

Article

Tripartite Relationships in Legume Crops Are Plant-Microorganism-Specific and Strongly Influenced by Salinity

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Abstract: This study investigated the effects of specific strains of two arbuscular mycorrhizae fungi (AMF) (*Rhizophagus irregularis* and *Claroideoglomus claroideum*) and of two plant growth-promoting bacteria (PGPB) (*Rhizobium leguminosarum* and *Burkholderia* spp.), supplied either individually or as combination of a mixture of both arbuscular mycorrhizae fungi with each bacteria on root morphology, growth and fresh grain yield in pea (*Pisum sativum* L.) plants. Inoculated and non-inoculated pea plants were subjected to two levels of salinity (0 and 50 mM) by the addition of sodium chloride into tap water. Prior to fresh grain harvesting the morphology of root system was analyzed and the dry matter of roots and shoots were individually measured in randomly selected plants. Fresh pods were individually harvested per each plant; fresh (green) grains were separately counted and weighted per each pod at each individual plant, and the average grain weight was calculated by dividing total grain weight of plant with the respective number of green grains. The raise of salinity in the irrigation water strongly diminished the growth of pea plants by significantly reducing weight, length, surface area and root volume of pea plants. The relationships of pea plants with beneficiary fungi and bacteria were specific to each microorganism and highly depended on the environment. We found that under saline conditions, *Rhizophagus irregularis* provided a better vegetative growth and a higher yield than *Claroideoglomus claroideum*. Although, single application of *Burkholderia* spp. provides a better vegetative growth than single application of *Rhizobium leguminosarum* the best results, in terms of growth and harvested yield, were still obtained by combined application of AM fungi with *Rhizobium leguminosarum*. This combination was able to sustain the average grain weight at the level of non-saline plants and provided a significantly higher yield than the control plants.

Keywords: tripartite; *Pisum sativum*; *Rhizophagus irregularis*; *Claroideoglomus claroideum*; *Rhizobium leguminosarum*; *Burkholderia* spp.

1. Introduction

With the increase in the world's population, the demand for agriculture yield has increased tremendously, thereby leading to large-scale production of chemical fertilizers [1]. Since one of the most important factors in the generation of high yields from crop plants is nitrogen, farmers are applying high amounts of the fertilizers which are very costly and make the environment hazardous. In addition, there is evidence that more than 50% of applied nitrogen and phosphorus fertilizer is somehow lost through different processes, which not only represents a cash loss to farmers, but also consequently pollutes the environment [2]. Indeed, progressively reduced N use efficiency due to increased fertilization doses has been reported since the early stages of plant growth [3].

Because the use of fertilizers and pesticides in the agricultural fields have caused degradation of soil quality and fertility, researchers and scientists have shifted their attention to safer and more productive means of agricultural practices [1]. Currently, increasing global concern regarding the production of enough food to uphold the growing human population has been reinforcing the importance of sustainable intensification of plant production [4], and the use of microbial consortia of plant-growth promoting bacteria (PGPB) is the current trend in agriculture [5]. It is already recognized that microbial communities are much more diverse than previously thought [6], and there are vast opportunities of using them.

Two types of soil bacteria have been shown to have the capacity to act as PGPB; rhizospheric bacteria, which are typically found around the roots of plants; and endophytic bacteria, which are found within the tissues of the plant itself (notwithstanding the fact that endophytic bacteria may also be found free-living in the soil) [7]. Conceptually, PGPB may affect plant growth either directly or indirectly. Direct promotion of plant growth occurs when either (i) PGPB facilitates the acquisition of resources from the environment including nitrogen, phosphorus, and iron; or (ii) modulates plant growth by providing or regulating various plant hormones, including auxin, cytokinin, or ethylene [7]. Additionally, there are also reports on rhizobia conferring increased resistance against plant pathogens [4]. Interestingly in these relationships, plants are not only affected by PGPRs, but also have important tools to manipulate gene expression and behavior in the bacteria that they encounter, for their own benefits [8].

In addition, the use of arbuscular mycorrhizae fungi (AMF) to alleviate soil stresses on plant growth is also receiving increased attention [9]. The symbiosis of plants with AMF often results in increased nutrient uptake, accumulation of osmoregulatory compounds, an increase in photosynthetic rates, and a decrease in root respiration and water use, suggesting that salt stress alleviation by AMF results from a combination of effects ranging from nutritional to molecular [10,11].

The symbiosis among legumes, plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) is one of the most important ecological mutualisms [2]. The majority of legumes form symbiotic associations with both phosphorus-acquiring AMF and nitrogen (N)-fixing rhizobia in tri-partite relationship. The microsymbionts in both associations are benefited by photoassimilates from the plant, and the macrosymbiont obtains fixed nitrogen in the case of bacterial symbiosis of root nodules, and immobile nutrients especially phosphate in the case of AM symbiosis [2].

