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Title: *Arachis hypogaea* PGPR isolated from Argentine soil modifies its lipids components in response to temperature and salinity

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PII: S0944-5013(14)00160-8
DOI: http://dx.doi.org/doi:10.1016/j.micres.2014.12.012
Reference: MICRES 25732

To appear in:

Received date: 18-11-2014
Revised date: 23-12-2014
Accepted date: 26-12-2014

Please cite this article as: Paulucci NS, Gallarato LA, Reguera YB, Vicario JC, Cesari AB, García de Lema MB, Dardanelli MS, *Arachis hypogaea* PGPR isolated from Argentine soil modifies its lipids components in response to temperature and salinity, *Microbiological Research* (2015), http://dx.doi.org/10.1016/j.micres.2014.12.012

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1 **_Arachis hypogaea_ PGPR isolated from Argentine soil modifies its lipids**
2 **components in response to temperature and salinity**

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26 **Abstract**

27 The aim of this work was to clarify the mechanism related to plant growth promoting of
28 a bacterial strain (L115) isolated from *Arachis hypogaea* rhizospheres and the effects of
29 high growth temperature and salinity on phospholipids and fatty acids composition.
30 L115 was isolated from peanut rhizospheres and identified according to the sequence
31 analysis of the 16S rRNA gene. Phenotypic, metabolic and plant growth promoting
32 rhizobacteria (PGPR) characteristics of L115 were tested. Inoculation test in plant
33 growth chamber was performed. In addition, L115 was exposed to a 37ºC and 300 mM
34 NaCl and phospholipids and fatty acid composition were evaluated.
35 L115 strain was identified as *Ochrobactrum intermedium* and was able to increase the
36 peanut shoot and root length as well as dry weight, indicating a PGPR role by being
37 able to produce indole acetic acid and siderophores and present ACC deaminase
38 activity. In addition, L115 showed tolerance to both high growth temperature and 300
39 mM NaCl. The most striking change was a decreased percentage of 18:1 fatty acid and
40 an increase in 16:0 and 18:0 fatty acids, under high growth temperature or a
41 combination of increased temperature and salinity. The most important change in
42 phospholipid levels was an increase in phosphatidylcholine biosynthesis in all growth
43 conditions.
44 L115 can promote the growth of peanut and can tolerate high growth temperature and
45 salinity modifying the fatty acid unsaturation degree and increasing phosphatidylcholine
46 levels.
47 This work is the first to report the importance of the genus *Ochrobactrum* as PGPR on
48 peanut growth as well as on the metabolic behaviour against abiotic stresses that occur
49 in soil. This knowledge will be useful for developing strategies to improve the growth

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50 of this bacterium under stress and to enhance its bioprocess for the production of
51 inoculants.
52

53 **Keywords**

54 Isolated rhizospheric bacteria, PGPR, stress, fatty acid, phospholipid, peanut

55

56 1. Introduction

57 The peanut (*Arachis hypogaea*) is an annual herbaceous plant of family *Fabaceae* and
58 thrives in tropical and subtropical climates. The peanut is an important oilseed crop of
59 rainfed areas and a rich source of essential phytonutrients like niacin, folate, fibre and
60 vitamin E and antioxidant polyphenols, among several others (Kris-Etherton et al.,
61 2008). Argentina is one of the main peanut producer countries in the world and the 87%
62 of its production is concentrated in Córdoba province (Ibañez et al., 2010).
63 The peanuts rhizosphere bacterial community is of great importance and provides
64 important insights into the formulation of unique bioinoculants for sustainable and
65 green agricultural practices (Yousuf et al., 2012). There is great interest in developing
66 sustainable agricultural practices through the use of bioinoculants in the production
67 system and therefore, an understanding of the microbial structure and activity within
68 plant rhizospheres is important in order to produce efficient bioinoculants (Haldar et al.,
69 2011). Ibañez et al. (2009) recovered several fast-growing bacterial isolates from
70 surface-sterilised peanut root nodules growing in soils from Córdoba, Argentina. The
71 phylogenetic analysis of the bacterial 16S rDNA sequences showed that they belonged
72 to the Phylum Proteobacteria, Class Gammaproteobacteria, and included *Pseudomonas*
73 spp., *Enterobacter* spp. and *Klebsiella* spp. After storage, these strains were unable to
74 induce nodule formation in *Arachis hypogaea* L. plants, but did enhanced plant yield. It

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75 can be speculated that rhizospheric soil would contain plenty of genes with PGPR-like
76 functions. Detailed knowledge about PGPR activities and the interactions that occur in
77 the rhizosphere using either functional or sequence based methods will help us to devise
78 novel uses for PGPR microorganisms for use in sustainable agriculture.

79 Several environmental conditions are limiting factors for the growth of plants, PGPR
80 activity, and PGPR-legume symbiosis. High soil temperatures in tropical and
81 subtropical areas are a major problem for biological nitrogen fixation of legume crops
82 (Michiels et al., 1994), and can affect from the bacterial attachment, root infection,
83 nodule development to nitrogenase activity (Zahran, 1999). In addition, the salinity of
84 the soil may inhibit cell division and the elongation growth state and therefore stunt the
85 growth of leaves and stems of affected plants (Principe et al., 2007) The application of
86 100 mM of NaCl to peanut plants inhibited the nodule formation by *Bradyrhizobium*
87 strains (Dardanelli et al., 2009).

88 Cellular envelopes are the first barriers that protect the bacterium against different
89 environmental stresses; the survival is determined by their capacity to adapt through the
90 alteration of the lipid membrane composition (Aricha et al., 2004; Bakholdina et al.,
91 2004). The primary lipid components of the membrane are phospholipids (PL), the lipid
92 composition of membranes can be changed upon exposure to environmental and
93 chemical factors, through alterations in the polar head groups of the PL (Finean and
94 Michell, 1981) in order to maintain membrane fluidity (Soltani et al., 2005). Previous
95 studies in our laboratory have demonstrated that the PL composition is modified by salt
96 and temperature stresses in *Bradyrhizobium* TAL 1000 (Paulucci et al., 2011). Another
97 mechanism used to stabilise membrane fluidity in bacteria involve changes in fatty acid
98 (FA) components, changing the ratio of saturated to unsaturated, *cis* to *trans*

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99 unsaturation, branched to unbranched structures, acyl chain length, and formation of
100 cyclopropane FA (Ramos et al., 1997; Donato et al., 2000).
101 The immediate response to soil inoculation with PGPR varies considerably depending
102 on the bacteria, plant species, soil type, inoculum density, and environmental
103 conditions. The inoculated bacteria sometimes do not survive in the soil when
104 competing with better-adapted indigenous microflora (Bashan, 1998). An effective
105 biological control strain isolated from one region may not perform in the same way in
106 other soil and climate conditions (Capper and Higgins, 1993; Duffy et al., 1997;
107 Johnsson et al., 1998). For this reason, one important factor to be considered when
108 screening new isolates is their activity in a range of environments in which they would
109 be expected to be used and, in particular, in different soil types (Ross et al., 2000). Not
110 only is it important to find new bacteria that are tolerant to various conditions, but also
111 to understand what the mechanisms are that generate such tolerance. In addition, little is
112 known regarding the behaviour of the PL and FA in native bacteria under abiotic stress.
113 The aims of the present study were i) isolate a native strain associated with an *A.*
114 *hypogaea* roots, tolerant to high temperature and salinity, ii) evaluate PGPR
115 characteristics and behaviour symbiosis with peanut plants and iii) investigate the basis
116 of this tolerance at level of FA and PL.
117 The results may identify new strategies for increasing efficiency between rhizobacteria
118 and peanut plants. Since these microorganisms would be used for major inoculant
119 industries in Argentina and must be stable at room temperature for long periods in order
120 to obtain a high-quality production for export, our findings will be of great practical
121 importance.
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124 # 2. Materials and methods

125 ## 2.1. Isolation of native peanut rhizospheric bacteria

126 Bacterial strains were isolated from the rhizosphere of peanut plants cultivated in the
127 main peanut producing areas of central and south Córdoba, Argentina
128 (33°01′30″S 64°02′50″O). Rhizobacteria were isolated by placing one root in a 500 mL
129 Erlenmeyer flask containing 25 g of 0.1 cm diameter glass beads and 50 mL of
130 phosphate buffered saline (PBS, pH 7.4) and agitating the flasks at 150 r.p.m. at 28°C
131 for 1 h. After agitation, appropriate dilutions from the flasks were plated on 10%
132 trypticase soy agar (TSA) supplemented with cycloheximide and the plates incubated at
133 28°C for 2–15 days, after which, colonies were picked off the plates, inoculated on 10%
134 TSA agar slants, incubated at 28ºC for 2 days, and finally stored at 4ºC. These colonies
135 were also cultivated in 10% TSA media, incubated at 28°C for 18 h and finally, each
136 culture was suspended in a 20% glycerol solution and stored at -80°C.

