due to the use of a
114 liquid culture system that avoids solidification problems of the gelling agent (Thorpe et al., 2008). Based
115 on our results, the micropropagation of RP-20 explants in GreenTray® bioreactors could be performed
116 using culture medium with pH 3 since no differences in the number of shoots, shoot length, shoot FW
117 were observed, in comparison with those using culture medium with pH 5.7 as considered optimum for
118 plants growth. These results are consistent with those obtained by Martins et al. (2011) who reported that
119 micropropagation of *Plantago* spp. could be efficiently carried out at pH 4 instead of the commonly used
120 for *in vitro* tissue culture (pH 5.7). In contrast, other authors concluded that apple micropropagation could
121 be carried out at a broad range of pH ranging between 5.5 and 7.5 (Shi et al., 2017). However, this was
122 not possible for RP-20 micropropagation since it was negatively affected at pH 7, observing reductions of
123 86.5 and 83.9% the number of shoots as well as the shoot FW, respectively, compared to the culture at pH
124 5.7. Under pH 7 conditions, endophytes growth was favoured, leading to higher log CFU mL^{-1} regarding
125 the medium at pH 5.7 at 1, 3, 6 and 8 days of culture. In contrast, it is noteworthy to mention that when
126 the pH of the micropropagation medium was adjusted to 3, endophytes population were somehow
127 controlled, registering lower values of log CFU mL^{-1}. As mentioned, in the previous study conducted in
128 the presence of microorganisms, a relationship between the pH of the culture medium and endophytes
129 growth was established, being the bacterial population considerably reduced at low pH (approximately
130 2.5 log CFU mL^{-1}) when the inoculation with *C. ramotenellum* PGP02 took place (Cantabella et al.,
131 unpublished data). This microorganism significantly decrease the level of culture pH. In this sense, it is
132 widely known that while bacterial growth is favoured at pH values ranging 6.5-7.0, more acidic pH values
133 below 5.0 seriously compromised bacterial performance (Mossel et al., 1995). In the present study, the
134 uncontrolled growth of bacterial endophytes in the RP-20 cultures as well as the negative effects
135 provoked by the culture at pH 7 are most probably the responsible for the negative effects in *in vitro*
136 micropropagation. However, further experiments will be required to corroborate this assumption. All the
137 previous results were supported by those obtained in the content of chlorophyll pigments in *in vitro* RP-
138 20 leaves. After identification, it was revealed that four different microbial species were responsible of
139 these endophytic contaminations. The bacterial species *Bacillus subtilis*, *Roseomonas mucosa* and
140 *Microbacterium oxydans* were identified by MALDI-TOF, and the species *Luteibacter yeojensis* was
141 detected by the sequencing of nucleotides of the 16S rDNA. In this regard, many authors have previously

142 provided evidence concerning the endophytic origin of some of these bacterial species. However, not all
143 of them are described as endophytes in *in vitro* culture. In addition to the endophytic nature of these
144 microorganisms, positive effects in plant growth have been reported. In most cases, these endophytes
145 strains have shown beneficial effects in plant growth, increasing plant growth parameters or inhibiting the
146 growth of pathogen microorganisms (Comby et al., 2017; Hernández-Pacheco et al., 2021). For instance,
147 *R. mucosa* have been found as an *in vitro* endophyte bacterial species in walnut cultures obtained from
148 embryonic tissue (Pham et al., 2017). In a recent work, the endophyte bacterial species *M. oxydans* was
149 isolated from tomato roots (Hernández-Pacheco et al., 2021). This bacterial isolates displayed a growth
150 promoting ability in tomato plants as well as antifungal activity against *Botrytis cinerea*, *Fusarium*
151 *oxysporum* and *Rhizoctonia solani*. In *in vitro* tissue culture, bacterial species belonging to the
152 *Microbacterium* genus have been previously associated with a higher propagation success in cherry
153 (*Prunus avium* L.) genotypes (Quambusch et al., 2014). In addition, many studies are available about the
154 endophytic origin of *B. subtilis* in many plant species, most of them reporting its role as plant-growth
155 promoting bacteria and biological control agent against plant pathogens (Comby et al., 2017; Fouda et al.,
156 2021). In contrast, very little information is available about the role of *L. yeojuensis* as bacterial
157 endophyte. Nevertheless, other species belonging to this genus have been reported as endophytes in
158 *Quercus* spp., contributing at different levels to carbon, phosphorous and nitrogen cycles (Lasa et al.,
159 2019). Due to the abovementioned, the isolated microorganisms will be stored for further experiments.
160 Therefore, the presented results might represent a paradigm shift in the plant *in vitro* tissue culture that
161 help to mitigate the losses occasioned by the presence of bacterial endophytes. However, further
162 investigations are required in this regard since it is reported that pH requirements for optimal growth are
163 highly depending on the plant species (Leifert et al., 1992). Altogether, it has been demonstrated that
164 endophytes populations in micropropagated explants might be controlled by modulations in the pH of the
165 culture medium, replacing the addition of antibiotics and contributing to a more sustainable *in vitro* plant
166 production.