Although there are a considerable number of publications, the information available about the effect of combined inoculation of PGPB and mixed inocula of AMF to alleviate the noxious effect of salinity stress on legume crops is far from being completed. Furthermore, there is a high variability in the performance of AM fungi [12] and PGPB that may be due to various environmental factors that might affect their growth and proliferation in plants [1]. Therefore, the objective of this study was to investigate the variation between different bacteria (*Rhizobium leguminosarum* and *Burkholderia* sp.) inocula and different AMF (*Rhizophagus irregularis* and *Claroideoglossum claroideum*) inocula, as well as interactions between each bacteria with the mixture of AMF (*Rhizophagus irregularis* + *Claroideoglossum claroideum*) regarding their influence on root morphology, growth, and yield of pea (*Pisum sativum* L.) plants, in order to provide a better understanding of this tripartite association and its impact on legume crops.

2. Materials and Methods

2.1. Plant Material and Experimental Set-Up

The experiment was conducted in a non-heated greenhouse in Tirana, Albania during the spring of 2017. Graded seeds of a commercial pea cultivar (Progress 9), two strains of distinguished AM fungi (*Rhizophagus irregularis*—originally isolated from soils of La Manche canal banks, and *Claroideoglossum claroideum*—originally isolated from Mongolian soils; both provided by Symbiom, s.r.o., Sázava, Czech Republic) and two bacterial strains (a *Rhizobium leguminosarum* *bv.* *Viciae* and

a *Burkholderia* spp., both isolated and provided by UTAD, Portugal) were used in the experiment. The seeds were sown in large plastic pots (0.6 m × 0.2 m × 0.2 m) filled with a mixture of vermiculite (Agra-Vermiculite, Pull Rhenen B.V., The Netherlands) + river sand (1:3). Several experimental variants were applied; (i) purely mixture substrate (control, Ctr); (ii) mixture substrate and 10% (v/v) crushed, expanded clay particles coated with AM-fungal spores of *Rhizophagus irregularis* (Rh.ir+); (iii) mixture substrate and 10% (v/v) crushed, expanded clay particles coated with AM-fungal spores of *Claroideoglossum claroideum* (Cl.cl+); (iv) mixture substrate and 10% (v/v) peat moss inoculated with a *Rhizobium leguminosarum* strain (10^2 colony-forming units (CFU) g⁻¹) (Rh.l+); (v) mixture substrate and 10% (v/v) peat moss inoculated with a *Burkholderia* sp. strain (10^2 CFU g⁻¹) (Brh+); (vi) mixture substrate and mixture of AM fungi (*Rhizophagus irregularis* + *Claroideoglossum claroideum*; 5% v/v) with *Rh. leguminosum* (5% v/v) (AMF + Rh.l) and (vii) mixture substrate and mixture of AMF (*Rhizophagus irregularis* + *Claroideoglossum claroideum*; 5% v/v) with *Burkholderia* sp. (5% v/v each) (AMF + Brh). AMF-inoculated clay particles and respective inocula of *Rh. leguminosarum* or *Burkholderia* spp. strains were homogeneously mixed with the substrate before sowing. To each pot, 20 graded seeds were sown 4 cm apart from each other at 2 cm depth, in two parallel lines. The seeds were sown in February 14, 2017 and the experiment lasted till May 20, 2017.

Two different levels of salt-stress (0 and 50 mM) were established by the addition of different amounts of sodium chloride (NaCl) to the irrigation water. All plants were equally distributed to both salinity treatments, according to a full factorial design. Each treatment was represented by six pots; each of them represented a replication. The total number of pots of all treatments was arranged in eight parallel rows (split in half between the non-saline and saline conditions), six pots per each row. The treatments were randomly distributed among the rows, in groups of two pots. Six other pots, randomly distributed, were additionally used outside the experimental design, in order to have equal space distribution among all treatments. Plants were watered during the whole experimental period with equal amounts of either tap water (0 mM NaCl) or saline water (50 mM NaCl). The irrigation was conducted by a gravity driven drip irrigation system (two drippers per pot, with 0.2 L hrs⁻¹ discharge rate). The time, frequency, and length of irrigation cycles were automatically controlled by an electronic irrigation controller (Itec 8, Netafim Ltd, Tel Aviv, Israel).

2.2. Plant Sampling and Measurements

At DAS (day after sowing) 95, 10 plants from each treatment were randomly selected and harvested. Roots were gently washed free of adhering substrate particles, and plants were dissected and separated into roots and shoots. The root system was scanned with an Epson Expression/STD 4800 Scanner. Subsequently, the acquired root images were analyzed with WinRHIZO Arabidopsis software (Regent Instruments Inc., Sainte-Foy, QC, Canada) and root morphology parameters, total root length (RL), surface area (RSA), and root volume (RootV) were measured and recorded. In addition, saved root images were later used to count the number of nodules to each plant. The plant organs were subsequently dried (65 °C, 48 h) and weighted separately to an accuracy of 0.001 g (TP 303; Denver Instruments GmbH, Göttingen, Germany).

