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# Quantitative effects of soil salinity on the symbiosis of wild lima bean (*Phaseolus lunatus* L.) and *Bradyrhizobium* in Costa Rica

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## Summary

Global climate change and local anthropogenic activities are increasing soil salinization with permanent negative effects on agricultural and ecosystem productivity. While salt stress is known to affect plant performance, its effects on the association with key microbial plant symbionts, such as legume-associated nitrogen-fixing rhizobia, are less understood. In this field study conducted in Costa Rica (Puntarenas), we used sympatrically-occurring wild lima bean (*Phaseolus lunatus* L.) and *Bradyrhizobium* to quantify biomass production of unfertilized rhizobial (R+) and fertilized rhizobia-free (R-) plants at different levels of experimentally manipulated salinity in native soil. In response to salt stress, nodulation was significantly reduced even at slightly increased salt levels. Plants growing at soil salinity levels of 2, 4, 6, and 8 mS/cm showed a mean reduction of nodules by 60.22, 76.52, 83.98, and 92.5% compared to the controls. Similarly, we also observed a significant decline in plant biomass at elevated salinity. However, biomass accumulation of R- plants was significantly less impacted compared to R+ plants, suggesting that the plant-microbe symbiosis is more salt-sensitive than the plant host itself. We suggest that the search for more salt-tolerant, crop plant-compatible rhizobial strains may provide a sustainable approach to maintain agricultural productivity on low to moderately saline soils.

## Introduction

Human activities have increased the prevalence of soil salinization in arable land, with significant detrimental impact on crop plant productivity (SINGLETON et al., 1982; SCHOFIELD et al., 2001). Soil salinization is a wide-spread form of land degradation that affects between 20-30% of agricultural land (LI et al., 2014; SINGH, 2015), and occurs when excess salts accumulate in the upper layers of otherwise productive soils (ROJAS et al., 2016). Salt deposition in soils can occur naturally through erosion, periodic flooding, saltwater intrusion, and leaching; however, human activities including intensive irrigation, deforestation, and industrial emissions also introduce a significant amount of salt into environments (RENGASAMY, 2006). Elevated salt levels can be difficult to ameliorate in soils, and if unchecked, soil is irreparably degraded, causing ecosystem disturbances and reducing crop yield (CABOT et al., 2014; BIRGÉ et al., 2016).

Salt stress manifests incrementally, causing difficulties with plant water and nutrient uptake, thus inhibiting biomass accumulation and fruit production, root hair growth, and causes premature leaf senescence (TU, 1981; KAUSHAL and WANI, 2016). Plant physiology is directly altered by unfavorable shifts of ionic balances and osmotic stress (BORSANI et al., 2003; BALLHORN and ELIAS, 2014). Certain plant families have evolved adaptations to this osmotic stress through specific traits such as salt excreting glands or suites of xerophobic traits (BARHOUMI et al., 2007). Other plants show a relatively broad range of salt tolerance, but these levels of tolerance vary considerably among plant families and species (YADAV et al., 2011). In addition to plant intrinsic factors, the interaction with plant-associated microbes may affect the salt tolerance of a given species. For example, SHENG et al. (2008) found that the salt tolerance of *Zea mays* improved when inoculated with the arbuscular mycorrhizal species *Glomus mosseae*. Similarly, certain soil microbes have been shown to confer salt tolerance by emitting volatile organic compounds that regulate plant stress responses (YANG et al., 2009). In nature, most vascular plants form complex associations with multiple microbial symbionts – including bacterial and fungal endophytes, mycorrhizal fungi, and nitrogen-fixing bacteria. These interactions can complicate studies on host plant salt stress tolerance through the added symbiont response and host-symbiont interaction.