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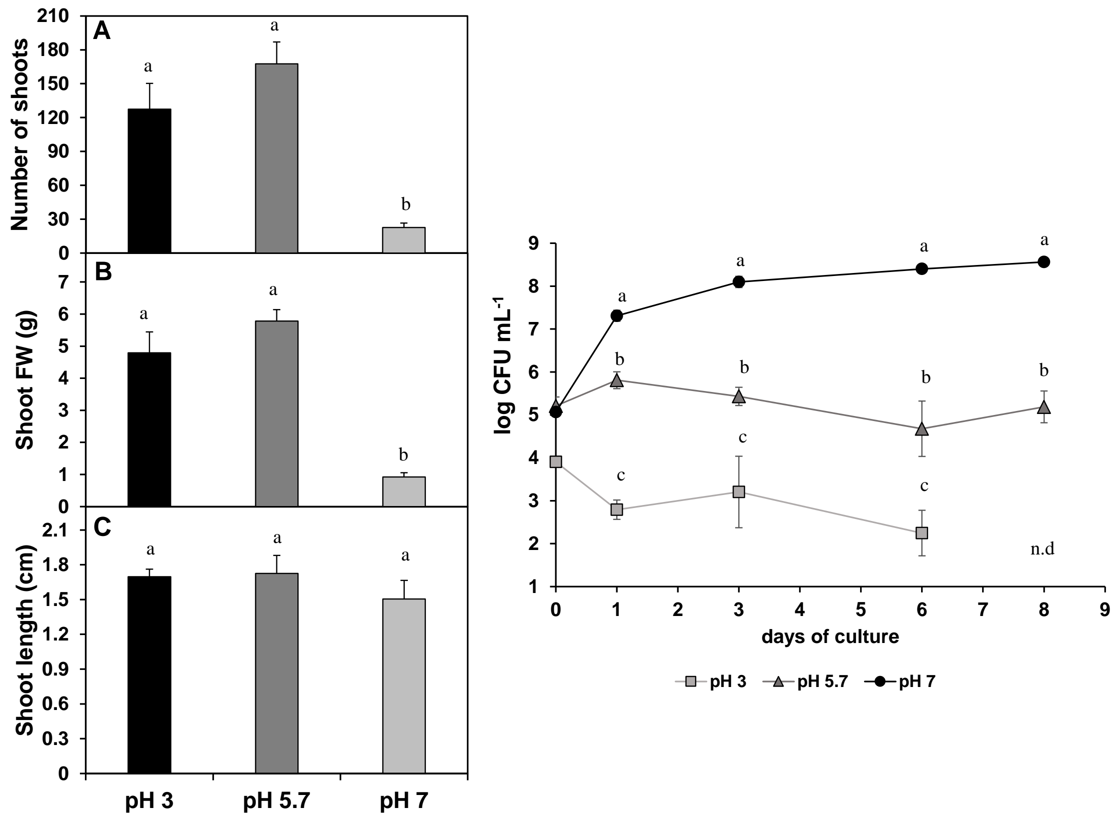
249 FIGURE LEGENDS



250

251 **Figure 1.- Effect of the culture of RP-20 *in vitro* explants in GreenTray® TIS bioreactors at pH 3,**
252 **pH 5.7 and pH 7 on *in vitro* micropropagation parameters (number of shoots, shoot FW and shoot**
253 **length) after 8 days of culture (A) and population dynamics of endophytes (B).** For *in vitro*
254 micropropagation parameters, data represents the mean ± standard error (SE) of the measures taken in
255 three bioreactors per treatment. For population dynamics data, the showed values for each treatment
256 represents the mean ± SE of samples taken in three bioreactors. In both cases, different letters indicate
257 significant differences among treatments according to Tukey HSD test ($P < 0.05$).