At the technical maturity stage (DAS 95), fresh pods were individually harvested per each plant; fresh (green) grains were separately counted (GN) and weighted per pod and per individual plant. The average grain weight (AvgGW) was calculated by dividing total grain weight (GW) of each plant with the respective number of green grains. Pearson correlation coefficients between each of the above root morphology parameter (RL, RSA, RootV) with dry matter of roots, shoots, and the entire plant (DM_{Root} , DM_{Shoot} , DM_{Plant}) and GW, as well as the respective significance levels of the respective correlation coefficients were calculated by SigmaPlot13.

2.3. Statistics

Differences in DM (DM_{Root} , DM_{Shoot} , DM_{Plant}), root morphology parameters (RL, RSA, RootV), the number of nodules per plant and yield parameters (GN, AvgGW, GW) were tested by

two-way ANOVA, using the personal computer (PC) program SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA). Each significant ANOVA result ($p < 0.05$) was followed by a Tukey test at $p < 0.05$ as a post-hoc test to compare pair wise means within and among treatments. Values given throughout the text are means \pm SD.

3. Results

The architecture of root system of pea plants (*Pisum sativum* L.) was largely affected by the quality of growing environment (non-saline or saline) and the presence of microorganisms. Under non-saline conditions, RL, RSA and RootV were significantly enhanced due to the inoculation of *Rhizophagus irregularis* (Rh.ir+) and bacteria (*Rhizobium leguminosarum* and *Burkholderia* sp.), while although slightly higher compared to control plants, no significant influence was noticed due to the inoculation of *Claroideoglomus claroideum* (Cl.cl+). The combination of mixtures of AM fungi (*Rhizophagus irregularis* + *Claroideoglomus claroideum*) with each bacteria have also contributed to significantly higher values of each of analysed root parameters (RL, RSA, RV) compared to control plants (Table 1). The longest root length was obtained in Brh+ and Rh.l+ plants (respectively 1200 and 1094 cm), followed by Rh.ir+ and combined applications of AM fungi with each bacteria. A quite similar picture was seen regarding root surface area (RSA) and RootV; Brh+ and Rh.l+ plants did have the highest respective values, followed by single applications of *Rhizophagus irregularis* and AMF + bacteria variants (Table 1).

Under non-saline conditions, the inoculation of bacteria was followed by a significant increase in the number of nodules of Rh.l + and Brh + plants compared with controls (Figure 1). A significantly higher number of nodules than the control was also recorded in AMF + Rh.l and AMF + Brh plants, although significantly less than single applications of *Rhizobium leguminosarum* or *Burkholderia* spp. Although not significant, the raise of salinity reduced the number of nodules per plant. Interestingly, single applications of *Rhizobium leguminosarum* or *Burkholderia* spp. showed a significantly higher number of nodules than the control plants. In addition, the number of nodules in Brh+ plants was higher than in Rh.l+ plants. Different from the non-saline conditions, no difference was found between combined applications of fungi and bacteria, (AMF + Rh.l, AMF + Brh) and control plants (Figure 1).

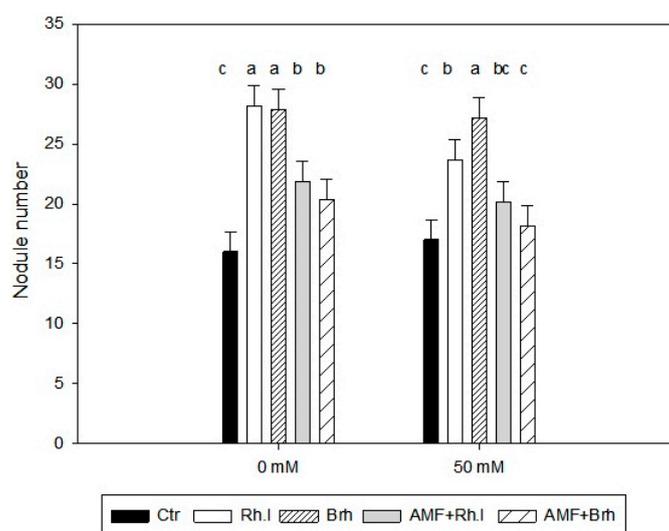


Figure 1. Average number of nodules per plant in control (Ctr), *Rhizobium leguminosarum*-inoculated (Rh.l), *Burkholderia* sp.-inoculated (Brh), *Rhizophagus irregularis* + *Claroideoglomus claroideum* + *Rhizobium leguminosarum*-inoculated (AMF + Rh.l) and *Rhizophagus irregularis* + *Claroideoglomus claroideum* + *Burkholderia* spp.-inoculated (AMF + Brh) pea plants under two levels of salinity (0 and 50 mM NaCl). Different letters indicate significant differences within following parameters (Tukey test, $p < 0.05$; mean \pm SD).

The raise of salinity significantly reduced the values of analyzed root morphology parameters in Cl.cl+, Rh.l+, Brh+ variants. Although characterized by a trend of reduced values due to the raise of salinity, Rh.ir+ and combined applications of AMF and bacteria were able to sustain RL, RSA, and RootV at the level of non-saline plants and importantly, significantly higher than control plants. On the contrary, like in the non-saline conditions, no difference was found between Cl.cl+ and control plants (Table 1).

