ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Hortorum Cultus, 17(5) 2018, 73–86

www.acta.media.pl 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2018.5.7

ORIGINAL PAPER

Accepted: 13.02.2018

COMBINED APPLICATION OF ARBUSCULAR MYCORRHIZAE FUNGI AND PLANT GROWTH PROMOTING BACTERIA IMPROVES GROWTH AND NUTRIENT UPTAKE EFFICIENCY OF PEA (*Pisum sativum* L.) PLANTS

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ABSTRACT

The study aimed to investigate the effects of commercially available AMF inoculate (a mixture of *Rhizophagus intraradices*, *Claroideoglomus etunicatum*, *Funneliformis mossea*, *Funneliformis geosporum*, *Rhizophagus clarus*) and plant growth promoting bacteria (*Rhizobium leguminosarum* and *Burkholderia* sp.), either supplied individually or in combination with each other, on growth, root morphology and nutrient uptake capabilities in field pea (*Pisum sativum* L.) plants. Inoculated and non-inoculated pea plants were subjected to three levels of salinity (0, 20 and 50 mM) by the addition of sodium chloride into tap water. Morphology of root system was analyzed and dry matter of roots and shoots were individually measured several times during the growing cycle in randomly selected plants. The dry matter of roots and shoots was mixed together and concentration of N, P, K and Na was analytically determined. The raise of salinity in the irrigation water has strongly diminished the growth of pea plants by significantly reducing the weight, length, and surface area of root system, and deteriorating its nutrient capabilities. The inoculation of either AM fungi or PGPB in the growing substrate has contributed to alleviating the salinity stress effects through promoting growth and enhancing nutrient uptake capabilities of the root system. The combined application of AM fungi and PGPB could further enhance the nutrient uptake capabilities of pea plants under adverse salinity conditions.

Key words: AMF, *Rhizobium leguminosarum*, *Burkholderia* sp., salinity, dry matter, root length, root surface area, nutrient uptake rate

INTRODUCTION

Salinity is a severe environmental stressor in agriculture [Flowers 2004, Shelden and Roessner 2013], which causes serious deleterious effects on plant growth and productivity [Edelstein et al. 2011, Porcel et al. 2012]. Plants growing in saline soils are subjected to toxic effects of specific ions such as sodium and chloride, that disrupt the structure of enzymes and other macromolecules, damage cell organelles and disrupt photosynthesis and respiration. Soil salinity could also induce the physiological drought in plants and result in a nutrient imbalance due to decreased nutrient uptake and/or transport to the shoot [Porcel et al. 2016].

Increasingly higher chemical fertilization doses are a common response of counteracting the deleterious effects of saline water in cultivated crops. However, stimulated by increasing public awareness re-

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garding environmental and human health damage induced by overuse of fertilizers, worldwide agricultural practice is moving to a more sustainable and environmental friendly approach. In this context, soil microorganisms with beneficial activity towards the plant growth represent an attractive alternative to conventional agriculture [Gamalero and Glick 2014]. Currently, there are many reports proving that the use of beneficial microbes can enhance plant growth, development, nutrient uptake, and yield of legume crops. Indeed, although all parts of the plant are colonized by microorganisms, the rhizosphere represents the main source of microorganisms with plant-beneficial activities. Commonly, they are arbuscular mychorrhizae fungi (AMF) and bacteria.

Arbuscular mycorrhizal fungi (AMF) have been frequently reported to improve crop plants' tolerance to stressful abiotic environments such as saline soils [Jahromi et al. 2008, Babaj et al. 2014, Vuksani et al. 2015]. The improved salt tolerance of AM plants has been attributed to a more efficient uptake of nutrients, increase in photosynthesis ability, facilitation of water uptake by plants and mitigation of ionic imbalance [Porcel et al. 2016]. Meantime, plant' beneficiary bacteria are commonly defined as plant growth-promoting bacteria (PGPB). They typically promote plant growth in two ways: direct stimulation and biocontrol [Gamalero and Glick 2014], which includes activities such as: nitrogen fixation, phosphate solubilization, iron sequestration, synthesis of phytohormones, modulation of plant ethylene levels, and control of phytopathogenic microorganisms [Pięta and Pastucha 2008, Gamalero and Glick 2014].

