Salt stress alleviation in citrus plants by plant growth promoting rhizobacteria *Pseudomonas putida* and *Novosphingobium* sp.

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Abstract

Detrimental salt stress effects on crops are likely to increase due to climate change reducing the quality of irrigation water. Plant growth promoting rhizobacteria (PGPRs) can mitigate stress induced damage in plants cultivated under high salinity conditions. In this work, *Citrus macrophylla* (alemow) plants inoculated with the rhizobacteria *Pseudomonas putida* KT2440 or *Novosphingobium* sp. HR1a were subjected to salt stress for 30 days. Results showed that in absence of salt stress, *Novosphingobium* sp. HR1a induced a decrease of transpiration (E) and stomatal conductance (g_s). Both rhizobacteria reduced salt-stress induced damage. Levels of abscisic acid (ABA) and salicylic acid (SA) were lower in inoculated plants under salt stress conditions. Similarly, under stress conditions maximum efficiency of photosystem II (F_v/F_m) in inoculated plants decreased to a lower extent than in non-inoculated ones. In stressed plants, *Novosphingobium* sp. HR1a also induced leaf accumulation of 3-indole acetic acid (IAA) and a delay in the decrease of quantum yield (ΦPSII). *P. putida* KT2440 inhibited root chloride and proline accumulation in response to salt stress. Although both bacterial species had beneficial effects on salt-stressed citrus plants, *Novosphingobium* sp. HR1a induced a better plant performance. Therefore, both strains could be candidates to be used as PGPRs in programs of inoculation for citrus protection against salt stress.

Keywords: citrus, *Novosphingobium*, plant growth promoting rhizobacteria, *Pseudomonas*, salt stress

Abbreviations: ABA: Abscisic acid; ACC: 1-aminocyclopropane-1-carboxylate; CFU: Colony Forming Units; E: Transpiration; F_v/F_m: Maximum efficiency of photosystem II; g_s: Stomatal conductance; IAA: 3-

28 Indole acetic acid; NI: Non-Inoculated; PGPR: Plant growth promoting rhizobacteria; Φ_{PSII}: Quantum
29 efficiency of PSII photochemistry; SA: Salicylic acid.

30 ***Key message:*** This work reveals the protective role of two rhizobacteria, *Pseudomonas putida* and
31 *Novosphingobium* sp., on citrus plants subjected to salt stress conditions.

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39 ***Author contribution statement***

40 VV-P performed the experiments and wrote the manuscript. AG-C and RMP-C contributed in the design
41 of the experiments and the supervision of the work, as well as the correction of the manuscript.

42 ***Compliance with ethical standards***

43 The authors declare that they have no conflict of interest.

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46

47 ***Introduction***

48 In nature, plants are constantly subjected to a wide variety of both abiotic and biotic stress conditions which
49 can reduce their growth and productivity. Moreover, climate change exacerbates these adverse conditions,
50 affecting mainly to abiotic stress conditions as drought, heat stress or salinity (Zandalinas et al. 2018). In
51 this context, salt concentration in groundwater of coastal regions is increasing because of saltwater intrusion
52 due to overexploitation of aquifers, leading to reductions of yield and plant performance (Klassen and
53 Allen, 2017). High substrate salinity has two different effects which trigger plant damage: the osmotic
54 component, that appears in the early stage of stress and restricts water absorption, producing plant
55 dehydration and turgor loss; and the ionic component, due to the accumulation of Na^+ and Cl^- ions, that
56 reach toxic levels in tissues (Gupta and Huang, 2014). For most species Na^+ appears to reach toxic
57 concentrations in plant tissues before Cl^- does. On the contrary, in the case of citrus, Cl^- is considered the
58 most toxic ion (Moya et al. 2002, 2003; Montoliu et al. 2009). Consequently, it is necessary to explore new
59 strategies to maximize plant tolerance to this stress condition to improve plant productivity in affected
60 zones, as occurs in the Mediterranean region where citrus is one of the main crops.

61 Several strategies to mitigate salt stress-induced damages in plants have been proposed, including chemical
62 treatments, such as 24-epibrassinolide (Ekinci et al. 2012) or abscisic acid (ABA) analogues (Arbona et al.
63 2006), improvement of mineral fertilization (Rady, 2012), modification of gene expression (Zhao et al.
64 2017; Vives-Peris et al. 2018a), and modification of soil microbiota communities to potentiate plant
65 colonization by beneficial microorganisms such as arbuscular mycorrhizal fungi or plant growth promoting
66 rhizobacteria (PGPR) (Qin et al. 2016). PGPR can benefit plant growth and alleviate salt stress by multiple
67 mechanisms: i) producing biofilms that favour humidity accumulation around the roots and avoid the
68 entrance of toxic ions or pathogens (Wang et al. 2017), ii) increasing nutrient availability in the substrate
69 due to phosphate solubilization or atmospheric nitrogen fixation (Pereira and Castro 2014; Wang et al.
70 2017), iii) inducing root growth and development through the release of phytohormones and secondary
71 metabolites to the rhizosphere such as indole-3-acetic acid (IAA) or other signalling molecules as nitric
72 oxide (Cassán et al. 2014), iv) producing siderophores (Sayyed et al. 2013) and v) decreasing 1-
73 aminocyclopropane-1-carboxylate (ACC) levels, by increasing ACC deaminase activity in the rhizosphere,
74 which consequently derives in a decrease in ethylene concentration in plant tissues (Vacheron et al. 2013;
75 Singh et al. 2015; Nadeem et al. 2016). It has been also reported that PGPR induces an increase in water
76 use efficiency by modulating transpiration and stomatal conductance, and a decrease in the content of

77 reactive oxygen species in inoculated plants (Vejan et al. 2016). The effectiveness of these stress-mitigating
78 effects depends on the type of plants, bacteria, and their putative interaction.

79 Although the palliative effects of PGPR on plants subjected to abiotic stress conditions have been studied
80 in a wide variety of herbaceous plants, including tomato, rice, lettuce wheat, potato, cotton, soybean, maize,
81 chickpea, lentil or pea (Dimkpa et al. 2009; Nadeem et al. 2014, Vurukonda et al. 2016), the effect of these
82 beneficial microorganisms in woody plants is less well-known and reports are limited to *Vitis vinifera*
83 subjected to chilling (Barka et al. 2006), or *Pinus halepensis* and *Quercus coccifera* trees subjected to water
84 stress (Rincón et al. 2008). In citrus, the information on the stress-mitigating role of soil microorganisms is
85 mainly focused on the beneficial effects of mycorrhizal fungi in plants growing under different biotic or
86 abiotic stresses such as those caused by *Phytophthora* (Watanarojanaporn et al. 2011), drought (Wu and
87 Zou, 2009), salinity (Satir et al. 2016; Zhang et al. 2017) and low temperatures (Wu and Zou, 2010). In
88 relation to PGPRs, only the effect of *Pseudomonas putida* FCA-8 on citrus cultivated in the absence of any
89 stress condition has been reported (Chiquito-Contreras et al. 2012). Moreover, although *P. putida* KT2440
90 has been described as a PGPR in *Zea mays* (Planchamp et al. 2015), the positive role on plant growth of
91 the strain *Novosphingobium* sp. HR1a has not been proved, being only considered as a PGPR as with other
92 strains in this genus (e.g., *Novosphingobium oryzae* sp. nov., Zhang et al. 2016).

93 Consequently, in this work, the putative palliative effect of two rhizobacterial strains, *P. putida* KT2440,
94 and *Novosphingobium* sp. HR1a, on damage caused by salt stress conditions in alemow plants was
95 evaluated. The main objective of this investigation has been to test the beneficial effect of both strains under
96 stressful situations, regarding on different plant biochemical and physiological parameters.