No growth promotion effects of AM fungi, or bacteria were found under non-saline conditions regarding DM_{Root} . Even, significantly smaller DM_{Root} values were recorded in case of Rh.l+ and AMF + Brh variants (Table 2). Contrary to that, significant growth promotion effects were noticed regarding DM_{Shoot} . The highest DM_{Shoot} values were obtained by the inoculation of *Rhizophagus irregularis* and *Burkholderia*. Overall, this resulted in significantly higher DM_{Plant} in Rh.ir+ and Br+ variants, but no growth promotion was proved in case of single inoculation of *Claroideoglomus claroideum*, or *Rhizobium leguminosarum* (Table 2). Overall, *Rhizobium* and *Burkholderia* shows the smallest $DM_{Root}:DM_{Shoot}$ ratio, indicating that the growth enhancement effects of bacteria in pea plants were mostly related with the growth promotion of the above ground biomass (Table 2).

The rise of salinity to 50 mM L⁻¹ has sharply reduced DM_{Root} of control plants and all other variants. However, all AMF and bacteria-inoculated variants were able to have significantly higher DM_{Root} values than control plants. Contrary to that, when comparing non-saline and saline conditions, no changes were evidenced in the majority of variants regarding DM_{Shoot} . The only exceptions were Rh.l+ and Brh+ plants, which under saline conditions also reflected a significant reduction in DM_{Shoot} . Therefore, the significant reduction in DM_{Plant} under saline conditions was mostly the consequence of drastic reduction in root dry matter (Table 2). The rise of salinity was followed by a significant reduction of $DM_{Root}:DM_{Shoot}$ ratio (Table 2). This reached its minimum values in Brh+ and Ctr plants (respectively 0.06 and 0.07) and generally confirmed that the deleterious salinity effects were stronger on root systems rather than on the above-ground plant biomass.

No influence of either microorganisms, or salinity was found regarding the number of pods per plant (data not shown). No statistical differences were also found regarding average seed number per pod and average seed weight under non-saline conditions (Table 3). Surprisingly, single applications of *Claroideoglomus claroideum* (Cl.cl+) negatively influenced the fresh grain yield by significantly reducing it (1.46 gr plant⁻¹) versus the control plants (1.95 g plant⁻¹). The only variant that provided a significantly higher yield compared to control plants was the combination of an AMF mixture (*Rhizophagus irregularis* + *Claroideoglomus claroideum*) with *Rhizobium leguminosarum* (2.38 g plant⁻¹) (Table 3).

The effects of beneficiary microorganisms were much more visible under saline conditions. Rh.ir+ and Brh+ plants did have significantly higher numbers of average seeds per pod (respectively; 4.5 and 4.2) than the control plant (2.8) and the rest of the variants. More interestingly, while in the control plants and single applications of AMF or bacteria, the average seed weight was significantly reduced due to raised salinity, the combined applications of fungi and bacteria had weights at the level of non-saline conditions. Indeed, the most deleterious effect of raised salinity over the control plants was found regarding the yield, which dropped down from 1.95 g plant⁻¹ to only 0.6 g plant⁻¹ (Table 3). The same happened with single applications of *Claroideoglomus claroideum* and *Rhizobium leguminosarum*, which decreased to the same statistical group with control plants. Although a significant yield reduction due to raised salinity was also noticed in case of single applications of *Rhizophagus irregularis* and *Burkholderia*, they were still able to achieve a significantly higher yield than the control plants. Interestingly, the highest grain yield was obtained by a combination of a mixture of AM fungi (*Rhizophagus irregularis* + *Claroideoglomus claroideum*) with N-fixing bacteria (*Rhizobium leguminosarum*) (2.01 g plant⁻¹), which was significantly higher than all other variants (Table 3). A significantly higher grain yield than the control plants was also obtained in the case of combined applications of AM fungi with *Burkholderia* (Table 3).

Table 1. Root length (RL, cm), root surface area (RSA, cm²) and root volume (RootV, cm³) of control (Ctr), *Rhizophagus irregularis*—inoculated (Rh.ir+), *Claroideoglossum claroideum*—inoculated (Cl.cl+), *Rhizobium leguminosarum*—inoculated (Rh.l+), *Burkholderia* spp.—inoculated (Brh+), *Rhizophagus irregularis* + *Claroideoglossum claroideum* + *Rhizobium leguminosarum*—inoculated (AMF + Rhl), and *Rhizophagus irregularis* + *Claroideoglossum claroideum* + *Burkholderia* spp.—inoculated (AMF + Brh) pea plants under two levels of salinity (0 and 50 mM NaCl). Different letters indicate significant differences within following parameters (Tukey test, $p < 0.05$; mean \pm SD).