Legumes (Fabaceae) play critical roles in determining the productivity, sustainability, and diversity in many terrestrial ecosystems due to their association with nitrogen-fixing rhizobia (CREWS and PEOPLES, 2004; BAREA et al., 2005; VALIENTE-BANUET and VERDÚ, 2007; PEOPLES et al., 2009). At the same time, this plant family includes some of the globally most important crop plants such as soybeans, alfalfa, and common bean (SINGLETON et al., 1982; ZAHRAN, 1999; PEOPLES et al., 2009). In both crops and wild legumes several factors have been identified as having significant impacts on the belowground rhizobial symbiosis, and thus the vigor of the host plant. These include abiotic factors such pH, soil moisture, and salinity (BOONKERD and WEAVER, 1982; GRAHAM, 1992) as well as biotic factors such plant disease and herbivory (ZAHRAN, 1999; HEATH and LAU, 2011). By focusing on soil salinity as one of the most detrimental abiotic factors on plant productivity, research has shown that the establishment and maintenance of rhizobial symbiosis is impacted through reduced root-hair infection and nodule development, which subsequently results in reduced nitrogen-fixation capacity and plant growth (ZAHRAN and SPRENT, 1986; DELGADO et al., 1994; YANNI et al., 2016). Indeed, this phenomenon has been observed in several important crop plant species. In soybean (*Glycine max*), 26.6 mM NaCl was sufficient to inhibit nodulation (SINGLETON and BOHLOOL, 1984), while fertilized chickpea plants (*Cicer arietinum*) were still able to nodulate when treated with 75 mM NaCl (LAUTER et al., 1981). Despite using salt-tolerant strains of *Rhizobium* and *Bradyrhizobium*, DELGADO et al. (1994) corroborated that salinity decreased nodular dry weight in soybean, as well as in pea (*Pisum sativum*), faba bean (*Vicia faba*), and common bean (*Phaseolus vulgaris*). In alfalfa, SERRAJ and DREVON (1998) attributed impaired plant growth following salinity treatments to inhibited nitrogenase activity. While matching the salt tolerances of both the rhizobial and crop plant strains may help optimize the symbiosis (BOUHMOUCH et al., 2005), the observed negative effects of soil salinity on the bacterial symbiosis, and thus the symbiotic nitrogen-fixation, raise the question of whether or not supplementing legume host plants with additional nitrogen may alleviate the negative effects of saline soils. Although evidence exists that legumes relying exclusively on symbiotically-fixed N supplies are more sensitive to salinity than

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those grown with additional or only mineral nitrogen, quantitative information on these interactions is scarce. Overall, much unknown variation exists on the effects of soil salinity on the legume-rhizobia symbiosis (RAO et al., 2002; BOUHMOUCH et al., 2005) – which is surprising given its vast impact on productivity, stability, and diversity of natural and agricultural ecosystems (BALLHORN et al., 2011, 2013). In our study, we used wild lima bean (*Phaseolus lunatus* L.) as an experimental plant to analyze quantitative effects of soil salinity on rhizobial nodulation and plant performance. While cultivated genotypes of lima bean are grown as important grain legumes in the Americas (BROUGHTON et al., 2003), wild lima bean represents an emerging model plant to study the chemical ecology of plant-herbivore and plant-microbe interactions (BALLHORN et al., 2011, 2013). Although lima bean has been extensively studied for its interactions with the biotic and abiotic environment, the tolerance of this species and its rhizobial symbiosis to saline soils is unknown. Here, we focused on the symbiosis between Costa Rican wild lima bean and a *Bradyrhizobium* strain (Genbank accession ID MF871645) both naturally occurring at the field site (Puntarenas, Pacific Coast), and tested quantitative effects of soil salinity on unfertilized rhizobial plants vs. fertilized rhizobia-free plants. Specifically, we aimed to answer the following questions: 1) How salt tolerant is wild lima bean and its association with a native rhizobial strain? 2) Do observed negative effects of soil salinity on plant growth result primarily from direct effects on the host plant, or from effects on the microbial symbiosis? and 3) Does the addition of mineral nitrogen fertilizer alleviate the negative consequences of the declining symbiosis in saline soils?