97

98 ***Materials and methods***

99 Plant material and treatments

100 Six-month-old alemow (*Citrus macrophylla* Wester) plants were acclimated in a greenhouse for two
101 months under natural photoperiod and temperatures of 25 ± 3.0°C and 18 ± 2.0°C (day/night respectively).
102 A non-sterilized mixture of peat moss, perlite and vermiculite (80:10:10) was used as substrate. Plants were
103 watered with half-strength Hoagland solution three times a week (Arbona et al. 2009).

104 A first experiment was carried out to optimize the inoculation. Plants about 50 cm height, were transferred
105 to plastic pots containing 400 mL of substrate (sterilized three times with an autoclave at 121º C for 30 min
106 each time),. Inoculated plants were watered with a *P. putida* KT2440 solution containing the necessary
107 bacteria to inoculate the pot volume to an OD_{660nm} of 0.1 (Franklin et al. 1981), while control plants were
108 watered with the same volume of water, without inoculum. Ten days after the inoculation, salt stress was
109 applied by adding 60 and 90 mM NaCl to the watering solution twice a week. Both, non-inoculated plants,
110 and plants watered without NaCl were added as controls. Leaf and root samples were randomly collected
111 30 days after salt stress onset (Fig. 1.A). This experiment was realized with ten plants per group at the
112 beginning of spring and it was not repeated since it was performed for stablishing the conditions for the
113 second experiment.

114 In the second experiment, *C. macrophylla* plants (similar in height and age to those used in the first
115 experiment) were inoculated with two different rhizobacterial strains, *P. putida* KT2440 and
116 *Novosphingobium* sp. HR1a up to a final OD_{660nm} of 0.1 (Franklin et al. 1981; Segura et al. 2017). Ten days
117 after the inoculation with bacteria, salt stress was applied, by adding 90 mM NaCl to the watering solution
118 twice a week (Fig. 1.B). Leaf and root tissues were randomly sampled after 30 days of stress. Non-
119 destructive analyses, including gas exchange and chlorophyll fluorescence parameters were determined at
120 10, 20 and 30 days after the stress onset. This experiment was performed with ten plants per group and it
121 was repeated twice during the summer period with similar results

122 Visual leaf damage

123 In the first experiment damage apparition was determined by counting the percentage of abscessed leaves
124 in each one of the ten plants of every group after 30 days of stress. In the second experiment different
125 degrees of salt stress-induced damage were stablished, including non-damaged leaves, mild-damaged
126 leaves, intermediate-damage leaves, severe-damaged leaves and abscessed leaves (Fig. 2). The percentage
127 of apparition of each symptom was counted 30 days after salt stress imposition in ten plants per group.

128 Chloride analysis

129 Quantification of chloride ions was performed in plant tissue and soil saturated extract. Measurements in
130 leaves and roots were performed by automatic titration with a chloride meter (Model 626, Sherwood
131 Scientific Ltd., Cambridge, UK) as described in López-Climent et al. (2008). Three replicates of each
132 sample were extracted by adding 25 mL of the chloride extraction buffer, consisting in 0.1 N HNO_3

133 (Panreac, Barcelona, Spain) and 10% glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA) to 0.25 g of
134 fresh tissue, and were incubated for 12 hours at room temperature. Chloride concentration was measured
135 by titrating 0.5 mL of the solution with the chloride meter

136 Chloride measurements of soil saturated extract were performed by adding water to 2 g of soil until
137 saturation. After 24 hours at room temperature, the water was collected from the soil with a vacuum pump,
138 and chloride concentration was measured in the chloride meter. Chloride content was measured in three
139 independent samples of soil for each treatment.

140 Determination of colonization rate

141 In the first experiment, plant colonization by the rhizobacteria was determined by counting colony forming
142 units (CFU). Roots were washed with 5 mL of sterile deionized water in agitation at 200 rpm for 1 hour.
143 Serial dilutions of the wash water were plated on a selective lysogeny broth (LB) medium (Bertani, 1951)
144 supplemented with chloramphenicol, an antibiotic to which *P. putida* KT2440 is resistant (Franklin et al.
145 1981). CFU were counted after 24 h of incubation at 30°C (Goldman and Green, 2008). For each treatment,
146 three root samples from different plants were washed with sterile deionized water as described previously
147 and three replicates of each one were plated.

148 Proline analysis

149 The concentration of proline was determined in leaf and root samples as indicated in Bates et al. (1973)
150 with some modifications. Briefly, three replicates of fresh material was extracted by sonication in 3%
151 sulfosalicylic acid (Panreac) in distilled water. After centrifugation, supernatant was mixed with glacial
152 acetic acid (Sigma-Aldrich) and ninhydrin reagent (prepared as in Vives-Peris et al. 2017). Samples were
153 incubated at 100 ºC for one hour and after centrifugation, proline concentration was spectrophotometrically
154 determined at 520 nm.

155 Phytohormone analysis

156 Concentration of ABA, salicylic acid (SA) and IAA was determined in leaves collected after 30 days by
157 high performance liquid chromatography coupled online to a triple quadrupole mass spectrometer
158 (Micromass, Manchester, UK) through an orthogonal Z-spray electrospray ion source (Durgbanshi et al.
159 2005). Three replicates of each sample were extracted with water from 0.2 g of fresh material reduced to
160 fine powder by using a mill ball equipment (MillMix20, Domel, Železniki, Slovenija). [$^{2}H_6$]-ABA, [$^{13}C_6$]-

161 SA and [$^2H_2$]-IAA were used as internal standards. Samples were centrifuged after the extraction, and the
162 supernatant was recovered, and pH adjusted to 2.8 to 3.2 with acetic acid. A liquid-liquid partition was
163 performed twice with diethyl ether and the supernatant was evaporated under vacuum in a centrifuge
164 concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The solid residue was diluted in 0.5 mL of
165 water:methanol 90:10 and filtered through 0.22 μM PTFE filters. Finally, 20 μL of this solution was
166 injected into the HPLC-MS system (Acquity SDS, Waters Corp., Milford, MA, USA).

167 Chromatography separation was achieved by using a reversed-phase C18 column (Gravity, 50 × 2.1mm
168 1.8-μm particle size, Macherey-Nagel GmbH, Germany) as stationary phase, and a methanol:water
169 gradient, both supplemented with 0.1% acetic acid, at a flow rate of 300 μL min^{-1} as mobile phase. Standard
170 curves with the commercial standards of the different phytohormones were used for quantifying sample
171 phytohormone concentrations. Results were processed using Masslynx v4.1 software.

172 Chlorophyll fluorescence parameters

173 Quantum yield (Φ_{PSII}) and maximum efficiency of photosystem II photochemistry, as F_v/F_m ratio, were
174 measured 10, 20 and 30 days after the onstet of the experiment between 9 and 11 h AM in twelve randomly
175 chosen undamaged leaves from three plants per treatment using a portable fluorometer (FluorPen FP-MAX
176 100, Photon Systems Instruments, Czech Republic). Four measurements were taken per leaf (Zandalinas et
177 al. 2016).

178 Leaf gas exchange parameters

179 Transpiration (E) and stomatal conductance (g_s) were measured with a LCpro+ portable infrared gas
180 analyzer (ADC Bioscientific Ltd., Hoddesdon, UK) under ambient CO_2 and humidity. Light was provided
181 by a photosynthetically active radiation lamp at 1000 μmol m^{-2} s^{-1} photon flux density. Air flow was set at
182 150 μmol mol^{-1} and all measurements were performed between 9 and 11 h AM. Three undamaged leaves
183 of three different plants were analyzed per treatment, and after instrument stabilization, ten measures were
184 consecutively performed in every leaf after 10, 20 or 30 days of treatment (Zandalinas et al. 2016).

185 Statistical analyses

186 Statgraphics Plus v.5.1. Software (Statistical Graphics Corp., Herndon, VA, USA) was used for statistical
187 analyses. Represented data are means of independent determinations and were subjected to one- or two-

188 way analysis of variance (ANOVA) and a Tukey posthoc test ($p \leq 0.05$) when significant differences were
189 detected.