137 A bacterial strain (called L115) was selected based upon its Gram stain, fast growth
138 rate, ability to produce exopolysaccharides, and tolerance of high temperature and
139 salinity.

140

141 ## 2.2. Culture Conditions

142 The bacterial strain was maintained on yeast extract mannitol (YEM) plates
143 (Somasegaran and Hoben, 1994) at 28ºC. The pH of the media was adjusted to 7 before
144 autoclaving. For the examination of bacterial growth, viability, and biochemical
145 parameters, the strain was grown in B media (Spaink et al., 1992) for 24 h in a shaking
146 water bath at 37ºC and 28ºC for temperature stress treatment. The media was
147 supplemented with 300 mM NaCl for saline stress treatment. Growth of the bacterial
148 was followed by measurement of the optical density at 620 nm (OD620) with three

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149 replicates. Viable cells were counted by removing samples at various times. Serial
150 dilutions of each sample were spotted on to YEM plates in triplicate and incubated at
151 28ºC. Viable cells were counted after 24 h.
152

153 **2.3. Sequences of 16S rDNA**

154 The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal
155 primers (Weisburg et al., 1991). Double strand sequencing was performed by custom
156 service with MACROGEN Inc. (Korea). Sequences were analysed using the BLASTN
157 algorithm available in GeneBank (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997).
158 Sequences were aligned using the Clustal W program (Thompson et al., 1997), with the
159 sequences retrieved from the GenBank database. A phylogenetic tree was reconstructed
160 using the neighbour-joining method (Saidtou and Nei, 1987) and p-distance model in
161 the MEGA4 program (Tamura et al., 2007) with bootstrap values based on 1000
162 replications (Felsenstein, 1985). The sequence obtained in this study was deposited in
163 the GenBank nucleotide sequence database under the accession number KM355982.
164

165 **2.4. Phenotypic, metabolic and PGPR characteristics from the isolated**
166 **L115 strain**

167 The L115 culture used was purified from a single colony after overnight incubation at
168 28ºC on YEM plates. The cell morphology was observed by microscope at 100X
169 magnification. The Gram stain was determined using a kit (Britania) and cytochrome
170 oxidase activity was tested according to the manufacturer's recommendations (Britania).
171 The mega plasmid profile from the L115 strain was assayed by a modification to the
172 methods described by Eckhardt (1978).

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173 Catalase activity was tested by the addition of 3% H_2O_2 to bacterial smears on a glass
174 slide.
175 Aerobic acid production from different carbohydrates was studied using base agar
176 media (YEM without mannitol) supplemented with a different carbon source (1%) and
177 bromothymol blue as a pH indicator. The tubes containing the media supplement with a
178 different carbon source was incubated for 48 h at 28ºC and acid production was
179 evaluated by changes in the pH indicator. Growth at 28ºC and 37ºC was determined by
180 growth on YEM plates after 24 h of incubation.
181 The ability to hydrolyse starch was determined by the appearance of a clear halo around
182 the colony on agar plates containing soluble starch (3 g beef extract, 10 g soluble starch,
183 12 g agar, and 1000 mL distilled water, pH7). The presence of starch can be indicated
184 by the development of a blue colour and therefore, a clear halo indicates starch
185 hydrolysis. The ability to hydrolyse casein was determined by the appearance of a clear
186 halo around the colony on agar plates containing milk (5 g peptone, 3 g yeast extract, 1
187 g skim milk, 12 g agar, and 1000 mL distilled water, pH7).
188 The ability of L115 to form biofilms was assessed by the techniques described by
189 Lerner et al. (2009). Briefly, glass tubes were inoculated with 800 µl of YEM medium
190 and incubated with shaking at 30°C to $O.D_{600}$=0.5. Cells were removed, and the tubes
191 were washed three times with saline solution, stained with crystal violet 0.1% (w/v) for
192 15 min, and rinsed to remove the dye excess. Biofilm formation was quantified by
193 solubilization of crystal violet with 1ml of ethanol 95% (v/v) for 20 min and posterior
194 measurement of absorbance at $O.D._{590}$.
195 The aggregates formation was evaluated as described by Burdman *et al*. (1998), for
196 aggregation assays L115 was grown in 250 ml Erlenmeyer flasks, in 100 ml of YEM
197 liquid medium. Flasks were inoculated with exponential-phase cultures to an initial

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198 O.D.$_{540}$ of approximately 0.05 (about lo7 c.f.u. ml-l) and incubated on a rotary shaker
199 (150 r.p.m.) at 30 ºC. Aliquots of liquid culture containing aggregates were transferred
200 to a conical tube and allowed to stand. After 20 min, aggregates had settled to the
201 bottom of the tube and the suspension was mostly composed of free cells. Turbidity was
202 measured from the suspension using a Genesis 5 spectrophotometer (Spectronic
203 Instruments) at 540 nm (O.D$_S$). The culture was then dispersed by treatment in a tissue
204 homogenizer (Heidolph RzR 50) for 1min and the total turbidity was measured (O.D$_t$).
205 The percentage aggregation was estimated as follows: % aggregation = [(O.D$_t$- OD$_s$) X
206 100]/O.D$_t$.
207 Indoleacetic acid (IAA) production was determined as described by Nuntagij *et al.*
208 (1997) used as positive control *Azospirillum brasilense* Az39 strain. 1-
209 Aminocyclopropane-1-Carboxylate (ACC) deaminase activity was determined by the
210 ability of strain L115 to grow on a plate of modified minimal media (MM$_9$) (60 g/L
211 Na_2HPO_4, 30 g/L KH_2PO_4, 5 g/L NaCl, 2 mL of 1 M $MgSO_4$, 5 g/L Mannitol, and 100
212 µL of 1 M CaCl2) supplemented with 3 mM ACC as the sole nitrogen source.
213 In order to evaluate the role of strain L115 within the nitrogen cycle of the soil, we
214 evaluated its ability to reduce nitrate. Tubes containing the appropriate media (5 g
215 peptone, 3 g yeast extract, 1 g potassium nitrate, and 1000 mL distilled water) were
216 incubated for 24 h at 28ºC and nitrate reduction was evaluated using the Griess reactive
217 (0.2% naphthylethylenediamine dihydrochloride and 2% sulphanilamide in 5%
218 phosphoric acid). The chrome azurol sulfonate assay was used for qualitative detection
219 of siderophores (Schwyn and Neilands, 1987), *Pseudomonas protegens* CHA0 was used
220 as positive control. The ability of the isolated strain to fix atmospheric nitrogen was
221 evaluated by growth in liquid and solid NFb media (Döbereiiner et al., 1995), which is
222 free of nitrogen sources. The ability to move strain L115 in Swimming plates (YEM