## Materials and methods

### Field collection and processing of soil
Five hundred kilograms of soil were collected at a natural lima bean site in Puntarenas, Costa Rica at a depth of 0 – 30 cm corresponding to the major rooting depth of wild lima bean at this location. The soil was sieved to remove rocks and large organic debris and mixed thoroughly in an 8-horsepower concrete mixer for 30 min (Honda). The container of the concrete mixer was cleaned with a wire brush, 0.5% bleach solution, and was washed with sterile water prior to use [autoclaved in a bench top sterilizer (Benchmark Scientific BioClave 16™); 1260 mbar, 121 °C, 30 min)]. After mixing, the soil was separated into ten 50 kg batches and each batch was sampled three times for conductivity analysis (CDB-387 Conductivity Meter, OMEGA) according to the saturated paste extract method. After that, 2 kg of soil per batch were removed and unprocessed sea salt from a local salt producer (Solar Salinas) was added until the electrical conductivity (EC) of the subsamples were adjusted to 2, 4, 6 and 8 mS/cm. From the amount of salt used per 2 kg soil, the amount of salt required for adjusting the remaining 48 kg of soil to the different conductivity levels was calculated and added. Subsequent conductivity measurements were conducted to confirm the desired salt concentration. Additional salt or soil was added in case re-adjustment was necessary. Subsequently, soil was autoclaved in two identical benchtop sterilizers (see above) in batches of 5 kg (1260 mbar, 121 °C, 1 h). These batches were tested for variation in conductivity again but no further adjustment was required.

### Preparation of plants
The autoclaved, differently saline soil mixtures were added to bleach-sterilized 2-gallon plastic pots while still hot and pots were covered with sterile aluminum foil. Per salt treatment (EC: 1.4, 2, 4, 6, 8 mS/cm) pots were split into two groups and planted with pre-germinated sterile lima bean seeds collected from the natural local population. To produce sterile seedlings, surface-sterilized seeds were germi-

nated in sealed sterile Petri dishes (12 cm diameter) on autoclaved paper towels. When the radicle had reached a length between 0.1 and 0.5 cm, seedlings were transplanted to the pots in a sterile hood by punching a small hole in the aluminum foil cover and inserting the radicle. The aluminum foil remained on the pots for the experimental period to reduce spontaneous rhizobial colonization. Plants were watered and fertilized through a silicone tube with attached bacterial filter; sterile water and fertilizer solutions were applied with a syringe at 50 ml per day.

### Rhizobial inoculation
Plants were either inoculated with rhizobia (R+, N=12 plants per salinity treatment group) and unfertilized, or maintained rhizobia-free (R-, N=12 plants per salinity treatment group) and fertilized daily during the cultivation period with a 5.3 mmol $L^{-1}$ $CaNO_3$ solution made with sterilized water, a concentration previously used to compare nitrate-fed plants to those with nitrogen-fixing rhizobia (KIERS et al., 2006).

For rhizobial inoculation, a local *Bradyrhizobium* strain (Genbank accession ID MF871645) was isolated from wild plants, cultivated in liquid medium (yeast extract), and 10 ml of bacterial inoculum were applied to experimental plants ($10^7$ cells per ml) 5 days after planting the seedling. The bacterial solution was applied next to the plant with a pipette. This rhizobial strain is maintained in the Ballhorn lab and has been demonstrated to successfully colonize lima bean plants under field and laboratory conditions. Also, this strain was able to effectively fix nitrogen in preliminary experiments in which chlorotic, rhizobia-free lima bean plants turned dark green after inoculation and successful nodulation with this strain.

### Destructive harvest and processing of plant material
After a cultivation period of eight weeks, plants were destructively harvested and all nodules (visible under a stereo microscope at 6× magnification) were counted. To determine plant above- and below-ground biomass production, root and shoot portions were separated, roots were carefully washed, and all soil particles removed. Plant samples were dried in an oven at 72 °C for three days (Amerex Instruments, IncuMax CV250) and dry weight was determined with a fine balance (Mettler Toledo, XA DeltaRange®).

### Data analysis
Statistics were performed in R version 3.3.1 with an $\alpha$ = 0.05. Plant treatment differences within each soil electrical conductivity (EC) level were determined using two sample t-tests that assumed equal variance. To analyze differences in responses to soil EC within plant treatments, one-way analysis of variance (ANOVA) and pairwise Tukey's HSD post-hoc tests were used. Shapiro-Wilk and Levene's tests were used to determine if assumptions of ANOVA were met. Data were not transformed because only a small subset of treatment × response groups did not meet these assumptions.

### Accession number
Information about the *Bradyrhizobium* strain used in this study is available through GenBank under the accession number MF871645.