Like most of legumes, pea is very sensitive to salinity [Egamberdieva et al. 2013]. Severe growth retardation [Meça et al. 2016, 2017] and yield and protein reduction effects due to increased salinity are largely reported. Whereas, it is largely known that legume crops have the ability to establish symbiosis with bacteria, collectively known as rhizobia, which induce root nodules where biological nitrogen fixation takes place [Peix et al. 2014]. In addition, other PGPR species like *Burkholderia*, are able to increase root and shoot growth and the total biomass of plants [Egamberdieva et al. 2013].

The combined effect of AM fungi and PGPB and triple relationships (plant-AMF-bacteria) have been very intriguing and many researchers are devoted to understand these relationships. Indeed, there are already evidences confirming that the association of mycorrhizae fungi and *Rhizobium* sp., with pulse crops, increases the beneficial aspects compared with their single associations with the host plants [Havugimana et al. 2016]. Taking into account the above considerations, the objective of this study was to assess the impact of AMF inoculants, rhizobia and plant growth promoting rhizobacteria on growth, root morphology and nutrient uptake capabilities of field pea (*Pisum sativum* L.) plants grown under normal or saline irrigation water conditions.

MATERIAL AND METHODS

The experiment was conducted in an unheated greenhouse in Tirana, Albania. For that purpose, graded seeds of a commercial pea cultivar (Progress 9) were sown into 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) + gravel sand (2 : 1). Several experimental variants were applied: i) pure mixture of substrate (control, Ctr); ii) mixture of substrate and 10% (v/v) crushed, expanded clay particles coated with AM-fungal spores (~200 spores g^{-1}; mixture of *Rhizophagus intraradices*, *Claroideoglomus etunicatum*, *Funneliformis mossea*, *Funneliformis geosporum*, *and Rhizophagus clarus*, AMF+); iii) mixture of substrate and 10% v/v peat moss inoculated with *Rhizobium leguminosarum* spores (Rhl+); iv) mixture of substrate and 10% v/v peat moss inoculated with *Burkholderia* sp. spores (Brh+); v) mixture of substrate and AMF (5% v/v) + *Rh. leguminosum* (5% v/v) (AMF + Rhl) and vi) mixture of substrate and AMF (5% v/v) + *Burkhlderia* sp. (5% v/v each) (AMF + Brh). The clay particles with AMF spores and peat moss containing either *Rh. leguminosarum* or *Burkholderia* sp. spores were homogenously 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 January 10, 2016 and the experiment lasted till May 10, 2016.

Three different levels of salt-stress (0, 20 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 6 pots placed in a row alongside each other; each of them represented a replication. Plants were watered during the whole experimental period with equal amounts of either tap water (0 mM NaCl), or saline water (20 and 50 mM NaCl). The irrigation was conducted by a gravity driven drip irrigation system (2 drippers per pot, with 0.2 L h^{-1} discharge rate). For that purpose, individual 200 L deposits were placed over a 2 m high platform. Duration, frequency and length of irrigation cycles were automatically controlled by an electronic irrigation controller (Itec 8, Netafim Ltd, Israel).

On 35 and 60 DAS (day after sowing), ten plants from each treatment were randomly selected and harvested. Roots were gently washed to remove 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., Quebec, Canada) and root morphology parameters: length (RL), surface area (RSA), average diameter (AD) and tips (RT), were measured and recorded. 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).

The whole plant material (roots + shoots) was mixed together, grounded and analyzed for nutrient content: N (Khejldahl method), P (Mehlich 3 method) and K (Mehlich 3 method), respectively on DAS 35 and 60. Following that, the total amount (uptake) of each nutrient in plant (mg $plant^{-1}$) was calculated as the product of plant dry matter and nutrient concentration [Martinez et al. 2005]. Based on this, the specific absorption rate (SAR; g mg^{-1} d^{-1}) as the indicators of root absorption efficiency were calculated according to the following formula adopted by Martinez et al. [2005]:

$$SAR = \frac{Xt2 - Xt1}{t2 - t1} \cdot \frac{\ln RLt2 - \ln RLt1}{RLt2 - RLt1}$$

where:
Xt1 and Xt2 are the respective nutrient uptake (mg) at the start and at the end of analyzed period,
lnRLt1 and lnRLt2 are the respective natural logarithms of total root length at the start and at the end of analyzed period, t1 = start of analyzed period (DAS 35), and t2 = end of analyzed period (DAS 35).