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223 media with 0.3% agar) and Swarming plates (YEM medium with 0.5% agar) was
224 assessed as described by Deziel et al. (2001). Swimming motility was assessed
225 qualitatively by examining the circular turbid zone formed by the bacterial cells
226 migrating away from the point of inoculation.
227

228 **2.5. Inoculation test in plant growth chamber**

229 Seeds of *A. hypogaea* cv. were obtained from Criadero El Carmen (Córdoba,
230 Argentina). Seeds were surface-sterilised in 10% H_2O_2 for 15 min and rinsed five times
231 with sterilised distilled water, then germinated in darkness at room temperature on
232 inverted sterile water-agar Petri dishes for 2 days. Seedlings were grown in plastic pots
233 containing sterilised and nitrogen-free vermiculite as substrate. Pots were placed in a
234 growth chamber at 24ºC with illumination for 16 h/day (Albareda et al., 2006). Plants
235 were cultured with Jensen nitrogen-free nutrient solution (Vincent, 1970). The L115 and
236 *Bradyrhizobium* sp. C145 (recommended as a peanut inoculant by the Instituto Nacional
237 de Tecnologia Agropecuaria (INTA) in Argentina) strains were grown on YEM and
238 inoculated individually and in combination onto seedling roots with 1 mL of suspension
239 containing approximately 10^8cells/mL. *Azospirillum brasilense* Az39 (Rivera et al.,
240 2014) was grow on Luria-Bertani (Maniatis et al., 1982) broth and inoculated onto
241 seedling roots with 0.6 mL of suspension containing approximately 10^6 cells/mL.
242 Five treatments were used: (1) non-inoculated seeds (control); (2) seeds inoculated with
243 L115; (3) seeds inoculated with *Bradyrhizobium* sp. C145; (4) seeds coinoculated with
244 L115 and *Bradyrhizobium* sp. C145 and (5) seeds inoculated with *Azospirillum*
245 *brasilense* Az39.
246 Plants were removed from the jars after one month and the roots thoroughly rinsed with
247 water, blotted dry on filter paper, and nodules picked and counted. The following

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248 parameters were evaluated: nodule number, shoot and root dry weights (65ºC for 72 h)
249 and shoot and root length.
250

251 **2.6. Study of the effect of stress on membrane components of L115**

252 **Incorporation of radioactive substrates**

253 A total of 0.5 µCi of acetate was added to 25 mL of culture at the time of inoculation.
254 The culture was incubated at the appropriate temperature with shaking for 24 h. The
255 cells were then harvested by centrifugation at 6000 for 10 min at 4°C. Pellets were
256 washed twice with 0.9% NaCl and used for further studies.
257

258 **Lipid Extraction**

259 Lipids were extracted from the washed bacteria using a chloroform/methanol/water
260 extraction (Bligh and Dyer, 1959); 0.1 M KCl in 50% methanol was added to obtain a
261 lower chloroform phase and an upper phase. The lower phase, containing the lipids, was
262 washed once with the KCl solution, dried under N_2, and dissolved in a suitable volume
263 of chloroform/methanol 2:1 (vol/vol).
264

265 **Separation and Analysis of PL**

266 Thin layer chromatography (TLC) plates (silica gel HLF, 250 lm) were purchased from
267 Analtech. Aliquots of the total lipid extracts were analysed by TLC using a solution of
268 chloroform/acetone/methanol/acetic acid/water 40:15:14:12:7 (vol/vol/vol/vol/vol) as
269 solvent. All solvents were of analytical or high-performance liquid chromatography
270 grade. Lipids were detected with iodine vapours and the separated lipids were identified
271 by comparison with authentic purified standards purchased from Sigma. The TLC plates

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272 were scraped and the fractions quantified by radioactivity measured in a liquid
273 scintillation counter (Beckman LS 60001 C).
274
275 **Analysis of fatty acids by GC**
276 FA methyl esters (FAME) were prepared from total lipid extracts with 10% BF_3 in
277 methanol (Morrison and Smith, 1964) and analysed using a Hewlett Packard 5890 II gas
278 chromatograph equipped with a column as described in Paulucci et al. (2011). Gas
279 chromatograph conditions were as follows: 250°C injector temperature; 300°C detector
280 temperature; and nitrogen as carrier gas. The temperature was programmed at 120°C for
281 1 min and then increased by 10°C min^{-1} to 175°C for 10 min, 5°C min^{-1} to 210°C for 5
282 min and 5°C min^{-1} to 230°C for 5 min. The peak areas of carboxylic acid in total ions
283 were used to determine relative quantities. Fatty acids were identified by comparing
284 retention times to commercial standards (Sigma Chemical Co., St. Louis, MO, USA).
285
286 **2.7. Statistical Analyses**
287 Data were compared using a one-way analysis of variance (ANOVA) test followed by
288 Tukey's multiple comparison test to obtain a p-value. A $P<0.05$ was considered
289 statistically significant.
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296 # 3. Results

297 ## 3.1. Identification of isolated *Arachis hypogaea* rhizospheric bacteria

298 Based on our analysis of 16S rRNA gene sequences, L115 was identified as from the
299 *Ochrobactrum* genus. Comparative 16S rRNA gene sequence analysis (Fig. 1) showed
300 that strain L115 belonged to the family *Brucellaceae*, class Alphaproteobacteria, and
301 was most closely related to *Ochrobactrum intermedium* (GenBank accession number:
302 AJ242583, 99% identity).

303

304 ## 3.2. Phenotypic, metabolic and PGPR characteristics of isolated L115
305 strain

306 Cells of the L115 strain are Gram-negative, presenting with colonies that are circular,
307 off-white, convex, mucoid and translucent with entire margins. The L115 strain was
308 able to form a biofilm (Fig 2A) but not aggregates. The L115 strain showed both
309 swimming and swarming motility (Fig 2B-C). The plasmid profile indicated that the
310 isolate strain has two megaplasmids of molecular weight higher than 1000 Kb (Fig 2D).
311 The L115 strain grows on YEM agar at 28–37ºC under aerobic condition (oxidase and
312 catalase positive). Acid production from cellobiose, maltose, rhamnose and xylose was
313 observed (Table 1). In contrast to the reference strains (*O. intermedium* KACC 11952),
314 the isolate was positive for acid production from cellobiose but not from sucrose.
315 Similar to the reference strain (*O. intermedium* KACC 11952), L115 was negative for
316 starch and casein hydrolysis reactions (Table 1).

317 The L115 was able to produce IAA (80.2μg ml^{-1}). Nitrate to nitrite reduction and
318 siderophore (Fig 2E), as well as ACC deaminase activity was observed in strain L115.
319 In addition, the isolate grew in a nitrogen-free liquid media, suggesting that it may have
320 a nitrogen-fixing ability.

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321 The result of the analysis of the sequence of 16S rRNA gene and phenotypic
322 characterization would indicate that the L115 strain is *Ochrobactrum intermedium*.
323

324 **3.3. Symbiotic performance of L115 on *Arachis hypogaea***

325 The effect of individual inoculation and co-inoculation of L115 with *Bradyrhizobium*
326 sp. C145 (recommended as a peanut inoculant by INTA in Argentina) on *A. hypogaea*
327 seedling growth was evaluated (Fig. 3). The peanut plants inoculation with the L115
328 strain had an increase in shoot (34%) and root (39%) length when compared to
329 uninoculated plants (Fig. 3A), as well as in shoot (148%) and root (75%) dry weight
330 (Fig. 3B). Peanut plants co-inoculated with *Bradyrhizobium* sp. C145 and L115 strains
331 had enhanced root length relative to plants that were inoculated with only the individual
332 strains. In addition, co-inoculation with both strains increased production of nodules
333 when compared to the nodules produced by inoculation of peanut plants with single
334 strain *Bradyrhizobium* sp. C145, although the differences were not statistically
335 significant (Data not shown). In this regard, is important to highlight that the presence
336 of the L115 strain did not affect the nodulation by *Bradyrhizobium* sp. C145 when
337 peanut plants were co-inoculated with both strains.