190

191 ***Results***

192 Optimization of inoculation

193 *Leaf abscission*

194 After 30 days of treatment, leaf abscission increased in non-inoculated salt-stressed plants, reaching the
195 highest level in plants subjected to 90 mM NaCl, with a percentage of leaf abscission of 17.5%, whereas in
196 plants inoculated with *P. putida* KT2440, this percentage was of 2.6%, being similar to the observed in
197 control plants (Fig. 3).

198 *Chloride accumulation*

199 Chloride concentration was determined in the substrate, shoots and roots (Fig. 4). In the substrate, the
200 presence of bacteria did not modify chloride concentration, being this value only affected by high salinity,
201 with chloride levels 4.6 and 8.9 times higher than controls when soil was treated with 60 and 90 mM NaCl,
202 respectively (Fig. 4A). A similar trend was observed in leaves, with an increase in the concentration of this
203 ion depending on the stress severity exclusively, reaching values 1.8 and 3.0 times higher than those
204 observed in leaves of non-stressed plants when they were subjected to 60 and 90 mM NaCl, respectively
205 (Fig. 4B). The presence of the bacteria *P. putida* KT2440 reduced chloride accumulation in roots of plants
206 subjected to 90 mM NaCl (24.6% reduction compared to salt-stressed non-inoculated plants, Fig. 4C).

207 *Colonization rate*

208 The number of CFU was analysed at the end of the experimental period. Two different levels of bacterial
209 populations were observed (Fig. 5). In the case of non-inoculated plants, values of CFU were about 150,000
210 CFU g^{-1} root, whereas in plants inoculated with *P. putida* KT2440, this value was around 600,000 CFU g^{-}
211 ^{1} root. The increasing levels of NaCl did not affect the colonization rate.

212 Evaluation of the palliative effect of both strains in plants subjected to salt stress

213 *Appearance of symptoms induced by salt stress*

214 The presence of both rhizobacterial strains reduced salt stress-induced damage. Thus, whereas in non-
215 inoculated plants a 68.3% of leaves were affected, in plants inoculated with *P. putida* KT2440 or
216 *Novosphingobium* sp. HR1a, the percentages of damaged leaves were lower (57.0 and 45.0%), respectively
217 (Fig. 6). Moreover, in salt stress conditions, the severity of the damage in leaves from plants that had been
218 previously inoculated with any of both PGPR strains was lower than in non-inoculated plants subjected to
219 salt stress, with statistically significant diminutions of the appearance of severe-damaged leaves and
220 abscessed leaves (Sup. Mat. 1).

221 *Proline concentration*

222 An increase of leaf proline concentration was observed in all groups of plants due to salt stress application
223 (Fig. 7A) independently if plants had been previously inoculated or not. Proline concentration in plants
224 under salt stress was about 4.2 times higher than in their respective non-stressed controls. In roots of plants
225 subjected to salt stress, there was a 3.9-fold increase of proline content in salt-stressed non-inoculated plants
226 (Fig. 7B). Proline content also exhibited an increase (2.3-fold) in roots of plants inoculated with
227 *Novosphingobium* sp. HR1a. However, in roots of plants inoculated with *P. putida* KT2440, no statistical
228 differences in the content of the amino acid between stressed and non-stressed plants were found.

229 *Phytohormone concentration*

230 Endogenous levels of the phytohormones ABA, SA and IAA were measured in leaves after 30 days of
231 stress (Fig. 8). After this period, non-inoculated plants treated with 90 mM NaCl exhibited a 1.7-fold
232 increase in the leaf concentration of ABA (related to non-stressed plants). Contrarily, in plants inoculated
233 with *P. putida* KT2440 the concentration of this phytohormone decreased 40.4% after salt treatment. In
234 plants inoculated with *Novosphingobium* sp. HR1a, salt stress did not alter leaf ABA content (Fig. 8A).

235 In the absence of inoculation, salt stress induced a 2.3-fold increase of SA leaf concentration in comparison
236 with non-stressed plants. However, no differences were observed between salt-stressed plants and controls
237 in presence of any of the bacterial strains used in the experiment (Fig. 8B). Meanwhile, increases in IAA
238 content were only recorded in leaves of plants inoculated with *Novosphingobium* sp. HR1a and treated with
239 90 mM NaCl (2.8 fold with respect to controls, Fig. 8C).

240 *Chlorophyll fluorescence parameters*

241 Salt treatment and bacteria inoculation induced changes in chlorophyll fluorescence parameters (Fig. 9).
242 Salt stress clearly reduced Φ_{PSII} in non-inoculated plants, with a reduction of 36.4 and 55.5% related to
243 control plants after 20 and 30 days, respectively. In plants inoculated with *P. putida* KT2440, this reduction
244 was similar to that observed in non-inoculated plants, (reduction of 23.8 and 52.1% in Φ_{PSII} values related
245 to control after 20 and 30 of salt stress, respectively). In plants inoculated with *Novosphingobium* sp. HR1a,
246 this difference of Φ_{PSII} values between non-stressed and salt-stressed plants also decreased due to salt stress
247 (a diminution of 32.2% respect to control after 20 days from stress onset). However, after 30 days of salt
248 stress, the reduction of this parameter was not as marked as in non-inoculated plants (decrease of 39.0%,
249 Fig. 9A).

250 In line with the Φ_{PSII}, F_v/F_m decreased with the application of NaCl from 20 days of stress until the end of
251 the experiment (28.1 and 44.3% reduction in non-inoculated plants at 20 and 30 days, respectively).
252 However, in inoculated plants, levels of F_v/F_m were similar to controls for the first 20 days of stress. After
253 30 days of stress F_v/F_m values decreased a 52.9 and 38.3% in salt-stressed plants inoculated with *P. putida*
254 KT2440 and *Novosphingobium* sp. HR1a, respectively (in comparison with non-stressed inoculated plants,
255 Fig. 9B).

256 *Gas exchange parameters*

257 Gas exchange parameters, including E and g_s, were measured in leaves throughout the experimental period
258 but no differences were observed until 20 days of stress (Fig. 10). Salt stress induced a decline of E
259 (reduction of E values between 52.4 and 68.0% after 20 days and between 82.7 and 84.5% after 30 days,
260 related to control). Moreover, in absence of salt stress, *Novosphingobium* sp. HR1a also induced a
261 diminution of 35.5% in this parameter after 20 days in comparison with the value observed in non-
262 inoculated plants (Fig. 10A).

263 In addition, g_s exhibited a similar trend than that observed in E. Most of the differences observed in g_s were
264 in response to salt stress, with a decrease between 58.4 and 74.0% depending on the inoculum, at 20 days,
265 being more evident after 30 days, with values between 84.8 and 88.3% lower than those observed in non-
266 stressed plants. g_s was also influenced by the inoculation with *Novosphingobium* sp. HR1a in absence of
267 salt stress, exhibiting a decrease of 47.9 and 50.9% in comparison to control plants after 20 and 30 days
268 from the beginning of salt stress treatments respectively (Fig. 10B).

269

270 **Discussion**

271 Results presented in this work reveal that inoculation with *P. putida* KT2440 or *Novosphingobium* sp. HR1a
272 mitigates the negative effect of salt stress on alemow plants, indicating that both rhizobacteria have a role
273 as a PGPR. As far as we know, the beneficial effect of *Novosphingobium* sp. HR1a on plant performance
274 has not been described previously. Although both strains have positive effects on plant tolerance to this
275 stress condition, there are common and different responses depending on the inoculated rhizobacterium.

276 The number of soil total CFU increased because of the inoculation by *P. putida* KT2440, independently of
277 the salt treatment used. Although chloramphenicol was added to the selective bacteria culture medium, a
278 high number of CFU was also recorded in those cultures of rhizospheric soil from non-inoculated plants.
279 This fact could be due to the occurrence of antibiotic-resistant bacteria in soil. In fact, it has been reported
280 the existence of different bacterial species as *Bacillus subtilis*, *Escherichia coli*, *Streptococus* sp., *Klebsiella*
281 *mobilis* or *Pseudomonas aeruginosa* simultaneously resistant to different antibiotics including
282 chloramphenicol in different soils (Mindlin et al. 2008; Popowska et al. 2012; Eghomwanre et al. 2016),
283 what explains the results obtained in this work.