Variant	RL		RSA		RootV	
	0	50	0	50	0	50
Ctr	534.16 \pm 65.85c	653.81 \pm 65.85c	79.44 \pm 10.56d	102.16 \pm 10.56d	1.073 \pm 0.15c	1.273 \pm 0.15c
Rh.ir+	887.42 \pm 65.85b	686.37 \pm 65.85b	149.13 \pm 10.56b	112.05 \pm 10.56c	1.847 \pm 0.15b	1.464 \pm 0.15b
Cl.cl+	715.63 \pm 65.85b	590.05 \pm 65.85c	107.60 \pm 10.56c	88.21 \pm 10.56d	1.315 \pm 0.15c	1.051 \pm 0.15c
Rh.l+	1094.15 \pm 65.85a	830.85 \pm 65.85b	167.75 \pm 10.56a	139.21 \pm 10.56b	1.943 \pm 0.15b	1.869 \pm 0.15ab
Brh+	1200.28 \pm 65.85a	1025.02 \pm 65.85a	192.40 \pm 10.56a	152.95 \pm 10.56b	2.347 \pm 0.15a	1.799 \pm 0.15b
AMF + Rhl	843.99 \pm 65.85b	732.89 \pm 65.85b	128.30 \pm 10.56b	119.90 \pm 10.56c	1.591 \pm 0.15b	1.503 \pm 0.15b
AMF + Brh	929.84 \pm 65.85ab	659.00 \pm 65.85b	136.65 \pm 10.56b	109.42 \pm 10.56c	1.601 \pm 0.15b	1.448 \pm 0.15b
Significance						
Variant (V)	<0.001		<0.001		<0.001	
Salinity (S)	<0.001		<0.001		0.021	
V \times S	0.074		0.07		0.266	

Table 2. Dry matter of roots (DM_{Root} , g plant⁻¹), dry matter of shoot (DM_{Shoot} , g plant⁻¹) dry matter of plant (DM_{Plant} , g plant⁻¹) and $DM_{Root}:DM_{Shoot}$ ratio of control (Ctr), *Rhizophagus irregularis*—inoculated (Rh.ir+), *Claroideoglomus claroideum*—inoculated (Cl.cl+), *Rhizobium leguminosarum*—inoculated (Rh.l+), *Burkholderia* spp.—inoculated (Brh+), *Rhizophagus irregularis* + *Claroideoglomus claroideum* + *Rhizobium leguminosarum*—inoculated (AMF+Rhl) and *Rhizophagus irregularis* + *Claroideoglomus claroideum* + *Burkholderia* spp.—inoculated (AMF + Brh) pea plants under two levels of salinity (0 and 50 mM NaCl). Different letters indicate significant differences within following parameters (Tukey test, $p < 0.05$; mean \pm SD).

Variant	DM-Root		DM-Shoot		DM-Plant		DM-Root/DM-Shoot	
	0	50	0	50	0	50	0	50
Ctr	0.151 \pm 0.13a	0.037 \pm 0.13e	0.458 \pm 0.04c	0.427 \pm 0.04c	0.610 \pm 0.05b	0.464 \pm 0.05c	0.34 \pm 0.04ab	0.07 \pm 0.04c
Rh.ir+	0.175 \pm 0.13a	0.083 \pm 0.13c	0.505 \pm 0.04b	0.586 \pm 0.04b	0.680 \pm 0.05b	0.669 \pm 0.05b	0.38 \pm 0.04a	0.15 \pm 0.04bc
Cl.cl+	0.167 \pm 0.13a	0.043 \pm 0.13d	0.437 \pm 0.04c	0.447 \pm 0.04c	0.604 \pm 0.05b	0.490 \pm 0.05c	0.39 \pm 0.04a	0.23 \pm 0.04b
Rh.l+	0.134 \pm 0.13b	0.062 \pm 0.13c	0.486 \pm 0.04c	0.324 \pm 0.04d	0.620 \pm 0.05b	0.387 \pm 0.05c	0.28 \pm 0.04b	0.19 \pm 0.04b
Brh+	0.200 \pm 0.13a	0.033 \pm 0.13d	0.680 \pm 0.04a	0.537 \pm 0.04b	0.880 \pm 0.05a	0.571 \pm 0.05b	0.30 \pm 0.04b	0.06 \pm 0.04c
AMF + Rhl	0.147 \pm 0.13a	0.134 \pm 0.13b	0.455 \pm 0.04c	0.414 \pm 0.04c	0.602 \pm 0.05b	0.575 \pm 0.05b	0.41 \pm 0.04a	0.25 \pm 0.04b
AMF + Brh	0.123 \pm 0.13b	0.14 \pm 0.13b	0.485 \pm 0.04c	0.449 \pm 0.04c	0.608 \pm 0.05b	0.589 \pm 0.05b	0.35 \pm 0.04ab	0.30 \pm 0.04b
Significance								
Variant (V)	0.003		<0.001		<0.001		<0.001	
Salinity (S)	<0.001		0.413		0.012		<0.001	
V \times S	<0.001		<0.001		<0.001		0.048	

Table 3. Grain number (GN), average weight of fresh grains (AvgGW; g), and total fresh grain weight (GW; g plant⁻¹) of control (Ctr), *Rhizophagus irregularis*—inoculated (Rh.ir+), *Claroideoglossum claroideum*—inoculated (Cl.cl+), *Rhizobium leguminosarum*—inoculated (Rh.l+), *Burkholderia* spp.—inoculated (Brh+), *Rhizophagus irregularis* + *Claroideoglossum claroideum* + *Rhizobium leguminosarum*—inoculated (AMF+Rhl), and *Rhizophagus irregularis* + *Claroideoglossum claroideum* + *Burkholderia* spp.—inoculated (AMF + Brh) pea plants under two levels of salinity (0 and 50 mM NaCl). Different letters indicate significant differences within following parameters (Tukey test, $p < 0.05$; mean \pm SD).