338 In the Figure 3C can observe changes in the peanut root when they were inoculated with
339 L115 compared to uninoculated plants. *Azospirillum brasilense* Az39 is a
340 rhizobacterium isolated from surface-sterilized wheat seedlings in Marcos Juarez,
341 Argentina, may be associated with plant roots and promote growth of the same through
342 the production of plant growth regulators as IAA. Since L115 strain, similar to *A.*
343 *brasilense* Az39, was producing IAA, we decided to compare the effects of inoculation
344 of peanut with L115 and *A. brasilense* Az39 compared to uninoculated plants (Fig. 4).

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345 Peanut plants inoculated with *A. brasilense* Az39 showed an increase in the length and
346 the dry weight of shoot and root compared with uninoculated plants, however, these
347 were not statistically significant. The peanut inoculation with strain L115 showed a
348 better effect on growth parameters than when they were inoculated with *A. brasilense*
349 Az39 strain. The peanut plants inoculation with the L115 had an increase in shoot
350 (34%) and roots (16%) length compared to *A. brasilense* Az39 inoculated plants (Fig.
351 4A), as well as in shoot (98%) and root (37%) dry weight (Fig. 4B).
352

353 **3.4. Determination of the tolerance of L115 to high growth temperatures**
354 **and salinity**

355 Higher CFU values were obtained with both a higher growth temperature (37ºC) and a
356 combination of NaCl and high growth temperature compared to control. Viability of
357 L115 was not modified by 300 mM NaCl alone (Fig. 5).
358

359 **3.5. Effect of high growth temperature and salinity on L115 phospholipid**
360 **metabolism**

361 The predominant PL labelled from [1-14C] acetate sodium salt was
362 phosphatidylethanolamine (PE), followed by phosphatidylcholine (PC),
363 phosphatidylglycerol (PG), dimethyl phosphatidylethanolamine (DMPE), cardiolipin
364 (CL) and lysophosphatidylethanolamine (LPE) (Table 2).

365 Biosynthesis of PC and PE was highly modified by the conditions tested, thus PC
366 biosynthesis increased 22.4% by high salinity and 59% by high growth temperature and
367 105% by the combination of both. PE radioactive labelling decreased in all conditions
368 assayed (17.5% in high saline, 56% in high growth temperature, and 63% under
369 combined conditions).

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370 **3.6. Effect of growth conditions on L115 fatty acid composition**

371 The FA composition of L115 strain is shown in Table 3. The major FA was *cis*-
372 vaccenic (18:1Δ11), stearic acid (18:0), palmitic acid (16:0), eicosatrienoic acid (20:3)
373 and cyclopropane FA (19:0cyclo).
374 In L115, 18:1Δ11 was the FA that showed the greatest change in response to tested
375 conditions, declined from 51.3 to 9.1% under high growth temperature, and to 5.8%
376 under combined conditions. On the other hand, both saturated FA (16:0 and 18:0)
377 increased by the effect of high growth temperature. At 37ºC, 16:0 increased from 9.3 to
378 21.5% and to 17.4% under the combined conditions. The other saturated FA, 18:0,
379 increased from 25.5 to 43.9% at 37ºC and to 36.2% under combined conditions. These
380 conditions also caused changes in the percentage of 19:0cyclo whose increased from 6.9
381 to 17.9% at 37ºC and to 16.1% under combined conditions. High media salinity alone
382 produced an increased in 16:0 of 55%.
383 The growing conditions applied to L115 caused a shift in the ratio of unsaturated to
384 saturated FA (U/S in Table 2), which decreased in all experimental conditions.
385

386 **4. Discussion**

387 High saline conditions are known to suppress the growth of plants and result in a
388 diminished yield. In the southeast of the Cordoba province (Argentina), there are
389 $1.5X10^6$ ha affected by high salinity (Cantero et al., 1996). An important factor to be
390 considered when screening new bacterial isolates is their activity in the range of
391 environments in which it is expected they will be used.
392 With this consideration in mind, we isolated native bacteria from *Arachis hypogaea*
393 rhizospheres from the Cordoba province soil that were tolerant of 300 mM NaCl and

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394 high growth temperatures. We also analysed changes in the polar lipid membrane and
395 FA composition under these stress conditions.
396 16S rRNA gene sequence analysis identified the isolated strain as *Ochrobactrum*. Based
397 on results of the 16S rRNA gene sequence, the L115 strain and *Ochrobactrum*
398 *intermedium* are phylogenetic neighbours. In addition, metabolic and phenotypic
399 characteristic, as well as PL and FA profiles, support the placement of the L115 strain
400 within the genus *Ochrobactrum* (Woo et al., 2011). Based on the phylogenetic analysis
401 and phenotypic characteristics, strain L115 could be identified as *O. intermedium*.
402 However, some quantitative differences were found between the proportions of FA
403 components in strain L115 and several strains of the closest neighbours (*O.*
404 *intermedium*) (Imran et al., 2010; Woo et al., 2011, Ngom et al., 2004), which may
405 indicate that this is a new strain of *O.intermedium*. Yousuf et al. (2012) has also
406 reported the presence of strains of the *Ochrobactrum* genus in *A. hypogaea*
407 rhizospheres. These researchers found that a total of 108 *cbbL* clones were obtained
408 from the rhizospheres of *A. hypogaea,* which revealed predominance of the *cbbL*
409 sequence associated with *O. anthropi.* When L115 was used for *A. hypogaea*
410 inoculation experiments, we observed an increase in dry biomass compared to
411 uninoculated plants, indicating a PGPR role. This result shows, for the first time, that an
412 *Ochrobactrum* strain is able to promote the growth of *A. hypogaea* plants. This activity
413 could include production of IAA and siderophores, ACC deaminase activity and the
414 reduction of nitrates. In addition, inoculation of peanut plants with L115 caused marked
415 increases in growth parameters compared to those obtained when peanut plants were
416 inoculated with *Bradyrhizobium* sp. C145 (recommended as a peanut inoculant by
417 INTA in Argentina) and *Azospirillum brasilense* Az39 (model rhizosphere strain,
418 Argentine isolated), indicating its potential to promote the growth of peanut.

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419 Bacterial biofilm is a desired feature in PGPR strains, L115 showed capacity to produce
420 biofilm, reaching levels greater than that obtained with the strain used as control
421 (*Bradyrhizobium* sp. C145). This feature can enhance the beneficial effects on the plant,
422 since this allows greater number of viable cells for longer in direct contact with the
423 plant (Seneviratne et al., 2011).

424 *Ochrobactrum* species have been described as free-living Alphaproteobacteria, and have
425 been recovered from diverse habitats, including soil, plants and their rhizospheres,
426 animals, and humans. In soil, *Ochrobactrum* strains were found to constitute 2% of the
427 cultivable bacteria, and on the wheat rhizoplane this fraction was approximately 0.3%
428 (Lebuhn et al., 2000; Bathe et al., 2004), indicating that *Ochrobactrum* is a substantial
429 part of the currently cultivable soil and rhizosphere microbial communities. The
430 diazotrophy of *Ochrobactrum,* in concordance with our results, has previously been
431 included in the description of strains with complete symbiotic ability in *Acacia* and
432 *Lupinus* nodules (Ngom et al., 2004; Trujillo et al., 2005). In addition to its ability to
433 establish symbiotic relationships with legumes, some species of the genus
434 *Ochrobactrum*, as is the case of *O. antrhopi,* have been described as PGPR.

435 *Ochrobactrum* strains have been characterised by their ability to grow under high
436 concentrations of NaCl (Principe et al., 2007; Kesserü et al., 2002). In this study the
437 isolated bacterium was tolerant to high growth temperature (37ºC) and salinity (300 mM
438 NaCl).