Differences between DM, RL, RSA, AvgD and RT were tested by two way ANOVA, using the PC program SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA). Natural logarithms were used instead of original data for ANOVA analyses of RL, RSA and RT. Each significant ANOVA result ($p < 0.05$) was followed by Tukey test at $p < 0.05$ as post-hoc test to compare pair wise means within and among treatments. Values given throughout the text are means ± SD.

RESULTS

Effect of AM fungi and bacteria on growth of inoculated plants

Regardless of the fact whether there were control plants (Ctr), AMF inoculated (AMF+), or bacteria inoculated (Bc+; either *Rhizobium leguminosarum* inoculated, Rhl+, or *Burkholderia* sp., inoculated Brh+), the gradual increase of NaCl concentration in the irrigation water has steadily and significantly reduced the growth of both, the root system (DM_{root}) and the above ground plant biomass (DM_{shoot}) of pea plants (Tab. 1). Consequently, dry matter of the whole plant (DM_{plant}) followed the same trend (Tab. 1). However, the inoculation of AM fungi or bacteria has significantly increased the weight of DM of roots, shoots and the entire plant, either under control (0 mM NaCl) or saline (20 mM and 50 mM NaCl) conditions.

Table 1. Dry matter of roots (DM_{root}), dry matter of shoots ($DM_{stem + leaves}$), dry matter of Ctr, AMF+, Rhl+, Brh+, AMF + Rhl and AMF + Brh pea plants under three levels of salinity (0, 20 and 50 mM NaCl). Different letters indicate significant differences within following parameters (Tukey test, $p < 0.05$; mean ±SD)

Variant	Salinity	DM_{root}	DM_{shoot}	DM_{plant}	DM_{root} : DM_{plant}
Control	0	0.026 ±0.001c	0.150 ±0.01b	0.176 ±0.01b	0.25 ±0.02a
	20	0.024 ±0.001c	0.136 ±0.01c	0.160 ±0.01c	0.21 ±0.02b
	50	0.020 ±0.001d	0.103 ±0.01d	0.122 ±0.01d	0.17 ±0.02bc
AMF	0	0.028 ±0.001b	0.156 ±0.01b	0.184 ±0.01b	0.18 ±0.02bc
	20	0.027 ±0.001b	0.153 ±0.01b	0.180 ±0.01b	0.16 ±0.02c
	50	0.023 ±0.001c	0.132 ±0.01c	0.155 ±0.01c	0.17 ±0.02bc
RhL	0	0.034 ±0.001a	0.165 ±0.01a	0.199 ±0.01a	0.26 ±0.02a
	20	0.035 ±0.001a	0.144 ±0.01b	0.179 ±0.01b	0.19 ±0.02b
	50	0.029 ±0.001b	0.118 ±0.01c	0.147 ±0.01b	0.19 ±0.02b
Brh	0	0.036 ±0.001a	0.184 ±0.01a	0.225 ±0.01a	0.25 ±0.02a
	20	0.034 ±0.001a	0.157 ±0.01b	0.190 ±0.01b	0.18 ±0.02bc
	50	0.029 ±0.001b	0.144 ±0.01b	0.173 ±0.01b	0.16 ±0.02bc
AMF + RhL	0	0.030 ±0.001b	0.144 ±0.01b	0.174 ±0.01b	0.21 ±0.02b
	20	0.023 ±0.001c	0.141 ±0.01b	0.164 ±0.01c	0.17 ±0.02b
	50	0.022 ±0.001c	0.113 ±0.01d	0.135 ±0.01c	0.141 ±0.02c
AMF + Brh	0	0.030 ±0.001b	0.157 ±0.01b	0.188 ±0.01b	0.15 ±0.02c
	20	0.025 ±0.001c	0.125 ±0.01cd	0.150 ±0.01c	0.14 ±0.02c
	50	0.023 ±0.001c	0.125 ±0.01cd	0.148 ±0.01c	0.14 ±0.02c
Significance					
Variant		***	***	***	***
Salinity		***	***	***	ns
Variant × Salinity		ns	ns	ns	ns

Despite the significant difference in DM_{root}, no differences were found between AMF+ and Ctr plants regarding DM_{shoot} and DM_{plant} under non saline conditions (Tab. 1). On the contrary, the presence of AM fungi under mild (20 mM NaCl) and severe salinity stress (50 mM NaCl) has significantly increased the dry matter of roots (12.5%, 15%), shoots (12.5%, 28%) and the entire plant (12.5%, 27%), respectively (Tab. 1). Meanwhile, a significant increase in the weight of dry matter was recorded due to the inoculation of bacteria. Root, shoot and the whole plant dry matter of Bc+ plants was significantly higher than Ctr plants. The differences were statistically significant, in both the non-saline and saline (20 and 50 mM NaCl) conditions (Tab. 1).