Variant	GN		AvgGW (g)		GW (g Plant ⁻¹)	
	0	50	0	50	0	50
Ctr	3.33 \pm 0.34	2.83 \pm 0.34b	0.585 \pm 0.43a	0.274 \pm 0.43bc	1.95 \pm 0.16b	0.60 \pm 0.16d
Rh.ir+	3.66 \pm 0.34	4.50 \pm 0.34a	0.524 \pm 0.43a	0.383 \pm 0.43b	1.84 \pm 0.16b	1.73 \pm 0.16bc
Cl.cl+	2.75 \pm 0.34	2.75 \pm 0.34b	0.545 \pm 0.43a	0.26 \pm 0.43bc	1.46 \pm 0.16c	0.80 \pm 0.16d
Rh.l+	3.08 \pm 0.34	3.50 \pm 0.34b	0.558 \pm 0.43a	0.189 \pm 0.43c	1.7 \pm 0.16bc	0.77 \pm 0.16d
Brh+	3.58 \pm 0.34	4.25 \pm 0.34a	0.494 \pm 0.43a	0.377 \pm 0.43c	1.6 \pm 0.16bc	1.45 \pm 0.16c
AMF + Rhl	4.08 \pm 0.34	3.33 \pm 0.34b	0.651 \pm 0.43a	0.602 \pm 0.43a	2.38 \pm 0.16a	2.01 \pm 0.16b
AMF + Brh	3.33 \pm 0.34	2.58 \pm 0.34b	0.603 \pm 0.43a	0.557 \pm 0.43a	1.92 \pm 0.16b	1.64 \pm 0.16c
Significance						
Variant (V)	<0.001		<0.001		<0.001	
Salinity (S)	0.949		<0.001		<0.001	
V \times S	0.092		<0.001		<0.001	

Some mild correlations coefficients were found between root morphology parameters (RL, RSA, RootV) with the above ground (DM_{Shoot}) and the whole plant dry matter (DM_{Plant}), clearly indicating that root morphology parameters and plant biomass tend to increase together. Surprisingly, the correlations between root morphology parameters and the fresh grain yield were very weak and sometime even non-significant (Table 4).

Table 4. Pearson correlation coefficients and significance levels between root morphology parameters (RL, RSA, RootV), and dry matter (DM_{Root} , DM_{Shoot} , DM_{Plant}), and fresh grain yield of control (Ctr), *Rhizophagus irregularis*—inoculated (Rh.ir+), *Claroideoglossum claroideum*-inoculated (Cl.cl+), *Rhizobium leguminosarum*—inoculated (Rh.l+), *Burkholderia* spp.—inoculated (Brh+), *Rhizophagus irregularis* + *Claroideoglossum claroideum* + *Rhizobium leguminosarum*—inoculated (AMF + Rhl), and *Rhizophagus irregularis* + *Claroideoglossum claroideum* + *Burkholderia* spp.—inoculated (AMF + Brh) pea plants under two levels of salinity (0 and 50 mM NaCl).

		RSA	RootV	DM_{Root}	DM_{Shoot}	DM_{Plant}	Yield
RL	Corr.Coeff.	0.958	0.855	0.338	0.374	0.407	0.175
	p Value	0.000	0.000	0.000	0.000	0.000	0.023
RSA	Corr.Coeff.		0.909	0.326	0.367	0.395	0.147
	p Value		0.000	0.000	0.000	0.000	0.056
RootV	Corr.Coeff.			0.289	0.311	0.336	0.134
	p Value			0.000	0.000	0.000	0.082

4. Discussion

Significant growth promotion effects of AM fungi, i.e., enhanced growth of seedlings in nurseries and improved performance after planting in the field [13,14] improved stand establishment rate and enhanced plant resistance to soil salinity immediately after transplanting [15,16], and increased root and shoot dry matter [14], are reported in various vegetable crops. Similarly, growth-promoting capabilities of bacteria such as; nitrogen fixation, phosphate solubilization, iron sequestration, synthesis of phytohormones, modulation of plant ethylene levels ([17,18] are also largely documented. In addition to these, the architecture of root system seems also to be strongly affected by the presence of beneficiary microorganisms. Clearly, under normal (non-saline) conditions, the main root architecture parameters (RL, RSA, RootV) of pea (*Pisum sativum* L.) plants were enhanced by single inoculation of AM fungi (*Rhizophagus irregularis*) and bacteria (*Rhizobium leguminosarum* and *Burkholderia* sp.) or combined inoculation of an AMF mixture (*Rhizophagus irregularis* + *Claroideoglossum claroideum*) with each of bacteria. Indeed, the longest root length was found in Brh+ and Rh.l+ plants and both variants recorded also the highest RSA and RootV values. This is explained with enhanced root growth and increasing root length effects of indole-3-acetic acid (IAA) produced by plant-associated bacteria [19]. Similar to our results, enhanced growth effects due to inoculation of a certain strain of *Burkholderia* sp. were previously reported in tomato, cucumber, and sweet pepper [20].