439 Adaptive mechanisms induced in cells in response to changes in environmental
440 conditions in order to maintain membrane fluidity involve modification of PL
441 components (Ramos et al., 1997; Härtig et al., 2005). The L115 strain modified the PL
442 composition under all tested conditions by increasing PC and decreasing PE synthesis.
443 PC is a phospholipid that tends to form in the membrane bilayer structure, so that

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444 membranes with a greater amount of PC tend to be packaged more than those
445 containing greater amounts of PE (nonbilayer forming). Balancing bilayer and
446 nonbilayer forming lipids within the membrane is crucial in maintaining structural and
447 functional integrity (Denich et al., 2003). In strain L115, that PC increased at all
448 conditions tested, could mean an important adaptive mechanism for maintaining the
449 structure and function of the membrane under stress conditions.
450 The other adaptive mechanism often used by bacteria is alteration of membrane FA
451 (Ramos et al., 2002). The FA composition of total lipids in L115 was similar to those
452 reported by different *Ochrobactrum* strains (Ngom et al., 2004) containing principally
453 18:1Δ11, 18:0, 16:0 and 19:0cyclo FA. Of all the tested conditions, the growth
454 temperature increase was the one that caused the most significant changes in FA levels
455 in L115. High growth temperature and combined conditions caused a significant
456 reduction of 18:1Δ11 and an increase of 18:0 and 16:0. These changes provoked
457 modifications in the degree of FA unsaturation concurrent with results obtained for
458 rhizobia in which different environmental changes caused modifications in the U/S ratio
459 (Drouin et al., 2000; Boumahdi et al., 2001). 37ºC and combined conditions resulted in
460 a decrease in the U/S ratio and enhanced formation of 19:0cyclo. Cyclic FA in the
461 membranes of L115 could represent a mechanism to reduce membrane fluidity, similar
462 to rhizobia (Paulucci et al., 2011) and *lactobacillus* (Guerzoni et al., 2001).
463 We conclude that this new isolated strain is able to promote the growth of peanut plants
464 through the production of IAA and siderophores, likewise the strain is part of the
465 nitrogen cycle, reducing nitrate to nitrite. In addition the isolate is also able to adapt to
466 tested environmental conditions through PL and FA modification. The most important
467 mechanism for maintaining the physical properties of the plasma membrane is

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468 modification to the degree of FA unsaturation. The ability of L115 to alter its FA
469 19:0cyclo content may account for its tolerance of high temperature.
470 Understanding how native rhizobacteria tolerate the soil conditions in which they live
471 can be useful for generating strategies to improve plants survivability in a specific
472 niche. In addition, stress tolerant bacterial strains may have an important role in
473 improving the tolerance of plants and establish symbiosis.
474 Further studies in our laboratory are being conducted to determine whether the L115
475 bacterial strain can help mitigate the negative effect of salinity on peanut plants.
476

477 Acknowledgments

478 Financial assistance was provided by SECyT- UNRC/Argentina, CONICET PIP 112-
479 201101-00309 and PID 2010 MINCyT Córdoba.
480 N.S.P and M.S.D are a member of the Research Career of CONICET-Argentina. L.A.G,
481 Y.B.R, J.C.V and A.B.C are a fellow of CONICET-Argentina.
482

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660 **Figure legends**

661

662 **Fig. 1** A neighbour-joining tree based on 16SrRNA gene sequences showing the
663 phylogenetic position of strain L115 among other recognised members of the genus
664 *Ochrobactrum* and different PGPR strains.
665 Bootstrap values are based on 1000 replications and are shown at branch points. Bar
666 indicates 0.05% sequence divergence.

667

668 **Fig. 2** Relevant characteristics of L115 strain. (A) Biofilm production, (B) swimming
669 and (C) swarming motility, (D) plasmid profile and (E) siderophore production: 1 – 2:
670 *Pseudomonas protegens* CHA0; 3 – 4: *Bradyrhizobium* sp. C145; 5 – 6: L115 and 7 – 8:
671 *Azospirillum brasilense* Az39.

672

673 **Fig. 3** The effect of inoculation with L115 and *Bradyrhizobium* sp. C145 strains on
674 length (A) and dry weight (B) of shoot and root of *Arachis hypogaea.* Peanut plants
675 uninoculated and inoculated with strain L115 (C).
676 Values represent means ± SEM of three independent experiments.

677

678 **Fig. 4** The effect of inoculation with L115 and *Azospirillum brasilense* Az39 strains on
679 length (A) and dry weight (B) of shoot and root of *Arachis hypogaea.*

680

681 **Fig. 5** The effect of high growth temperature and salinity on viability of L115.

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682 Viability is expressed as CFU ml^{-1}. Values represent means ± SEM from three

683 independent experiments.

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Table1 Biochemical and physiological characteristics of strain L115 and other closely related strain in the genus *Ochrobactrum*

Characteristic	**1**	**2**
Gram reaction	_	_
Growth at:		
28ºC	+	ND
37ºC	+	+
Enzyme activities:		
Oxidase	+	+
Catalase	+	+
Hydrolysis of		
Starch	_	_
Casein	_	_
Acid production from:		
L-Arabinose	_	_
Cellobiose	+	_
D-Glucose	_	_
Fructose	+	ND
D-Mannose	_	_
Maltose	+	+
D-Mannitol	+	+
L-Rhamnose	+	+
Sucrose	_	+
Trehalose	_	_
Lactose	_	_
D-Xylose	+	+

Strains: **1**: strain L115; **2**: *O. intermedium* KACC 11952.

The table is arranged according to phylogenetic clustering.

Data taken from Woo et al. (2011).

+: Positive and _: negative

ND: not determined

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Table 2 Effect of high growth temperature and salinity stress on the incorporation of [14C] acetate into phospholipids of L115

PL (%)	Growth conditions			
	28ºC	28ºC+NaCl	37ºC	37ºC+NaCl
PC	21.4±3.4	26.2±1.4*	34.0±0,1*	44.0±1.7*
DMPE	9.20±0.5	10.0±1.0	14.6±2.0	11.0±0.8
LPE	2.00±0.8	2.00±1.2	3.00±1.4	1.80±0.5
PE	34.8±1.7	28.7±0.7*	15.0±1.3*	13.0±1.8*
PG	17.0±3.5	13.0±1.3	13.2±1.6	10.6±0.3
CL	7.40±1.0	7.80±0.7	6.60±1.0	7.40±0.1
NL	2.10±0.2	1.90±0.1	1.60±0,05	2.60±1.0
NI	6.00±3.6	10.4±1.2	12.0±3,6	9.60±2.0

Values represent means ± SEM of three independent experiments.

PL phospholipids, PC: phosphatidylcholine, DMPE: dimethylphosphatidylethanolamine, LPE: lysophosphatidylethanolamine, PE: phosphatidylethanolamine, CL: cardiolipin, PG: phosphatidylglycerol, NL: neutral lipids and NI: no identified.

* The difference with respect to the control value (28ºC) is statistically significant *P*<0.05.

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Table 3 Effects of high growth temperature and salinity on fatty acid composition of L115

Fatty acid type (%)	Growth conditions			
	28ºC	28ºC+NaCl	37ºC	37ºC+NaCl
Saturated				
18:0	25.5±3.3	27.0±3.8	43.9±7.0*	36.2±2.0*
16:0	9.30±0.8	14.4±1.4	21.5±1.8*	17.4±0.5*
Unsaturated				
18:1Δ11	51.3±5.5	44.7±2.5	9.10±2.3*	5.80±0.3*
20:3	6.30±2.0	6.90±3.2	8.30±0.4*	23.6±4.0*
Cyclopropane				
19:0cyclo	6.90±1.0	6.25±1.3	17.9±3.0*	16.1±3.3*
U/S [a]	1.3	1.06	0,2	0,4

Lipids were extracted and total lipid fatty acids were converted to methyl esters and analysed by GC as described in the text.