284 Salt stress had a negative effect on citrus performance, inducing leaf damage and abscission, Cl^-, and proline
285 accumulation, and a decrease in gas exchange and chlorophyll fluorescence parameters. These results are
286 in concordance with previous reports (López-Climent et al. 2008; Hussain et al. 2012). Moreover, in non-
287 inoculated plants, salt stress induced leaf accumulation of ABA and SA, which has been widely reported in
288 several species as *Arabidopsis thaliana* (Prerostova et al. 2017), *Cucumis sativus* (Chojak-Koźniewska et
289 al. 2017) or the citrus rootstock Carrizo citrange (*Citrus sinensis* L. Osbeck x *Poncirus trifoliata* L.
290 Raf.,Gómez-Cadenas et al. 1998). Although the results of leaf damage appearance follow a similar trend in
291 both experiments, the symptoms were more evident in the second experiment. This fact could be due to the
292 increase of citrus plants metabolism during summer, absorbing higher quantities of water (and the toxic
293 ions Na^+ and Cl^- in salt-stressed plants), causing a higher affection of salt stress, as it has been described in
294 other plant species as *Vigna radiata* (Sehrawat et al., 2015).

295 Among the palliative effects of the inoculation with rhizobacteria, a reduction of stress-induced proline
296 accumulation in roots of plants inoculated with *P. putida* KT2440 was observed. It has been reported that
297 levels of this amino acid generally increase in plant tissues under stress situations such as drought and
298 salinity. It has been suggested that this compound can act as an osmoprotectant that avoids plant dehydration

299 and turgor loss, as well as an oxidative damage inhibitor (Hayat et al. 2012). Other authors have indicated
300 that proline should be considered a stress marker although with a marginal role in plant tolerance (Arbona
301 et al. 2017). However, independently of its role, an increase of proline is generally associated to abiotic-
302 stress induced damage. Consequently, the decrease in proline levels in salt-stressed plants inoculated with
303 *P. putida* KT2440 would indicate that plants were suffering stress in a lower degree. Interestingly, whereas
304 non-inoculated plants increased leaf contents of ABA and SA in response to salt stress, those plants
305 inoculated with *Novosphingobium* sp. HR1a exhibited unaltered hormone levels in response to the adverse
306 situation and those inoculated with *P. putida* KT2440 showed even a decrease in ABA levels. This lack of
307 increase of ABA and SA levels would further support the lower impact of salt stress on inoculated plants.
308 It has been described previously that the ACC-deaminase activity produced by PGPRs inhibits ethylene
309 biosynthesis in plants (Dimkpa et al. 2009). Consequently, since ethylene crosstalks with ABA, this chain
310 could lead to a reduction of ABA levels (Arc et al. 2013). A lower increase in ABA has been also reported
311 in salt- and osmotic-stressed cucumber plants inoculated with the PGPRs *Burkholderia* sp., *Acinetobacter*
312 sp., and *Promicromonospora* sp. (Kang et al. 2014). In addition, IAA levels increased in plants inoculated
313 with *Novosphingobium* sp. HR1a and subjected to salt stress. This increase could be due to the presence of
314 the PGPR, since some rhizobacteria, including *Novosphingobium* genus, produce IAA (Krishnan et al.
315 2017). In any case, IAA has been reported as a salt stress reliever (Kaya et al. 2013) and could promote
316 root growth and lateral root development, facilitating root exploration of new soil zones with lower contents
317 of toxic elements or higher water availability (Bao et al. 2014).

318 The inoculation with both rhizobacteria allowed salt-stressed plants to keep higher F_v/F_m values than non-
319 inoculated ones for 20 days whereas *Novosphingobium* sp. HR1a also induced the maintenance of higher
320 levels of Φ_{PSII} in salinized plants even after 30 days of stress treatment. Although chlorophyll fluorescence
321 parameters, as well as chlorophyll and carotenoid contents, generally decrease with the stress application
322 (López-Climent et al. 2008), PGPRs help plants to delay the adverse effects. For example, in water stressed
323 *Ocimum basilicum* plants, inoculation with *Pseudomonades* sp. induced higher F_v/F_m values (Heidari and
324 Golpayegani, 2012). Moreover, PGPRs from *Bacillus megaterium* and *Enterobacter* sp. have been reported
325 as inducers of chlorophyll accumulation in *Abelmoschus esculentus*subjected to salt stress (Habib et al.
326 2016). Although most studies regarding the effect of PGPRs on photosystem II are focused in herbaceous
327 crops, Rincón et al. (2008) working with *Pinus halepensis* and *Quercus coccifera* inoculated with

328 *Pseudomonas fluorescens* reported similar results as those reported here, but under drought stress
329 conditions.

330 There is some controversy about the effect of PGPRs on E and g_s. Whereas a positive relationship among
331 PGPR inoculation and high E and g_s values has been reported in salt-stressed *Vigna radiata* inoculated with
332 *Enterobacter cloacae* and *Bacillus drentensis* (Mahmood et al. 2016), and *Triticum durum*treated with
333 PGPRs (Zhu et al. 2014), other works indicate that E and g_s decrease in presence of PGPRs, improving
334 water use efficiency and consequently improving plant tolerance to stress conditions (Bresson et al. 2013;
335 Yasmin et al. 2013). Results obtained in this work showed that E and g_s decreased in absence of stress in
336 plants inoculated with *Novosphingobium* sp. HR1a, although further work would be needed to explore the
337 metabolic consequences of this reduction.

338 In addition to the physiological parameters measured, chloride ion concentration decreased in roots of
339 plants inoculated with *P. putida* KT2440 and subjected to salt stress. The accumulation of this ion has been
340 previously reported as the critical component of salt stress toxicity in citrus plants, being a key marker to
341 quantify salt stress damage in this crop (Moya et al. 2003). Therefore, reductions in the absorption of this
342 toxic ion have been associated to a stress tolerance in citrus plants (López-Climent et al. 2008; Hussain et
343 al. 2012).

344 Previous studies have reported that *Novosphingobium* sp. HR1a is more tolerant to high NaCl
345 concentrations than *P. putida* KT2440 (Vives-Peris et al. 2018b). This better performance of
346 *Novosphingobium* sp. HR1a under high salinity could explain its higher beneficial effects on salt-stressed
347 citrus plants.

348 In conclusion, results presented in this work reveal that the rhizobacterial species *P. putida* KT2440 and
349 *Novosphingobium* sp. HR1a have a palliative effect on citrus plants subjected to salt stress, reducing the
350 damage caused by this adverse condition. Consequently, both rhizobacterial strains can be considered as
351 PGPRs and could be used in biofertilization and bioaugmentation programs in order to promote plant
352 growth and prevent the damage caused by salt stress. Finally, the positive effects caused by
353 *Novosphingobium* sp. HR1a on plant performance were more evident although this species has not been
354 described previously as a PGPR.