Inoculation of either *Rhizobium leguminosarum* or *Burkholderia* sp., resulted in up to 38% increase of DM_{root} under the non-saline conditions and even more, up to 46% under saline conditions vs. Ctr plants. In both Rhl+ and Brh+ plants, DM_{root} and DM_{plant} were significantly higher than AMF+ plants at any level of salinity (Tab. 1). However, differences were found between bacteria themselves: Brh+ plants showing the highest increase in DM_{shoot}, respectively up to 22% in non-saline conditions and up to 40% under 50mM NaCl stress. Meantime, the differences between them regarding DM_{root} and DM_{plant} were not statistically significant (Tab. 1). Surprisingly, though with slightly higher values than the Ctr plants, the combined inoculation of AM fungi with either *Rhizobium leguminosarum* (AMF + Rhl), or *Burkholderia* (AMF + Brh) did not resulted to any significant increase regarding DM_{root}, DM_{shoot} and DM_{plant} of co-inoculated plants vs. single application of either AM fungi (AMF+), or bacteria (Bc+) plants (Tab. 1).

Generally speaking, the ratio of root dry matter (DM_{root}) to plant dry matter (DM_{plant}) was not affected by the level of salinity (Tab. 1). In only few cases (control and bacteria inoculated plants), the increase in salinity level was followed by a decrease in DM_{root} : DM_{plant} ratio. Contrary to that, DM_{root} : DM_{plant} ratio was significantly affected by the category of inoculants used. The presence of bacteria (*Rhizobium leguminosarum* or *Burkholderia*) in the growing substrate did not affect the DM_{root} : DM_{plant} ratio. Similarly to Ctr plants, the root dry matter of Bc+ plants represented nearly 25% of the total dry weight in the plant (Tab. 1). Different from bacteria, the presence of AM fungi has significantly reduced the DM_{root} : DM_{plant} ratio to less than 18%. This ratio was even smaller in case of co-inoculation of AM fungi with *Burkholderia* sp. (14–15%), but on the contrary, it reached the level of control plants in the case of co-inoculation of AM fungi with *Rhizobium leguminosarum* (Tab. 1).

Effect of AM fungi and bacteria on root morphology of inoculated plants

Salinity has strongly affected the root morphology of pea plants as well. The increase in salinity in the irrigation water from 0 mM (control) to 20 and 50 mM sodium chloride was followed by a steady, though not always significant, reduction in root length (RL) and root surface area (RSA) (Tab. 2).

With only few exceptions, the raise of salinity did not influence the number of root tips and the average root diameter. The only cases where an effect of salinity on the average root diameter was found were in AMF+ and AMF + RhL inoculated plants (Tab. 2). Under 50 mM NaCl salinity stress, there was an increase of up to 23% in the average root diameter in AMF+ plants and nearly 40% in AMF + RhL inoculated plants. No effect of salinity was found in other cases. The increase in salinity to 50 mM NaCl has meanwhile reduced the number of root tips in Rhl+ plants (Tab. 2). That was the only case when a diminishing effect of increased salinity was found regarding the number of root tips in saline stressed pea plants.

There were strong, significant effects of different inoculants on root morphology parameters. In general, the root length in Brh+, AMF + RhL and AMF + Brh plants was significantly shorter than in Ctr plants at any level of salinity. In addition, the root length of AMF+ plants was shorter than Ctr or RhL+ plants under slight salinity conditions (20 mM NaCl), but not furthermore under a more severe salinity (50 mM NaCl) (Tab. 2). The pictures re-

garding RSA, AvgD and RT were very similar with RL. As a common attitude, under normal growing conditions, the inoculation of either AM fungi or bacteria alone, has not shown any significant effect on the root morphology parameters, while it has significantly reduced all of them under saline conditions (Tab. 2). Interestingly, the co-inoculation of AM fungi with either *Rhizobium leguminosarum* or *Burkholderia* has significantly reduced the root morphology parameters (RL, RSA, AvgD and RT) compared with AMF alone, either under non-saline or saline conditions (Tab. 2).