Similar to Aloni et al. (2011) [21] and Meça et al. (2016) [22] who reported severe inhibition of root development due to increased salinity, we found that the rise of salinity in the irrigation water from 0 to 50 mM sodium chloride was followed by a significant reduction of RL, RSA and RootV in Rh.l+, Brh+ and Cl.cl+ plants, but not in case of Rh.ir+ plants and combined applications of mixtures of AM fungi with bacteria. In agreement with previous findings of Ruiz-Lozano and Azcón, (2000) [23] who reported differences in fungal behavior and efficiency due to their origin, the results of this experiment proved that the growth-promoting effects of beneficiary microorganisms regarding root morphology parameters were highly depended on the individual characteristics of a certain inocula. In this experiment, Rh.ir+ plants recorded significantly higher RSA and RootV values than Cl.cl+ plants under no-saline conditions, and the differences between them were even larger under saline conditions. As such, these results are an indication of a higher salt sensitivity of *Claroideoglossum claroideum* compared with *Rhizophagus irregularis*. Resembling AM fungi, the rhizobia-plant association was

also highly specific. Because the symbiotic effectiveness of a bacterial strain is an estimation of host growth promotion and is usually based on the enhancement of plant shoot dry weight upon inoculation [4], considering its significantly higher RL, DM_{Shoot} , and DM_{Plant} values, we conclude that under saline conditions, single applications of *Burkholderia* spp. was more effective than using *Rhizobium leguminosarum*. It is already confirmed that a valuable feature of some *Burkholderia* species is their capacity for biological nitrogen fixation (BNF) [24]. This was also a distinguish feature of *Burkholderia* spp. that we used in this experiment (see Supplementary Material; nodule establishment in the roots of a pea plant due to the inoculation of a *Burkholderia* spp. strain). Considering that in our experiment the growing substrate was a mixture of vermiculate and river sand (therefore very poor regarding N content), our results are a confirmation of previous reports [25,26] which underlines that nitrogen-limited conditions favor the dominance of *Burkholderia* species over other rhizobial strains. Obviously, the higher number of nodules in Brh+ plants vs. Rh.l+ plants under saline conditions supports this finding. Still, since no significant difference was found regarding the number of nodules between combined applications of fungi and bacteria (AMF + Rh.l, AMF + Brh) and control plants, it seems that under our specific conditions (saline and very poor in nutrient elements substrate), rather than with bacteria, pea plants tend to develop a symbiotic relationship with AM fungi.

We found no significant growth effect with using beneficiary microorganisms on DM_{Root} under no-saline conditions. This result is in disagreement with previous reports [23,27] which showed that rather than enlarging the root system, AM fungi and bacteria contribute to increasing the nutrient uptake efficiency of the root system in colonized plants. On the contrary, there was a significant growth promotion effect of beneficiary microorganisms on DM_{Shoot} , and consequently on entire plant dry matter. The most visible results were obtained in Rh.ir+ and Br+ variants. Similar effects of AMF inoculation have been reported in many crops and have been related to increased nutrient uptake, accumulation of osmoregulatory compounds, increased photosynthetic rates [10], decrease in root respiration [28], facilitation of water uptake by plants, and mitigation of ionic imbalance [29]. Meanwhile, the growth promotion effects of bacteria found in various plant species (tomato, pepper, canola, bean, and lettuce) [30] were attributed to synthesis of phytohormones (such as auxins, cytokinins, and gibberellins) which promote shoot and entire plant growth [17,31].