355

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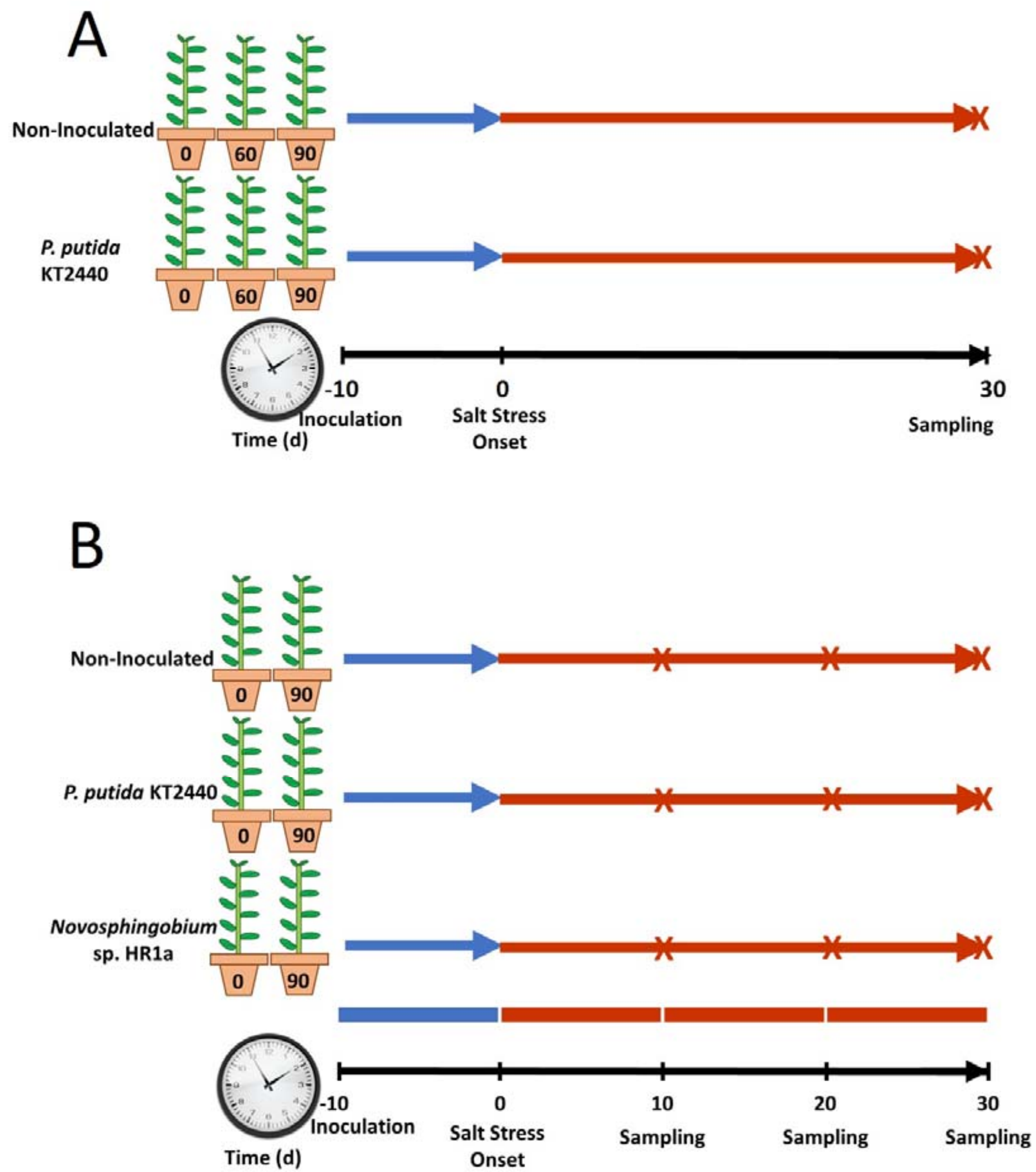

545 **Figure 1** Experimental design of the first (A) and the second set of experiments (B)

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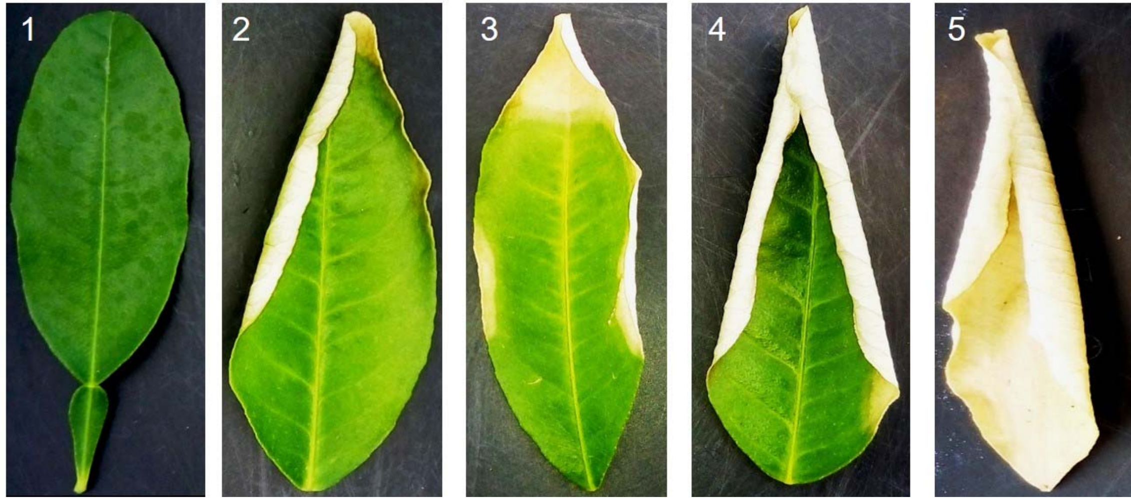

548 **Figure 2** Different levels of salt stress induced damage in leaves. 1: Non-damaged leaf; 2: Mild-damaged

549 leaf; 3: Intermediate-damaged leaf; 4: Severe-damaged leaf; 5: Leaf abscission

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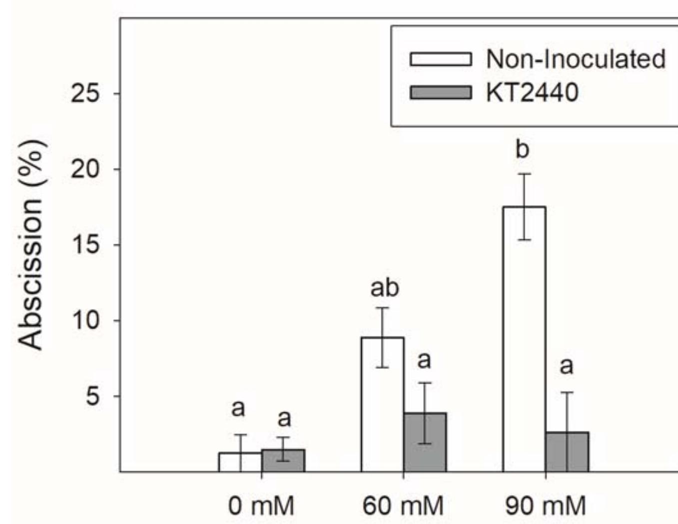

552 **Figure 3** Leaf abscission in non-inoculated plants (white bars) and plants inoculated with *P. putida* KT2440

553 (grey bars) exposed to 0, 60 and 90 mM NaCl for 30 days. Values indicate the mean of ten replicates ±

554 standard error. Different letters refer to statistically significant differences at $P \leq 0.05$