Percentage of each fatty acid is relative to total fatty acids defined as 100%. Values represent means ± SEM of three independent experiments.

a ratio between sums of unsaturated and sums of saturated fatty acids.

* The difference with respect to the control value (28ºC) is statistically significant $P<0.05$.

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Figure 1

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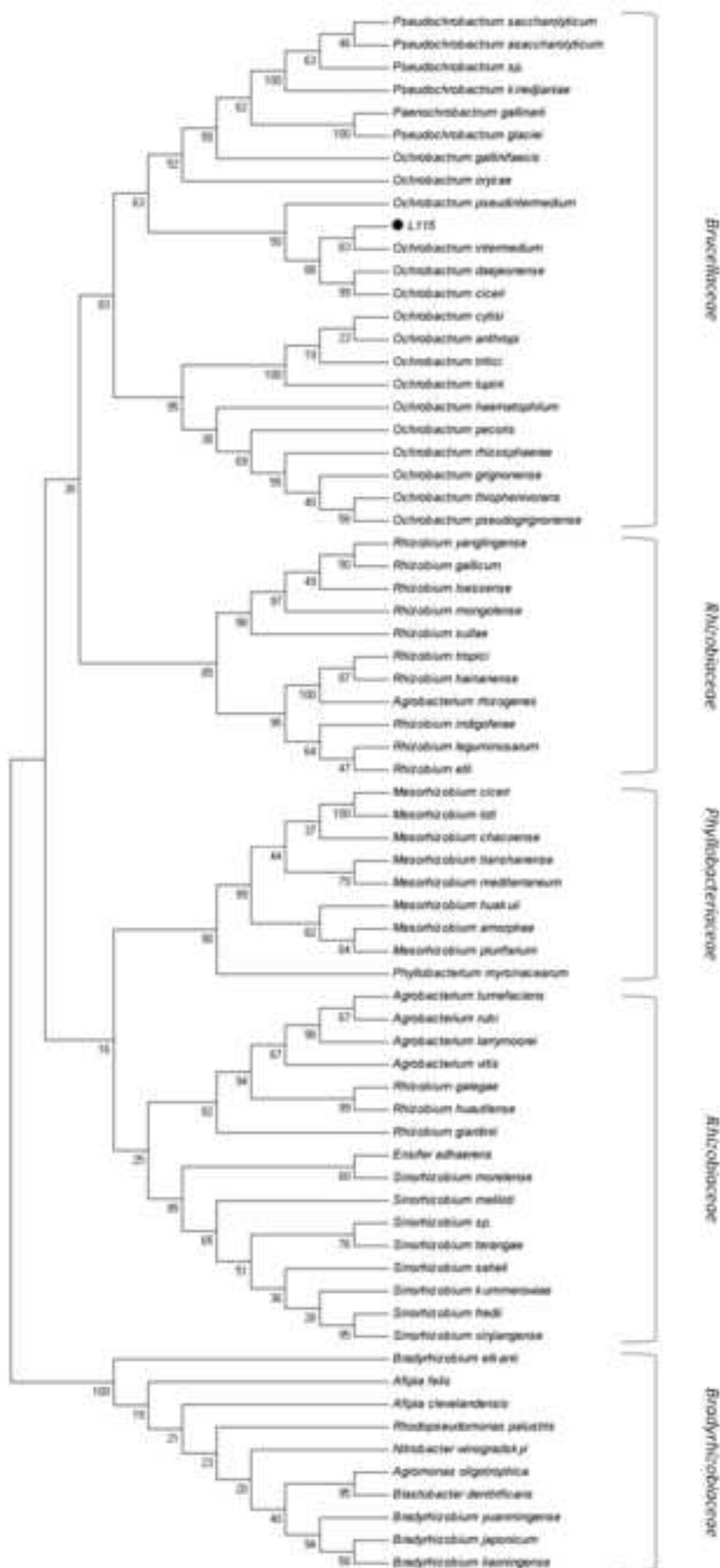