Table 2. Root length (RL, mm), root surface area (RSA, mm^2), average root diameter (AvgD, mm) and root tips (RT) of Ctr, AMF+, Rhl+, Brh+, AMF + Rhl and AMF + Brh pea plants under three levels of salinity (0, 20 and 50 mM NaCl). Natyral logarithms are used for RL, RSA and RT. Different letters indicate significant differences within following parameters (Tukey test, $p < 0.05$; mean ±SD)

Variant	Salinity	RL	RSA	AvgD	Tips
Ctr	0	7.423 ±0.06a	5.362 ±0.08a	0.839 ±0.04a	7.791 ±0.12a
	20	7.542 ±0.06a	5.485 ±0.08a	0.847 ±0.04a	7.454 ±0.12a
	50	6.912 ±0.06b	5.034 ±0.08b	0.922 ±0.04a	7.463 ±0.12a
AMF+	0	7.357 ±0.06a	5.543 ±0.08a	0.674 ±0.04b	7.099 ±0.12b
	20	6.962 ±0.06b	5.040 ±0.08b	0.764 ±0.04ab	6.733 ±0.12b
	50	6.806 ±0.06b	4.829 ±0.08b	0.829 ±0.04a	6.759 ±0.12b
Rhl+	0	7.204 ±0.06a	5.402 ±0.08a	0.645 ±0.04b	7.419 ±0.12a
	20	7.201 ±0.06a	5.346 ±0.08ab	0.697 ±0.04b	7.555 ±0.12a
	50	6.966 ±0.06b	5.126 ±0.08b	0.716 ±0.04b	7.135 ±0.12b
Brh+	0	7.092 ±0.06b	5.118 ±0.08a	0.684 ±0.04b	7.582 ±0.12a
	20	6.784 ±0.06c	5.058 ±0.08b	0.668 ±0.04b	7.266 ±0.12b
	50	6.864 ±0.06bc	4.946 ±0.08b	0.701 ±0.04b	7.412 ±0.12a
AMF + RhL	0	6.683 ±0.06b	4.876 ±0.08b	0.567 ±0.04b	6.971 ±0.12b
	20	6.915 ±0.06b	4.984 ±0.08b	0.602 ±0.04b	7.277 ±0.12b
	50	6.662 ±0.06c	5.016 ±0.08b	0.789 ±0.04a	7.334 ±0.12b
AMF + Brh	0	6.954 ±0.06b	4.994 ±0.08b	0.720 ±0.04b	7.253 ±0.12b
	20	6.665 ±0.06c	4.738 ±0.08b	0.741 ±0.04b	7.266 ±0.12b
	50	6.592 ±0.06c	4.523 ±0.08c	0.742 ±0.04b	6.957 ±0.12b
Significance					
Variant		***	***	***	***
Salinity		***	***	***	ns
Variant × Salinity		***	***	ns	***

Effect of AM fungi and bacteria on nutrient concentration and uptake of inoculated plants

Significant enhancing effects due to AM fungi inoculation were found regarding main nutrient concentration in the leaves of pea plants. Indeed, the presence of AM fungi in the growing substrate was followed by a significant increase of N, P and K in plant tissues (Tab. 3); N, P and K concentrations in AMF+ plants were significantly higher than Ctr and Bc+ plants. The inoculation of *Rh. leguminosarum* and *Burkholderia* sp. has significantly increased the tissue concentration of N and K compared to control plants as well, but not of P (Tab. 3). The concentration of each of these elements was, however strongly influenced by the level of salinity, showing a clear diminishing trend due to raised salinity. Surprisingly, though mostly significantly higher than Ctr plants, the concentration of main nutrient elements (N, P and K) of co-inoculated plants (AMF + RhL, AMF + Brh) was still significantly less than the respective concentration in AMF+ plants (Tab. 3).

Table 3. The concentration of main nutrient elements (N, P, K and Na) mg g^{-1} in plant dry matter by DAG 60 of Ctr, AMF+, Rhl+, Brh+, AMF + Rhl and AMF + Brh pea plants under three levels of salinity (0, 20 and 50 mM NaCl). Different letters indicate significant differences within following parameters (Tukey test, $p < 0.05$; mean ±SD)