## Results

Our study sought to identify quantitative effects of soil salinity on a natural legume-rhizobia symbiosis using Costa Rican wild lima bean and a local *Bradyrhizobium* strain as the experimental system. Experimentally elevated soil salinity levels resulted in a significant quantitative variation in nodulation ($F_{4,55}=113$, $p<0.001$). The num-

ber of nodules on plants with rhizobia (R+) significantly decreased with increasing salinity; more nodules were produced at the control EC level (1.4 mS/cm) than at plants growing at any other EC level (p<0.001; Fig. 1). Numbers of nodules produced by plants growing in soil with an EC of 4 and 6, and 6 and 8 mS/cm, respectively, were similar.
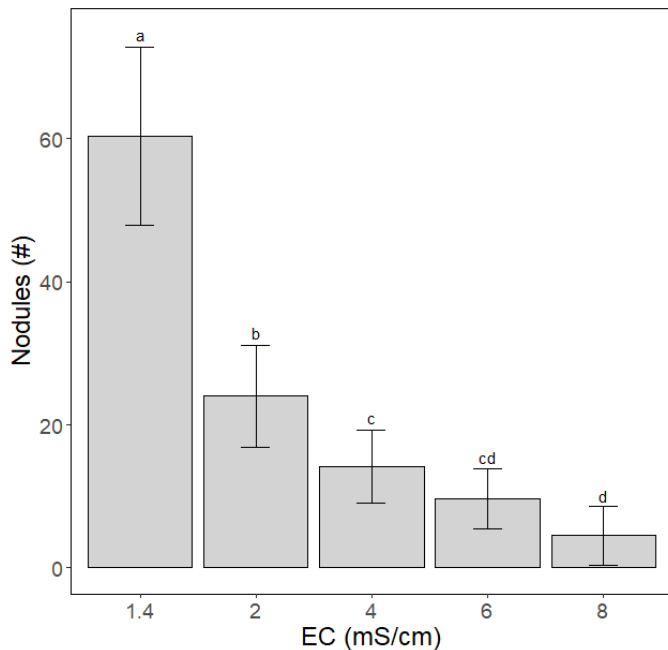


**Fig. 1:** Number of nodules in rhizobia-inoculated plants. Plants were grown in soils with different electrical conductivity (EC) and nodules were harvested and counted of 8 weeks of plant cultivation. Bars are means (±SD), stars represent statistical difference based on t-tests within EC treatment groups (significance: ** p<0.01, *** p<0.001). Lower case letters show significant differences among experimental plants according to post-hoc analysis (Tukey's HSD, p<0.01) after one-way ANOVA.

Total biomass production of both rhizobial (R+) and non-rhizobial (R-, nitrogen fertilized) plants was similar at the control EC level (1.4 mS/cm), but decreased in response to elevated soil salinity ($F_{4,55}=173$, p<0.001; $F_{4,55}=131$, p<0.001). When treated with salt, R+ plants (5.77 ± 2.78 g) produced significantly less biomass than the corresponding R- plants (8.24 ± 2.57 g) overall (t=5.07, df=117, p<0.001). At each EC level above the control (1.4 mS/cm), total biomass was significantly lower for R+ plants than for R- plants (two-sample t-test; 2: t=15.4, df=22, p<0.001; 4: t=11.8, df=22, p<0.001; 6: t=6.24, df=22, p<0.001; 8: t=4.00, df=22, p<0.001; Fig. 2). R+ plants were more sensitive to changes in the soil electrical conductivity as total biomass accumulation suffered at all EC levels when compared to the control EC level, except between 2 and 4, and 6 and 8 mS/cm (Tukey HSD, p<0.001). In R- plants supplied with nitrogen fertilizer, total biomass did not differ between 1.4 and 2 mS/cm, but differences were significant between 1.4 and 4, 6, and 8 mS/cm.