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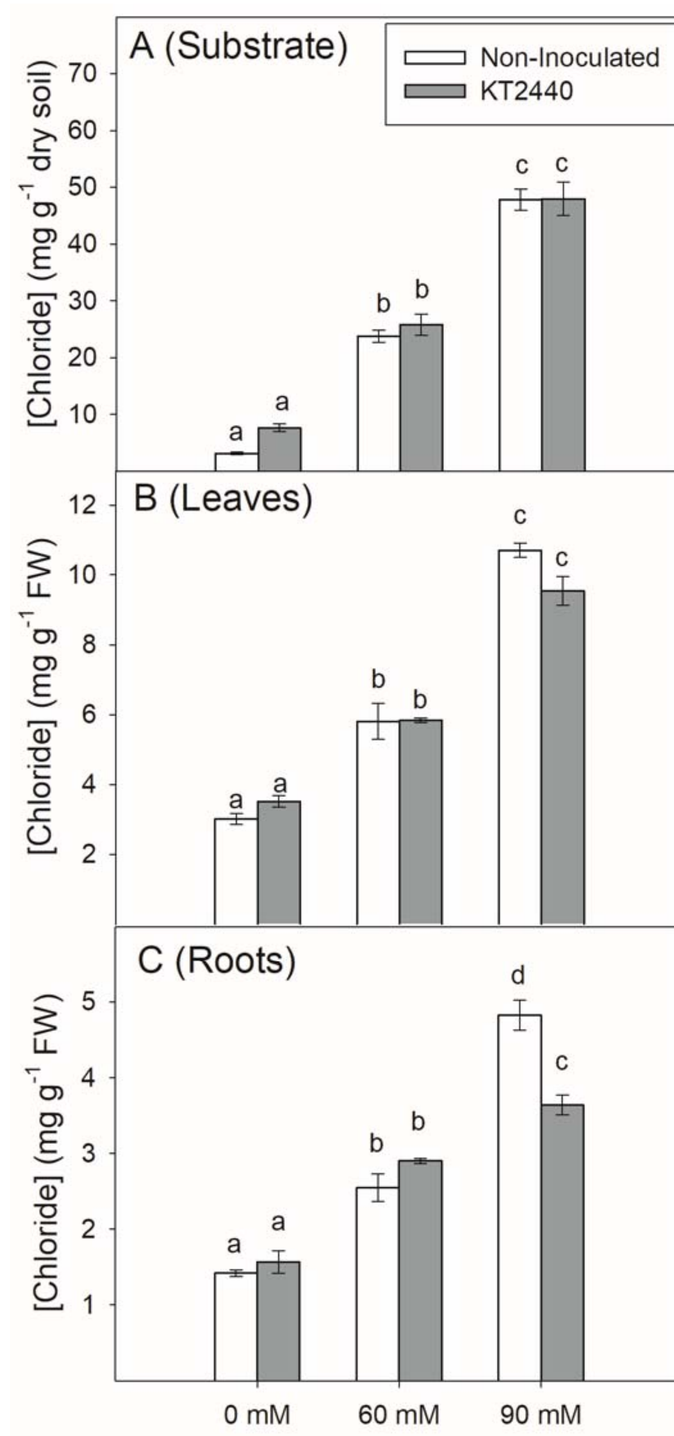

556 **Figure 4** Chloride contents in soil (A), leaves (B) and roots (C) in non-inoculated plants (white bars) and
557 plants inoculated with *P. putida* KT2440 (grey bars) exposed to 0, 60 and 90 mM NaCl for 30 days. Values
558 indicate the mean of three replicates ± standard error. Different letters refer to statistically significant
559 differences at $P \leq 0.05$

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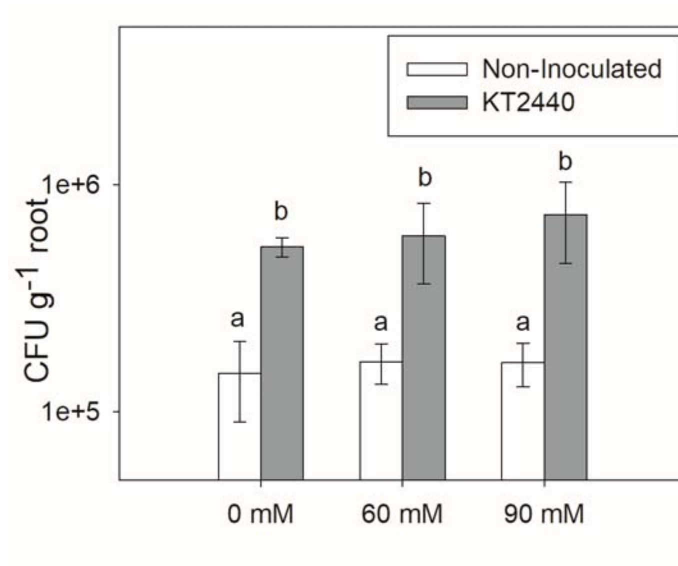

561

562 **Figure 5** Colony forming units in roots of non-inoculated plants (white bars) and plants inoculated with *P.*
563 *putida* KT2440 (grey bars) exposed to 0, 60 and 90 mM NaCl in the first set of experiments. Values indicate
564 the mean of nine replicates ± standard error. Different letters refer to statistically significant differences at
565 $P \leq 0.05$

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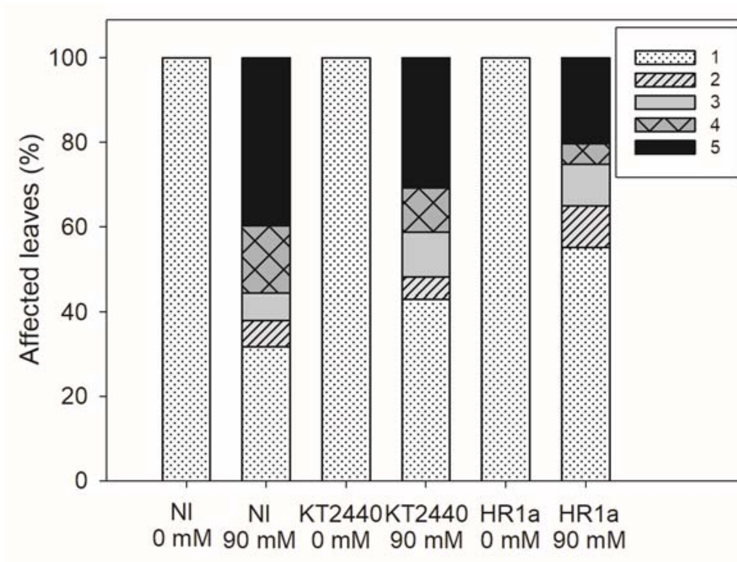
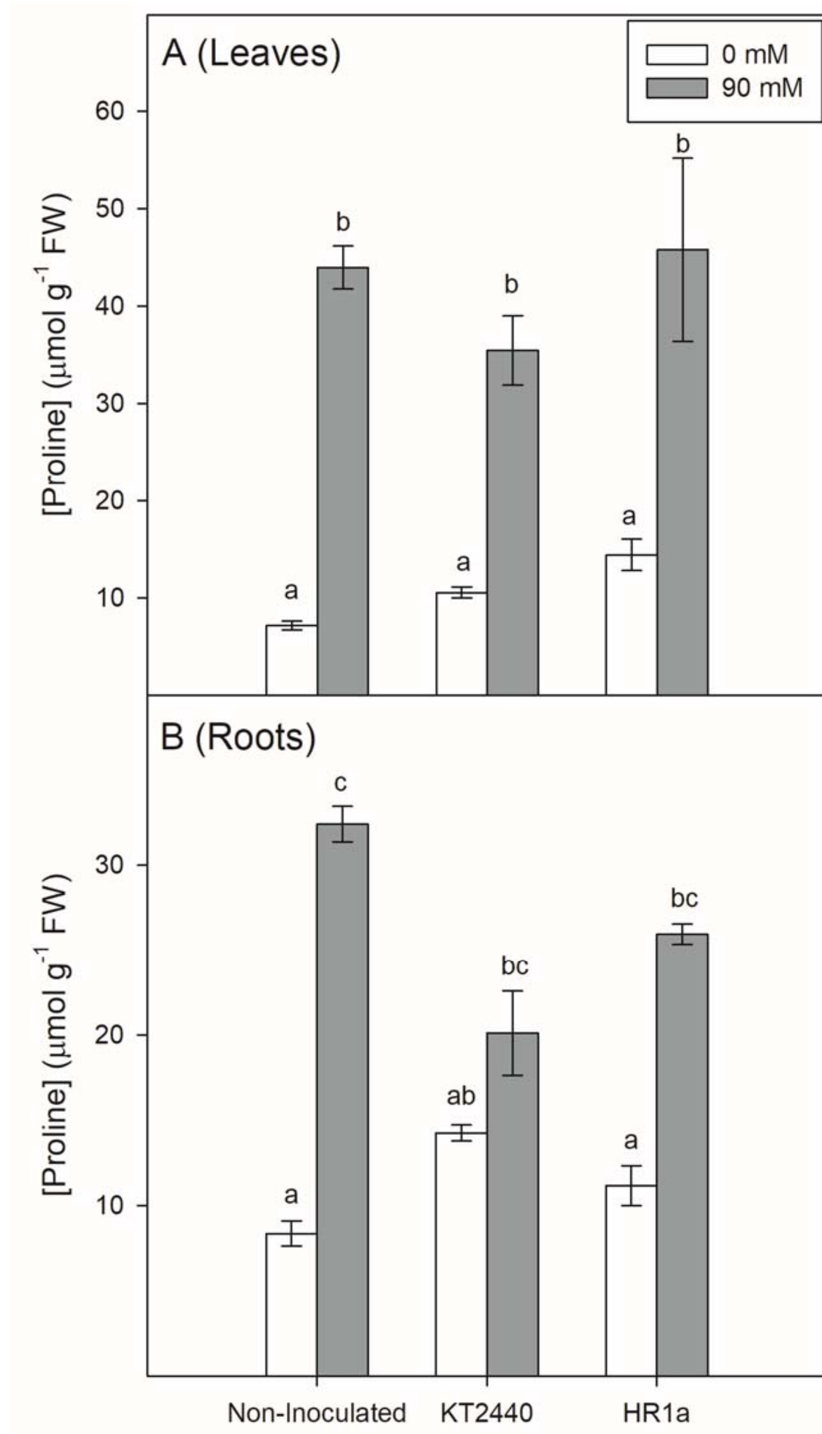