Figure 2

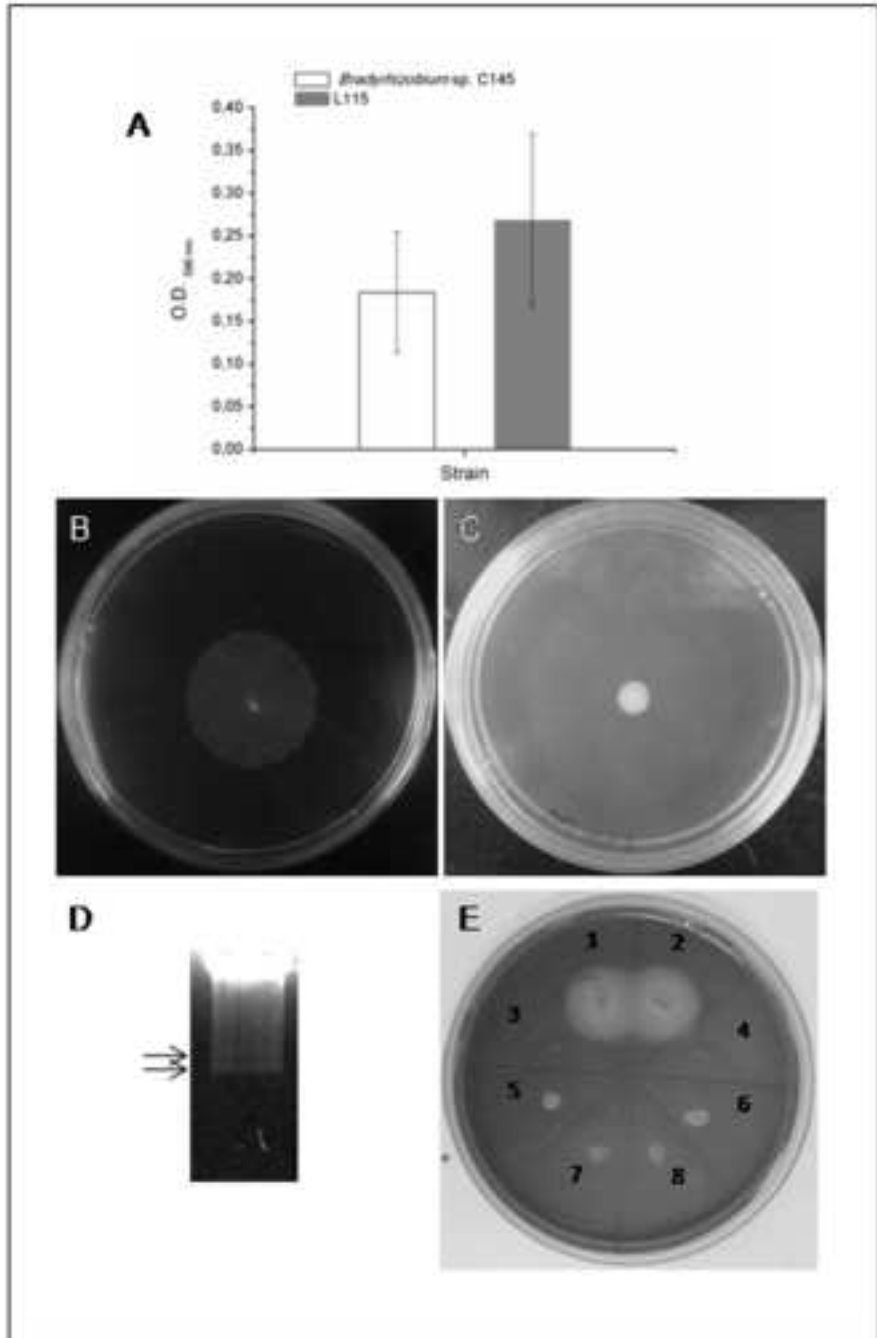

Figure 3A

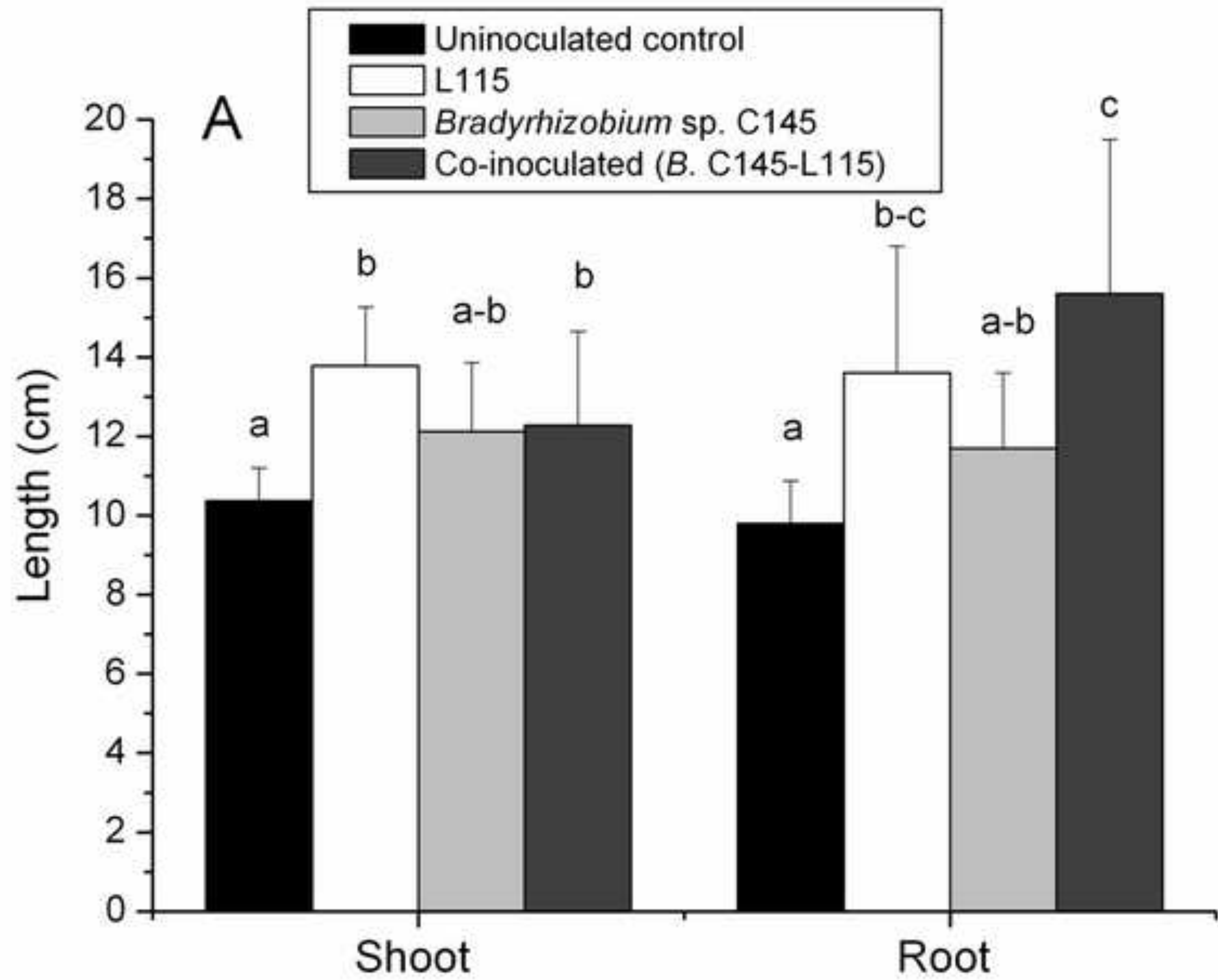

Figure 3B

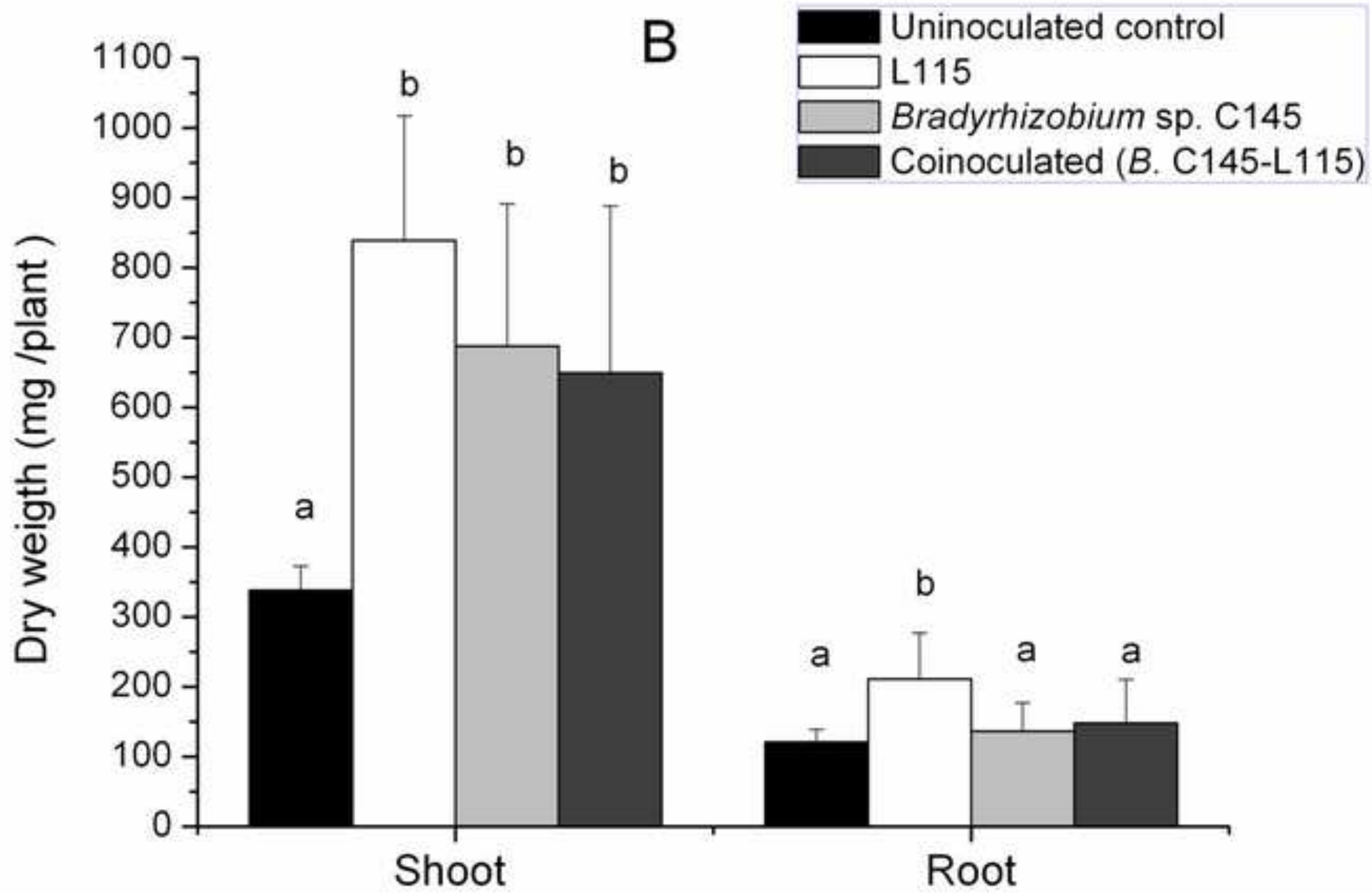