When looking at above- and belowground biomass separately, we observed significant differences depending on symbiotic state (R+ vs. R-) and salt treatment. Aboveground biomass decreased for both R+ and R- plants with increasing soil EC ($F_{4,115}=128$, p<0.001; Fig. 3). Plants without rhizobia produced significantly more aboveground biomass than R+ plants at 2, 4, and 8 mS/cm (two-sample t-test; 2: t=7.96, df=22, p<0.001; 4: t=3.99, df=22, p<0.001; 8: t=3.03, df=22, p<0.01). In R- plants, aboveground biomass differed significantly across salt treatments ($F_{4,55}=214$, p<0.001) and between all EC
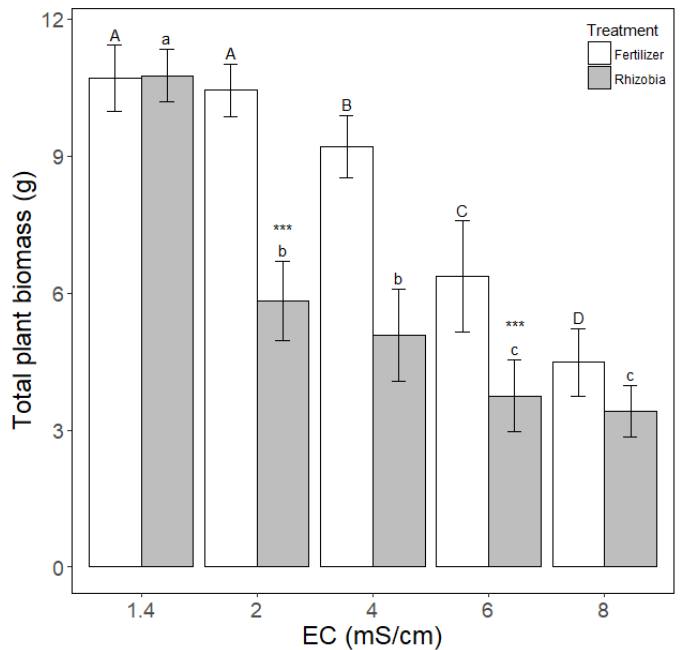


**Fig. 2:** Total biomass of plants with or without rhizobia. Plants were grown in soils with different electrical conductivity (EC) and total plant biomass (dry weight) was determined after 8 weeks of cultivation. Bars are means (±SD), lower (rhizobia) and upper case letters (fertilizer, no rhizobia) show significant differences among experimental plants according to post-hoc analysis (Tukey's HSD, p<0.01) after one-way ANOVA.
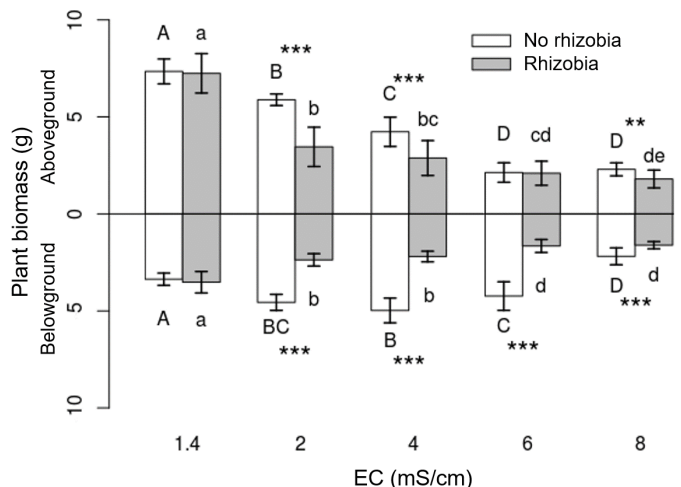


**Fig. 3:** Above- and belowground plant biomass. Plants with and without rhizobia were grown in soils with different electrical conductivity (EC) and plant above and below biomass (dry weight) was determined after 8 weeks of cultivation. Bars are means (±SD), stars represent statistical difference based on t-tests within EC treatment groups (significance: ** p<0.01, *** p<0.001). Lower (rhizobia) and upper case letters (fertilizer, no rhizobia) show significant differences among experimental plants according to post-hoc analysis (Tukey's HSD, p<0.01) after one-way ANOVA.

levels except between 6 and 8 mS/cm. In R+ plants, aboveground biomass also differed significantly across all salt treatments ($F_{4,55}=83.5$, p<0.001), as well as between plants growing in control soil (1.4 mS/cm) and all other EC levels; however, differences were insignificant between plants grown in 2 and 4, 4 and 6, and 6 and 8 mS/cm soil (p>0.05).