568 **Figure 6** Percentage of affected leaves. Different colors and patterns refer to the different levels of leaf

569 damage represented in the Figure 5. 1: Non-damaged leaf; 2: Mild-damaged leaf; 3: Intermediate-damaged

570 leaf; 4: Severe-damaged leaf; 5: Leaf abscission. Represented data refers to the mean of 10 plants.

571



572 **Figure 7** Proline concentration in leaves (A) and roots (B) of non-inoculated plants and plants inoculated
573 with *P. putida* KT2440 or *Novosphingobium* sp. HR1a in control conditions (white bars) and 90 mM NaCl
574 treatments (grey bars). Values indicate the mean of three replicates ± standard error. Different letters refer
575 to statistically significant differences at P ≤ 0.05

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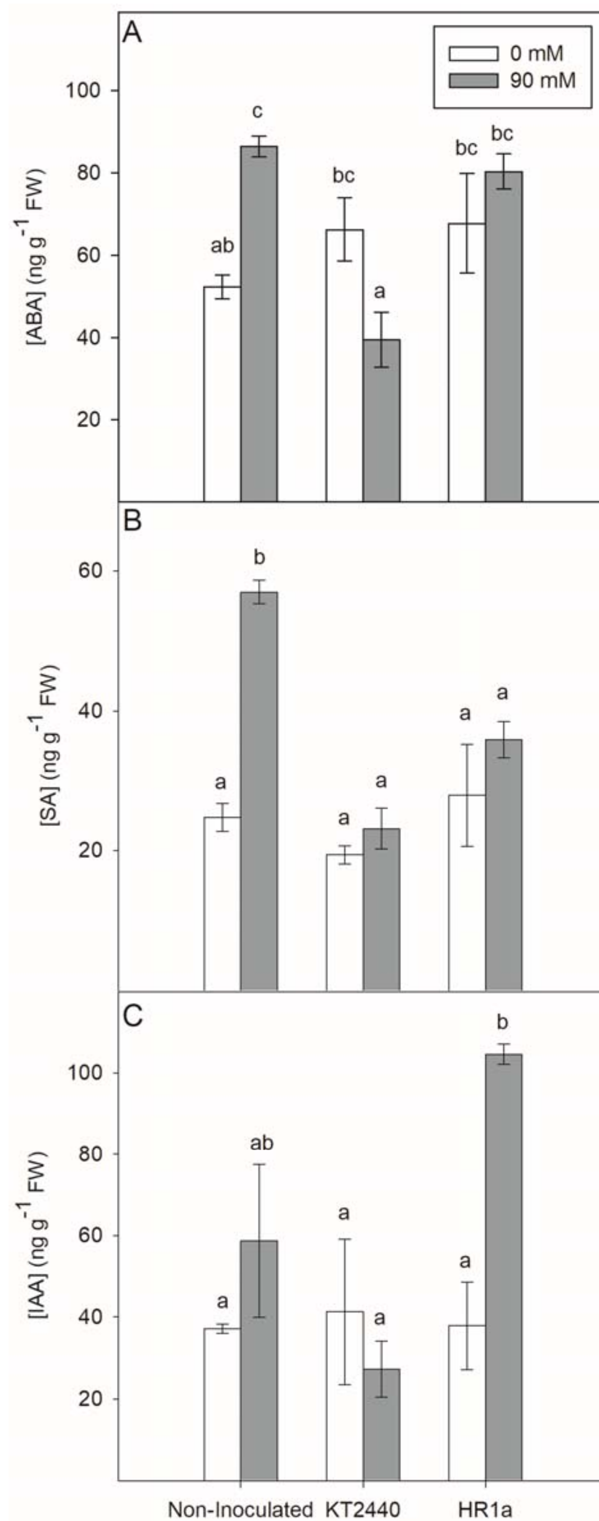
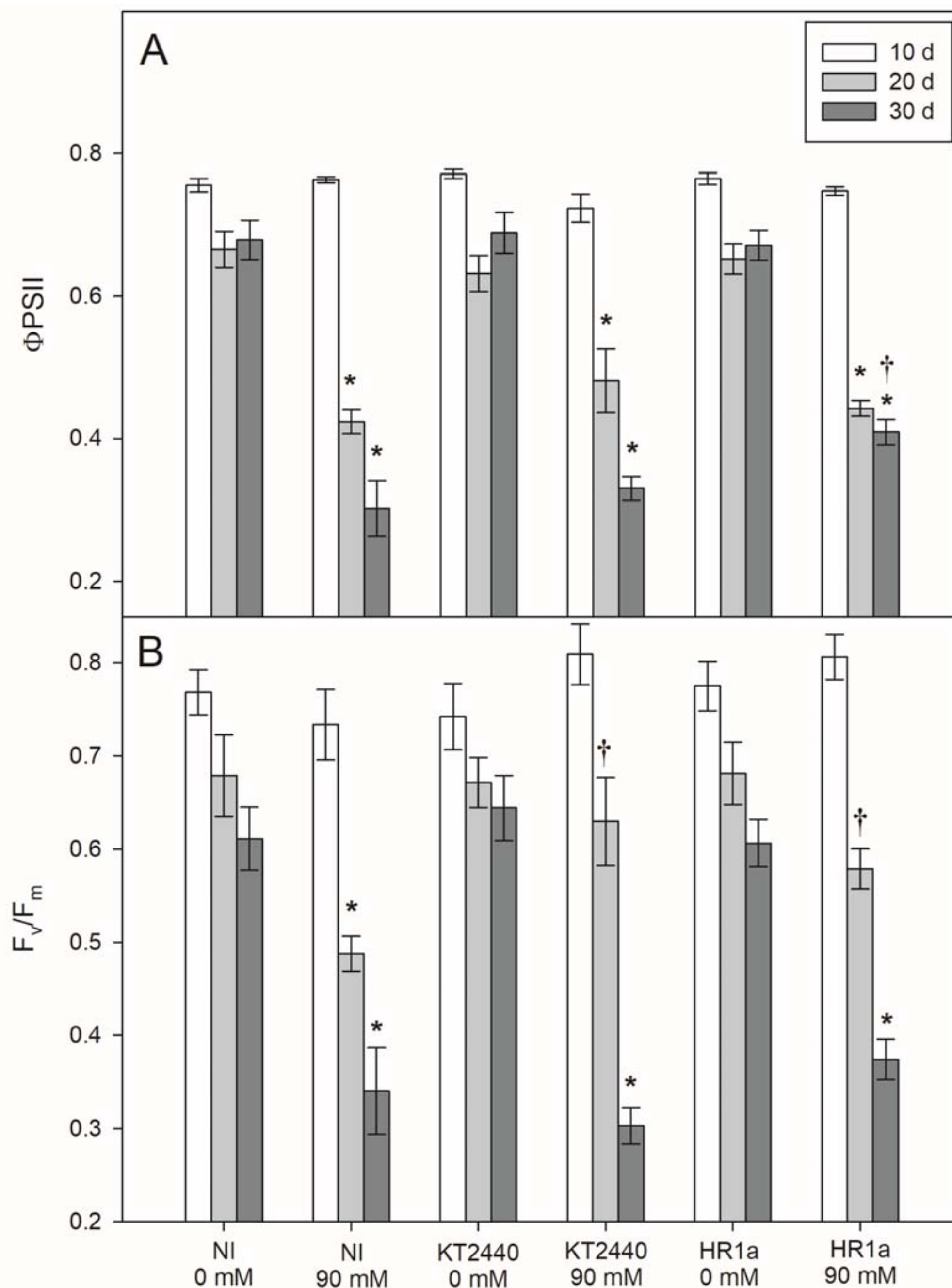