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Figure 4A

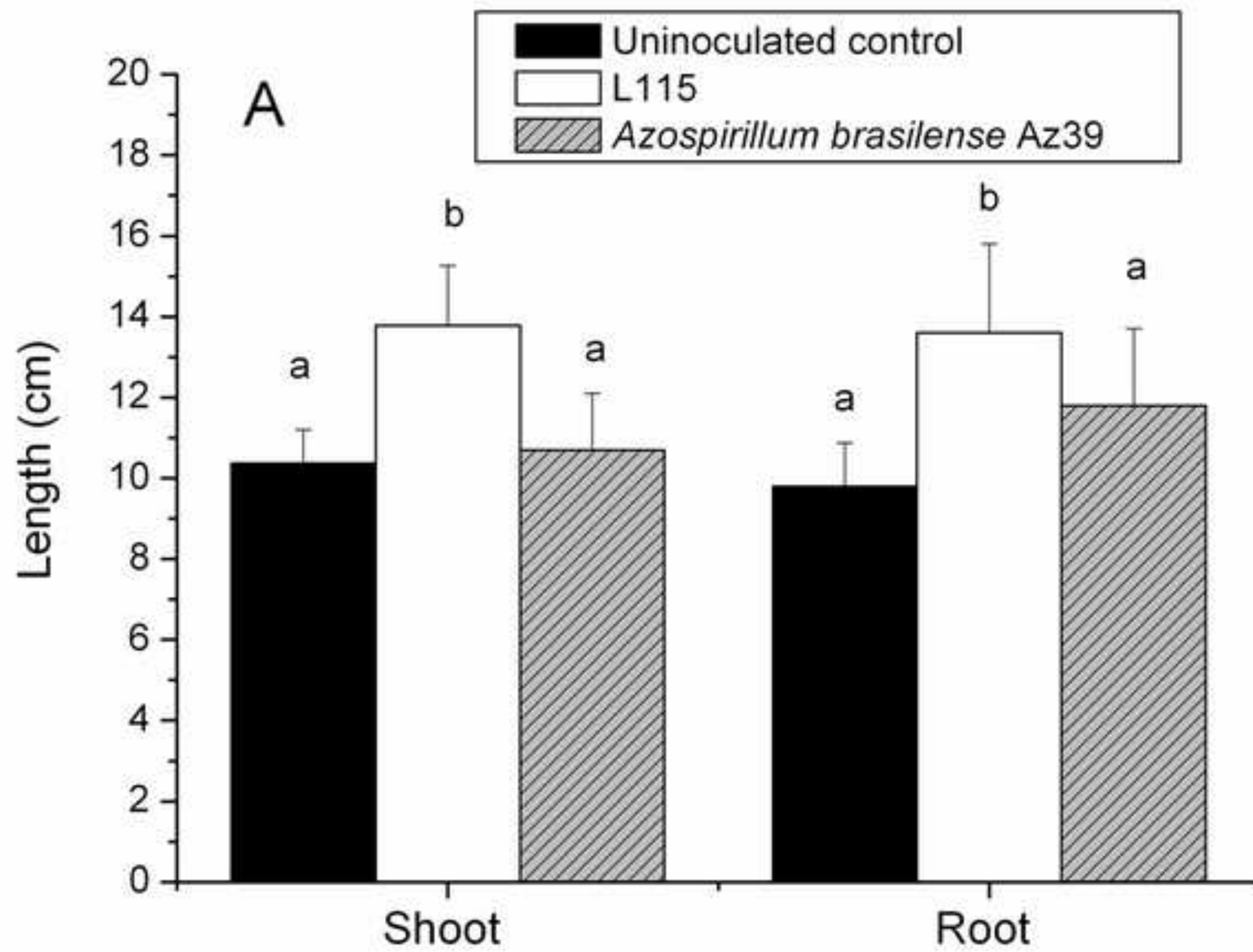

Figure 4B

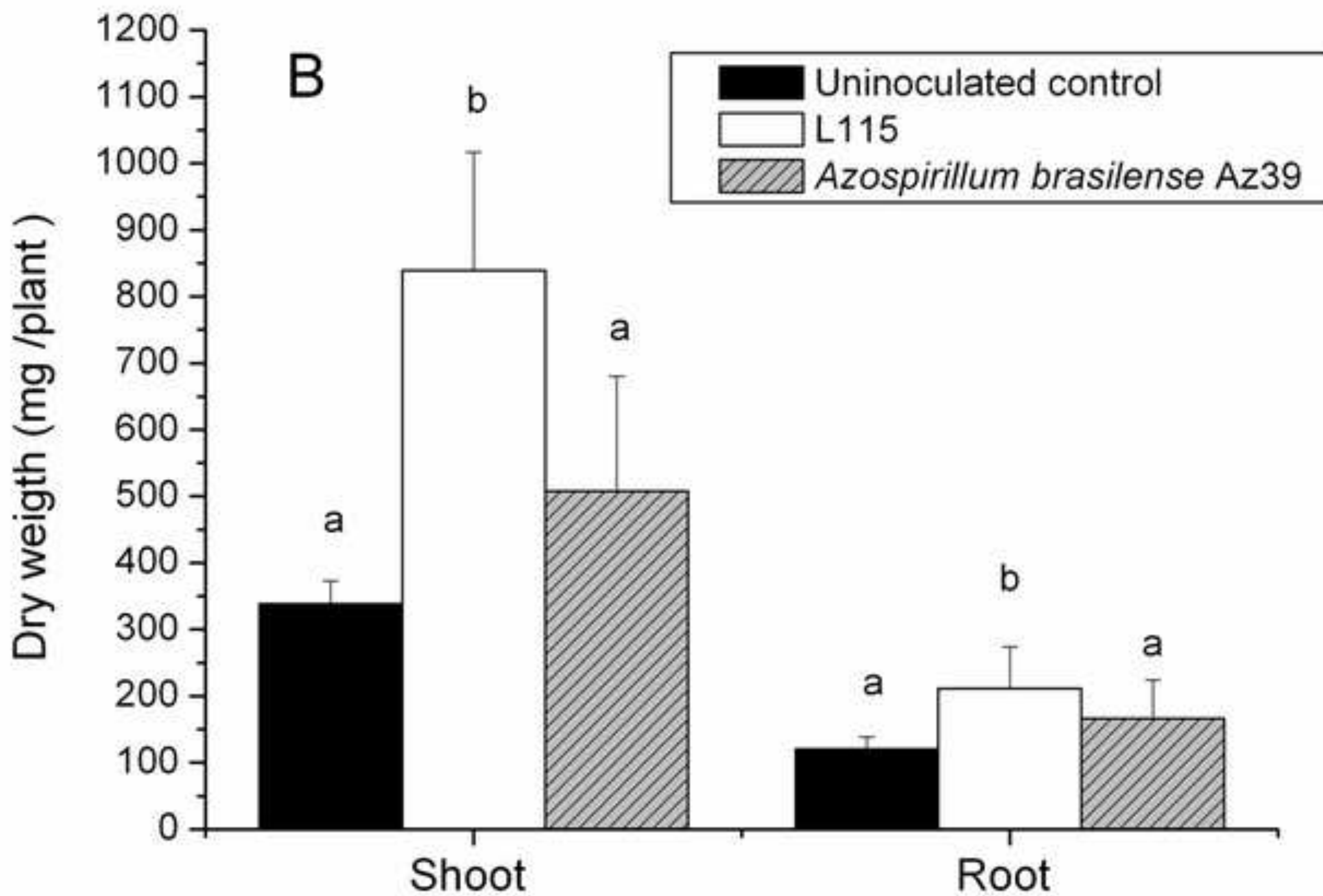

Figure 5