Belowground biomass increased through 4 mS/cm in R- plants, and was significantly higher in R- plants than in R+ plants (t=9.04, df=105, p<0.001) overall, particularly at all EC levels above the control (two-sample t-test; 2: t=14.5, df=22, p<0.001; 4: t=13.9, df=22, p<0.001; 6: t=11.1, df=22, p<0.001, 8: t=4.22, df=22, p<0.001; Fig. 3). Plants with rhizobia did not respond differently to soil salinity levels between 2 and 4, or 6 and 8 mS/cm; however, plants grown in control soil showed significantly different belowground biomass from all other EC levels. Plants without rhizobia showed similar belowground biomass when growing in soil with EC of 2 and 4, or 2 and 6 mS/cm, but had significantly different belowground biomass at all other EC levels when compared to control soil.

Overall, root-to-shoot ratio decreased significantly with increasing EC within each plant treatment (R+: $F_{4,55}$=5.75, p<0.001; R-: $F_{4,55}$=50.5, p<0.001), and only differed between plant treatments at the 4 mS/cm (t-test; t=2.26, df=22, p<0.05) and 6 mS/cm EC levels (t-test; t=12.03, df=22, p<0.001). In plants with rhizobia, the ratios at the control level were significantly different from those at 4, 6, and 8 mS/cm, which were all similar (Fig. 4). Ratios at the control level and 2, and 2 and 4 to 8 mS/cm were likewise similar. Plants grown without rhizobia showed root-to-shoot ratios differing between all EC levels except for 1.4 to 2, and 4 to 8 mS/cm (Fig. 4). Additionally, root-to-shoot ratios increased with EC, except for 8 mS/cm in R- plants, where it decreased significantly.
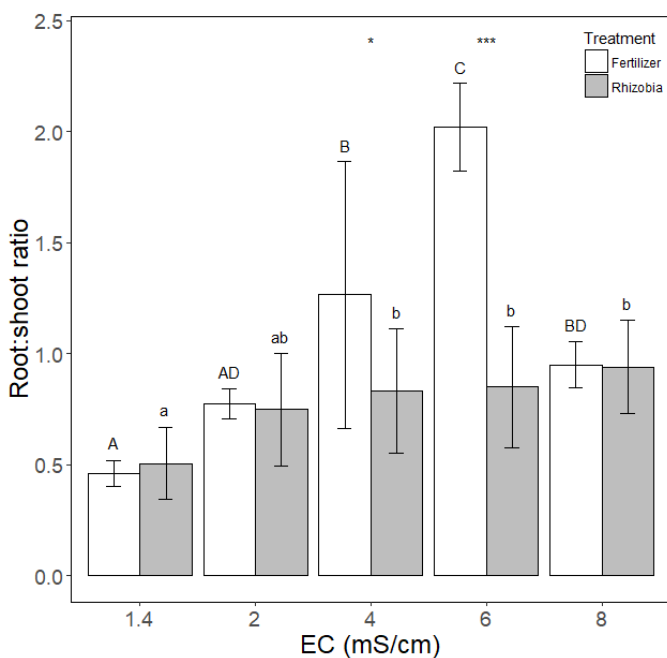


**Fig. 4:**  Root:shoot ratio for plants with and without rhizobia. Bars are means, stars represent statistical difference based on t-test within salinity treatment group (significance: * p<0.05, *** p<0.001). Lower (rhizobia) and uppercase letters (fertilizer, no rhizobia) show significant differences among experimental plants according to post-hoc analysis (Tukey's HSD, p<0.01) after one-way ANOVA.