Variant	Salinity	N	P	K	Na
Control	0	12.846 ±0.67c	0.650 ±0.03a	19.533 ±0.89c	1.553 ±0.66h
	20	12.223 ±0.67c	0.547 ±0.03b	18.768 ±0.89c	5.184 ±0.66e
	50	10.268 ±0.67d	0.538 ±0.03b	17.332 ±0.89c	14.917 ±0.66a
AMF	0	16.699 ±0.67a	0.638 ±0.03a	27.098 ±0.89a	1.114 ±0.66h
	20	17.750 ±0.67a	0.677 ±0.03a	24.117 ±0.89b	5.570 ±0.66e
	50	13.234 ±0.67b	0.580 ±0.03b	21.488 ±0.89b	12.554 ±0.66c
RhL	0	16.707 ±0.67a	0.546 ±0.03b	28.155 ±0.89a	1.579 ±0.66h
	20	13.817 ±0.67b	0.495 ±0.03b	27.814 ±0.89a	7.389 ±0.66d
	50	11.432 ±0.67c	0.487 ±0.03b	20.791 ±0.89b	11.552 ±0.66c
Brh	0	15.011 ±0.67b	0.558 ±0.03b	25.739 ±0.89b	1.572 ±0.66h
	20	14.883 ±0.67b	0.587 ±0.03b	24.016 ±0.89b	6.675 ±0.66e
	50	12.751 ±0.67c	0.565 ±0.03b	22.35 ±0.89bc	13.116 ±0.66b
AMF + RhL	0	14.388 ±0.67b	0.530 ±0.03b	28.129 ±0.89a	1.705 ±0.66h
	20	15.893 ±0.67b	0.681 ±0.03a	25.70 ±0.89ab	6.403 ±0.66e
	50	13.660 ±0.67b	0.522 ±0.03b	21.709 ±0.89b	11.922 ±0.66c
AMF + Brh	0	17.459 ±0.67a	0.612 ±0.03a	25.706 ±0.89b	2.507 ±0.66g
	20	12.205 ±0.67c	0.551 ±0.03b	20.995 ±0.89c	4.116 ±0.66f
	50	11.81 ±0.67cd	0.468 ±0.03b	21.166 ±0.89c	11.427 ±0.66c
Significance					
Variant		***	***	***	***
Salinity		***	ns	***	***
Variant × Salinity		***	ns	*	***

Sodium (Na^+) concentration in plant tissues was significantly influenced by the level of sodium chloride in the irrigation water. The relationship between salinity level in the irrigation water and leaf Na^+ concentration was quite linear, regardless the fact that was control or inoculated plant. There was, however quite distinguished positive effects of AM fungi or bacteria inoculation, either alone or co-inoculated, towards the reduction of sodium concentration in plant tissue (Tab. 3). While, there was no significant difference regarding Na concentration between control and inoculated plants in the non-saline conditions, the differences became very large at 50 mM NaCl stress conditions. Indeed, compared to nearly 15 mg g^{-1} dry matter in control plants, sodium concentration dropped down to nearly 12 mg g^{-1} dry matter in AMF+ and Bc+ plants (Tab. 3).

Specific absorption rates (SAR; mg of absorbed nutrient per cm of root length, daily) of main nutrient elements (N, P and K) were strongly influenced by the treatment and the level of salinity. Indeed, the increase in salinity has significantly reduced SAR-N, SAR-P and SAR-K. By raising the salinity from 0 mM NaCl to 50 mM NaCl, the average specific absorption rates of N, P and K were reduced by respectively 21%, 38% and 20% (data not shown). Instead, the average specific absorption rate of sodium (SAR-Na) was increased by nearly eight times.

The presence of AM fungi and bacteria has significantly improved the specific absorption rate of N, P and K. Either used alone, or in combination, AM fungi and bacteria provided significantly higher SAR-N values than Ctr plants in normal or slightly saline (20 mM NaCl) conditions. Interestingly, while bacteria alone (Bc+) were not able to provide higher SAR-N values than Ctr plants under more severe salinity conditions (50 mM NaCl), AMF+, AMF + RhL and AMF + Brh plants revealed significantly higher SAR-N values vs. Ctr plants (Fig. 1A). Obviously, combined application of AM fungi with bacteria (either RhL or Brh) provided the highest SAR-N values under either normal or saline conditions. Indeed, when used alone, AMF fungi provided a 47% increase in SAR-N vs. Ctr plants in non-saline conditions and up to 72% in combined application with either RhL or Brh. The difference remained very large even under saline conditions; respectively 62% when AM fungi were used alone and 65% in combined use with N fixing bacteria.