The rise of NaCl concentration in irrigation water has drastically reduced plant growth of either control or inoculated plants, indicating that AM fungi and bacteria were themselves negatively affected by salinity. The salt stress can affect AM fungi by slowing down root colonization, spore germination, and hyphal growth [32,33]. Meanwhile, raised salinity leads to a failure in the establishment of rhizobia, either by reducing the survival rate and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting root hair colonization [30]. The effect of raised salinity on DM_{Shoot} was mostly non-significant. Herewith, the significant reduction in DM_{Plant} was mostly a consequence of drastic reduction in DM_{Root} which consequently led to a drastically reduced $DM_{\text{Root}}:DM_{\text{Shoot}}$ ratio. Under these circumstances, the sustained growth of AMF-inoculated plants under salinity condition should be explained by the extensive hyphal network and the higher hyphae affinity to low nutrient concentrations than the plant roots themselves [34]. A similar reduction in $DM_{\text{Root}}:DM_{\text{Shoot}}$ ratio was previously reported under increased nutrients availability to the plant [35]. Interestingly, despite significant reduction due to raised salinity, combined applications of mixtures of AM fungi with either *Rhizobium leguminosarum*, or *Burkholderia* maintained to have significantly higher DM_{Root} values than control plants, or single applications of AM fungi. Similar to us, by investigating the combined inoculation of a mixture of AM fungi (*Acaulospora laevis*, *Glomus geosporum*, *Glomus mosseae*, and *Scutellospora armeniaca*) with *Rhizobium leguminosarum* bv. *viciae* on the growth of *Vicia faba* under alkaline soil conditions was also found that the dry matter accumulation of shoot and root of dually inoculated plants were significantly increased as compared with non-inoculated plants [2].

With the exception of combined application of AMF and *Rhizobium leguminosarum*, the effects of beneficiary microorganisms on fresh grain yield of pea plants under no-saline conditions were not very visible. Some negative effects were even noticed in the case of *Claroideoglomus claroideum*

inoculation. Indeed, it is an already known fact that mycorrhizal growth responses can range from highly beneficial to detrimental [12]. By analyzing the effects of three AMF species on two soybean genotypes, Wang et al. (2016) [12] found that only *R. irregularis* increased the plant biomass and phosphorus (P) uptake of both soybean genotypes under all P supply conditions. In contrast, *G. custos* and *G. aggregatum* did not lead to positive growth or P uptake benefits of both soybean genotype, and *G. aggregatum* even led to growth depression under medium P supply conditions.

As expected, a drastic reduction of fresh grain yield, due to both reduced numbers of seeds per pod and reduced average grain weights was recorded in the case of raised salinity. Interestingly, the efficiency of beneficiary microorganisms depended on the specific characteristics of a certain microorganism. Previously, Pollastri et al. (2018) were reported significant differences between *Funneliformis mosseae* and *Rhizophagus irregularis* in the potential impact of AM symbiosis on the performance of a bioenergy crop (*Arundo donax*) under saline conditions [36] and similarly, comparing three AMF species (*F. caledonius*, *F. mosseae*, and *R. irregularis*) regarding strawberry growth under severe salt conditions (100–200 mM), was found that *R. irregularis* mitigated salt stress to a higher degree than the other two AMF species [37]. Similarly, our data prove that *Rhizophagus irregularis* provided better vegetative growth and a higher yield than *Claroideoglossum claroideum*. As a matter of fact, differences between them were larger under saline conditions, thus indicating higher salt sensitivity for *Claroideoglossum claroideum*. This fits well with previous studies [38] which reported no presence of spores from *Claroideoglossum claroideum* in different sites of saline soils in Argentina. In addition, *Claroideoglossum claroideum* was reported as very sensitive to high concentrations of soil HMM (heavy metals and metalloids) [39]. Considering all of the above facts, it would be wise to avoid the use of *Claroideoglossum claroideum* under such conditions.

In agreement with previously reported increased beneficial aspects of the association of mycorrhizae fungi and *Rhizobium* sp., with pulse crops [40], we found that combined application of AM fungi (*Rhizophagus irregularis* + *Claroideoglossum claroideum*) and N-fixing bacteria (*Rhizobium leguminosarum*) were able to maintain average grain weight at the level of non-saline plants and having significantly higher yield than control plants. Improved plant nutrition due to the presence of AM fungi [14,41] and facilitation of the acquisition of nutrient resources or the alteration of plant hormone levels by root-colonizing bacteria [30,42,43], should be the reasons for better vegetation and highest yield in dually inoculated plants. Even though it was significantly less than AMF + Rh.i variant, the AMF + Brh combination and single application of *Rhizophagus irregularis* and *Burkholderia* were capable of providing significantly higher yields than the control plants. Similarly, either single inoculations of chickpea (*Cicer arietinum* L.) with *Rhizophagus irregularis* or *Mesorhizobium mediterraneum*, or dual inoculation with both (*M. mediterraneum* + *R. irregularis*) increased grain productivity under adverse environmental conditions [44]. Although, single applications of *Burkholderia* provide better vegetative growth especially for above ground plant biomass, and higher grain yield under saline conditions, than single applications of *Rhizobium leguminosarum*, combined applications of AM fungi with *Rhizobium leguminosarum* still seem to be more effective.

Since the specific characteristics of the root system, such as root length and surface area, which play an active role in ions and water uptake, would enhance salt tolerance of cultivated plants [45], it would be of great interest to take advantage of that effect from beneficiary microorganisms under saline conditions. However, since the correlation coefficients between RL, RSA, and RootV with fresh grain yield are weak, it seems to be true that the practical benefit to the association of pea plants with AM fungi or plant growth-promoting bacteria under saline conditions are mostly linked with improved plant nutrition [9,46], and more specifically with improved nutrient uptake of inoculated plants rather than with increasing their root length and root volume [27].

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