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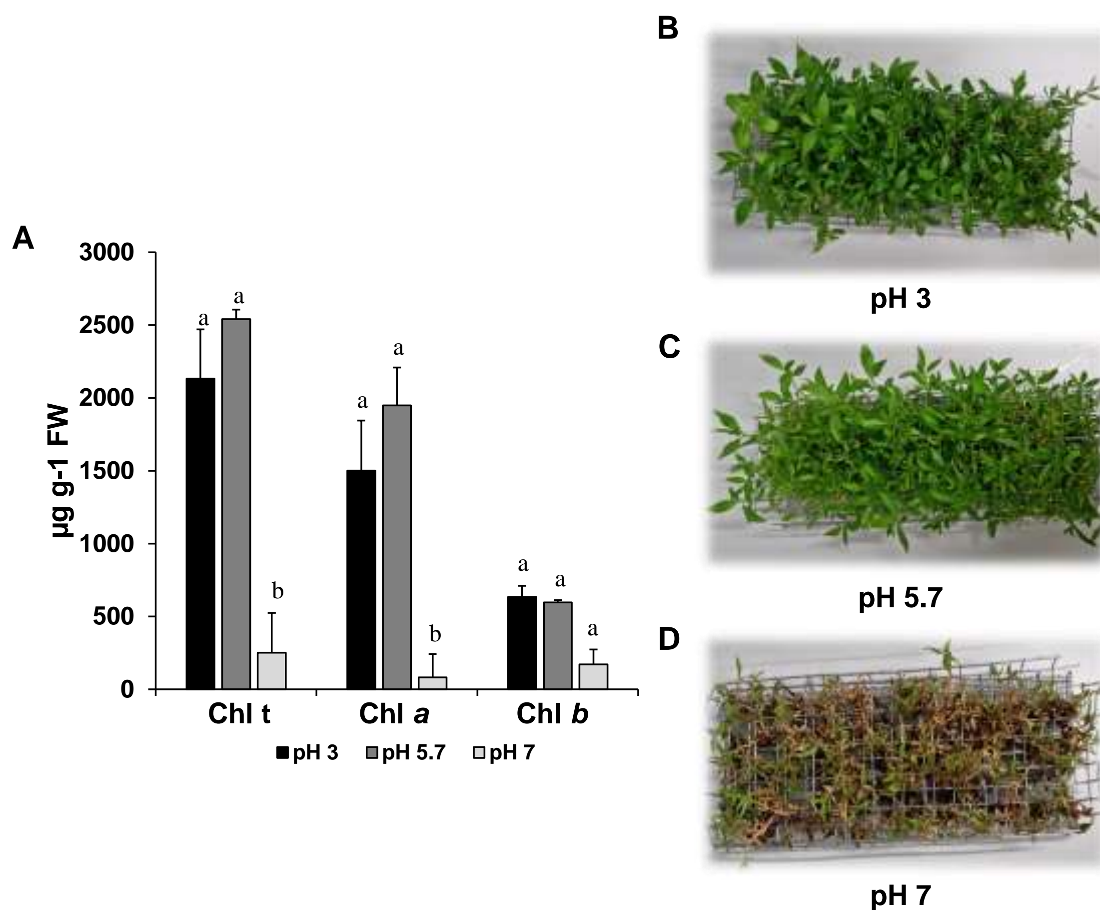

264

265 **Figure 2.- Chlorophyll content of RP-20 *in vitro* leaves of explants cultured in GreenTray® TIS**
266 **bioreactors at pH 3, pH 5.7 and pH 7 (A) and explants appearance after 8 days of culture (B).** Data
267 represents the mean ± standard error (SE) of the measures taken in three bioreactors per treatment.
268 Different letters within each chlorophyll pigment indicate significant differences among treatments
269 according to Tukey HSD test ($P < 0.05$).

270 **TABLES**

271 **Table 1.** Identification of the different isolated endophyte colonies in *Prunus* RP-20 *in vitro* explants.

272

MALDI-TOF identification	Score value	Interpretation
Bacillus subtilis	2.22	High confidence identification
Roseomonas mucosa	2.45	High confidence identification
Microbacterium oxydans	2.22	High confidence identification
16S DNAr identification	**% Similarity**	
Luteibacter yeojuensis	> 99%	High confidence identification

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