Similar to SAR-N, the presence of AM fungi and bacteria has significantly increased the SAR-K vs. Ctr plants. When used alone, AM fungi increased SAR-K by 54% under non-saline condition and 57% under 50 mM NaCl condition (Fig. 1C). Although slightly less, Bc+ plants provided similar results with AMF+ plants in non-saline and 20 mM NaCl stress. On the contrary, they did not show any advantage vs. Ctr plants under more severe sodium salinity (50 mM; Fig. 1C). Similarly to SAR-N, the combined application of AM fungi and *Rhizobium leguminosarum* provided the highest SAR-K value in non-saline conditions (3.3 mg $cm^{-1}day^{-1}$). Furthermore, combined AMF + Bc applications also maintained significant advantages vs. Ctr and Bc+ plants under saline conditions (Fig. 1C).

Table 4. Two-way ANOVA results including cross effects on specific absorption rate (SAR) of N, P, K and Na in the period DAS 35–DAS 60 of different treatments; Ctr, AMF+, Rhl+, Brh+, AMF + Rhl and AMF + Brh pea plants, under three levels of salinity (0, 20 and 50 mM NaCl). p values; significant ($p < 0.05$) values are highlighted in bold

Significance	SAR-N	SAR-P	SAR-K	SAR-Na
Treatment	**<0.001**	**<0.001**	**<0.001**	0.259
Salinity	**<0.001**	**0.040**	**<0.001**	**<0.001**
Treatment × Salinity	0.696	0.657	0.135	**0.031**