578 **Figure 8** Phytohormone contents in leaves of plants subjected to the different treatments after 30 days.
579 Endogenous ABA (A), SA (B) and IAA (C) in leaves of non-inoculated plants and plants inoculated with
580 *P. putida* KT2440 or *Novosphingobium* sp. HR1a in control conditions (white bars) and 90 mM NaCl
581 treatments (grey bars). Values indicate the mean of three replicates ± standard error. Different letters refer
582 to statistically significant differences at $P \leq 0.05$

583



584 **Figure 9** Chlorophyll fluorescence parameters in plants subjected to the different treatments. Quantum
585 efficiency (A) and maximum efficiency of PSII photochemistry (B) of non-inoculated plants and plants
586 inoculated with *P. putida* KT2440 or *Novosphingobium* sp. HR1a in control conditions and 90 mM
587 treatments after 10 (white bars), 20 (light grey bars) and 30 days (dark grey bars). Values indicate the mean
588 ± standard error. * refers to statistically significant differences among control and salt-stressed plants at P
589 ≤ 0.05, while † refers to statistically significant differences among non-inoculated and inoculated plants at
590 P ≤ 0.05

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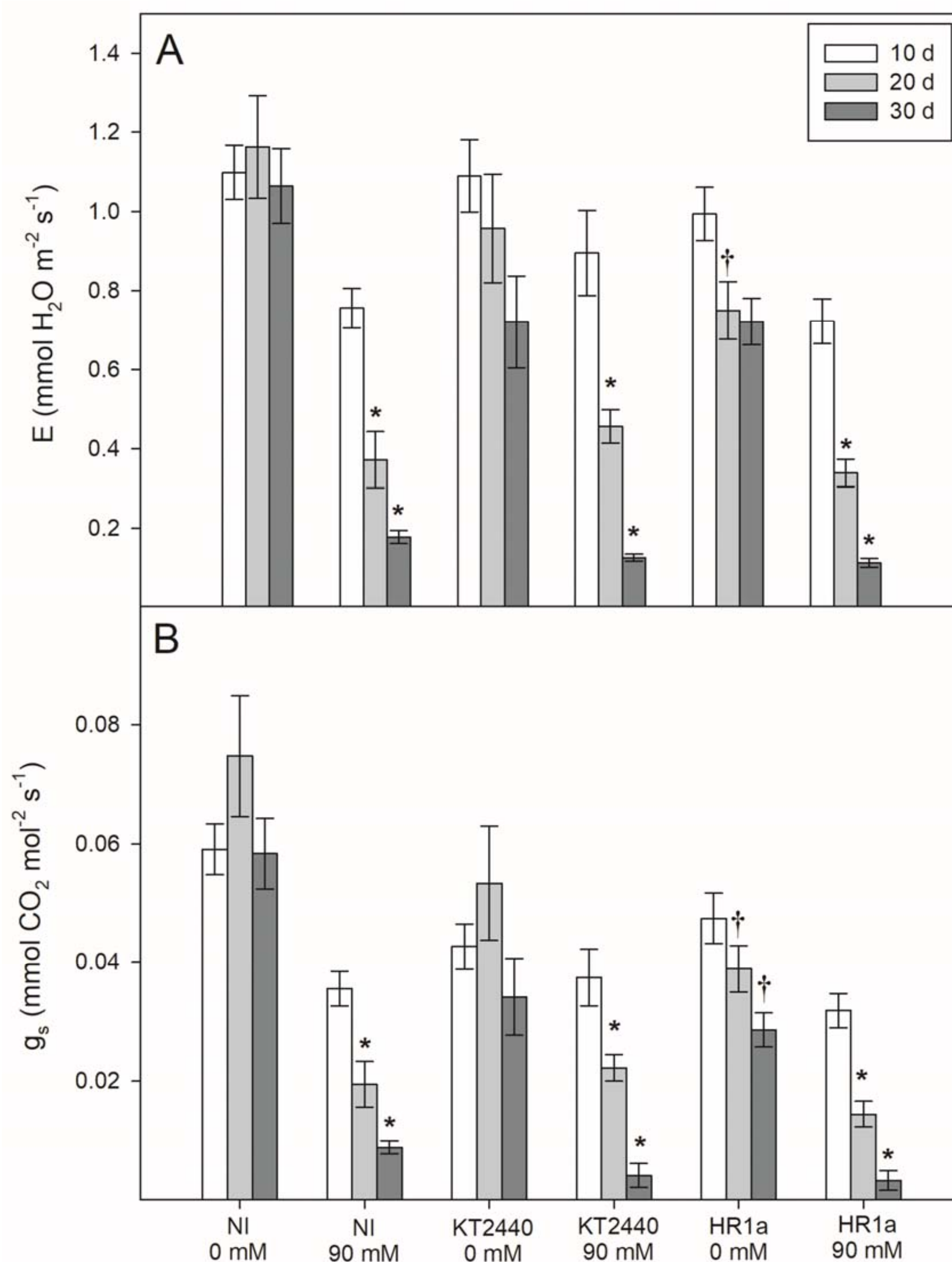

592 **Figure 10** Gas exchange parameters in plants subjected to the different treatments. Transpiration (A) and
593 stomatal conductance (B) of non-inoculated plants and plants inoculated with *P. putida* KT2440 or
594 *Novosphingobium* sp. HR1a in control conditions and 90 mM treatments after 10 (white bars), 20 (light
595 grey bars) and 30 days (dark grey bars). Values indicate the mean ± standard error. * refers to statistically
596 significant differences among control and salt-stressed plants at $P \leq 0.05$, while † refers to statistically
597 significant differences among non-inoculated and inoculated plants at $P \leq 0.05$

598 **Supplementary Material 1:** Numeric data, standard error and the significance letters
599 related to Figure 6.

600

		Non-damaged	Mild damage	Intermediate damage	Severe damage	Abscessed
Non-Inoculated	0 mM	100.0±0.0 c	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	90 mM	31.7±2.6 a	6.3±2.1 b	6.3±1.8 b	15.8±2.1 d	39.9±2.5 d
P. putida KT2440	0 mM	100.0±0.0 c	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0a
	90 mM	42.9±4.3 ab	5.3±2.1 b	10.5±3.1 ab	10.5±0.9 c	30.8±1.3 c
Novosphingobium sp. HR1a	0 mM	100.0±0.0 c	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	90 mM	55.1±3.7 b	9.9±2.4b	9.9±1.7 ab	4.9±1.9 b	20.3±3.7 b