## Discussion

The aim of this study was to quantify the response of a natural plant-rhizobia symbiosis to a gradient of soil salinity. We found that rhizobia-free but nitrogen-supplemented plants (R-) significantly outperformed plants with rhizobia (R+) at elevated soil EC levels, indicating that either the natural symbiosis or *Bradyrhizobium* performance is impaired – or both. Rhizobia colonize legume root-hairs through signaling interactions involving the production of Nod factors following recognition of legume-produced flavonoids (CÁR-DENAS et al., 2000). However, elevated salinity decreases growth of rhizobia and interactions with legume root-hairs (Tu, 1981), reducing nodulation and consequently decreasing the nitrogen-fixing capacity of inoculated host plants. Here, nodule production decreased by over 60% between the 1.4 (control) and 2 mS/cm treatment, and by over 90% between control plants and plants growing at 8 mS/cm, indicating high sensitivity of the symbiosis to even small increases in salinity. Since rhizobia and host plant genotypes vary in their relative salt tolerances, it is possible that the particular *Bradyrhizobium* strain (isolated from wild lima bean plants growing about 700-1000 meters inland) used in this study may be less salt-tolerant than other regional and more coastal strains, but the significant reduction in nodulation is still remarkable. While some information on negative effects of salinity on nodule and biomass production in legume hosts is available (LAUTER et al., 1981; SINGLETON and BOHLOOL, 1984; DELGADO et al., 1994; SERRAJ and DREVON, 1998; BOUHMOUCH et al., 2005; YANNI et al., 2016), future studies might seek to further define particular tolerance ranges. Total biomass was reduced significantly in R+ plants at already 2 mS/cm, unlike R- plants that did not show significant changes in biomass production until 4 mS/cm. Differences between R+ and R- treatments may have been driven by the opposite trends in belowground biomass, which actually increased slightly with increasing salinity for R- plants, while belowground biomass in R+ plants consistently decreased. Our study shows that even low (2-4 mS/cm) salinization negatively affects a natural legume-rhizobial symbiosis.

While we do have a good understanding of plant-rhizobia symbioses in many host plant systems – in particular crop plants – in other systems detailed information on the factors driving these interactions is lacking. In-depth research is still required to determine the impact of abiotic and biotic factors on the establishment, maintenance, and quantitative expression of this interaction. Such knowledge is important to estimate the impact of changing environmental conditions on natural and agricultural ecosystems. This study shows that the sensitivity of the symbiosis to environmental stressors such as soil salinity critically determines the performance of host plants growing on non-nitrogen supplemented soils. Our data point to future research possibilities in the breeding of legumes and their symbionts for higher salt tolerance; otherwise, in the absence of these optimized symbioses, our findings suggest that additional nutrient supplements are required to maintain productivity under increasing salinity. However, additional fertilization is not always a viable option for farmers (SANG-INGA, 2003), nor is it always benign for the environment. Runoff from fertilized fields can induce eutrophication of neighboring freshwater ecosystems (SMITH et al., 1999; HIREL et al., 2007), while WEESE et al. (2015) found that excessive N-addition to soils decreased rhizobia N-fixation capacity over time.

Rhizobia vary in their salt sensitivity, with many strains being inhibited only at levels well above what a plant could tolerate (BOUH-MOUCH et al., 2005). LAUTER et al. (1981) showed that rhizobia growth is only moderately inhibited at NaCl levels of 250mM. This implies that the plant-rhizobia interaction rather than the sensitivity of rhizobia themselves is a major limiting factor in crop salt tolerance. Legumes are major crops and forage plants throughout the world, and the symbiosis with rhizobia is crucial to their success in agriculture (DE ARAUJO et al., 2016). According to the Food and Agriculture Organization of the United Nations (2008), over 6% of arable land is currently considered saline or sodic. Problems have continued to arise due to outdated agricultural practices (crop rotations, irrigation practices, fertilizers, etc.) and many have been late to adopt eco-friendly policies to maintain sustainable growing conditions (RILEY and BARBER, 1970; TU, 1981). Better knowledge of the complex factors driving the plant-rhizobia symbiosis is required to establish, maintain, and protect natural and agricultural ecosystems.

Such future studies on the effects of salt stress on plant-rhizobia interface should include analysis of biochemical and molecular parameters on both sides of the interaction.

## Conclusions

In this study, we showed that the symbiosis between *Bradyrhizobium* and wild lima bean interactions displays sensitivity to even low level salt stress. Rhizobial inoculation is standard practice for growing legumes and the application of nitrogen can be lessened with appropriate combination of host plants and rhizobial strains. Our study suggests that the salt tolerance of such interactions is an important factor which needs to be considered for the development of sustainable agriculture approaches.

## Conflicts of interest
The authors declare that they have no conflicts of interest.

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