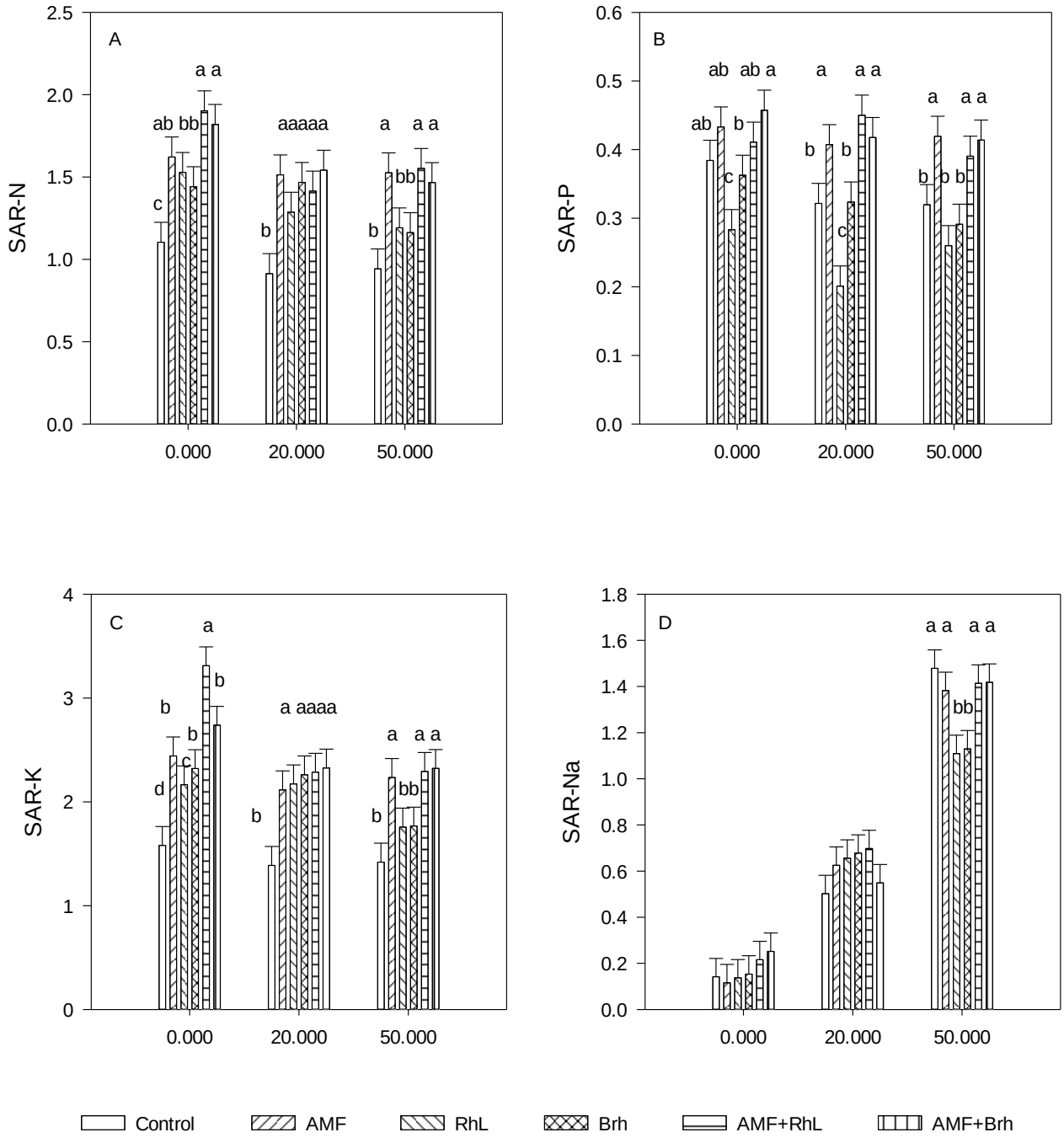

Fig. 1. Specific absorption rate (SAR) of N, P, K and Na in the period DAS 35–DAS 60 of Ctr, AMF+, Rhl+, Brh+, AMF + Rhl and AMF + Brh pea plants under three levels of salinity (0, 20 and 50 mM NaCl). Different letters indicate significant differences within following parameters (Tukey test, $p < 0.05$; mean ±SD)

Different from SAR-N and SAR-K, bacteria alone were not able to provide any advantage of the inoculated vs. control plants regarding SAR-P, either under normal or saline conditions (Fig. 1B). Instead, when used alone, AM+ showed a significant increase in SAR-P up to 12% under non-saline condition and up to 31% under saline (50 mM NaCl) conditions. Similarly, the combined use of AM fungi with either *Rhizobium leguminosarum* or *Burkholderia* sp. did also show significantly higher SAR-P values compared with Ctr plants (Fig. 1B). The most significant synergistic effect of combined use of AM fungi and bacteria regarding SAR-P was found in AMF + Brh plants.

Contrary to N, P and K, the presence of either AM fungi or bacteria did not show any effect regarding SAR-Na (Tab. 4). Significantly smaller SAR-Na values of Rhl+ and Brh+ plants under 50 mM NaCl stress (Fig. 1D) can be explained by the interaction found between the treatment and salinity.

DISCUSSION

Effect of AM fungi and bacteria on growth of inoculated plants

Gradual increase in NaCl concentration in the irrigation water has drastically reduced the growth of both the root system and the above ground biomass of pea plants. This is a conclusion largely confirmed by many publications [Cuartero et al. 2006, Huang et al. 2009, Edelstein et al. 2011, Porcel et al. 2012]. That deleterious effects of salinity over the plant growth is explained by Munns [2002] through a two-phase growth response to salinity: at the beginning of the growth, the reduction results essentially from a water stress or osmotic phase, and at the second phase, growth reduction is caused due to salts accumulation in transpiring leaves to excessive levels.

The decrease in root, shoot and the whole plant dry matter due to increased salinity was a common trend, clearly observed in all variants: Ctr, AMF+, Bc+ and AMF + Bc plants. It means that either AM fungi, or PGPB were themselves negatively affected by salinity. Indeed, as it was previously reported, the salt stress can affect the AM fungi by slowing down the root colonization, spore germination and hyphal growth [Jahromi et al. 2008, Abeer et al. 2015]. Meanwhile, 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 the root hair colonization [Egamberdieva et al. 2013].

We found, anyway, the strong growth promoting effects of either AM fungi or bacteria on plants growth. AMF+ and Bc+ remained the DM_{root} and DM_{shoot} under saline conditions (20 and even 50 mM NaCl) at the level of the control plants under non-saline conditions. We have also reported similar effects of AMF inoculation in tomato [Balliu et al. 2015] and field pea [Meça et al. 2016, 2017]. Mechanism of salinity alleviation in such cases is explained by Ruiz-Lozano and Azcón [2000] and with the stimulation of root growth.

In addition to improvements in mineral nutrition and suppression of soil-borne diseases, growth stimulation effects [Ekinci et al. 2014] and modification of root development [Gamalero and Glick 2014] are also accounted as potential mechanisms of salinity alleviation by PGPB. Indeed, the growth promoting effects of bacteria were stronger than AM fungi and differences with either AMF+ or Ctr plants were statistically significant in both non-saline and saline (20 and 50 mM NaCl) conditions. As a matter of fact, ameliorative effects of bacteria on plant growth under saline conditions have been reported for various plant species (tomato, pepper, canola, bean, and lettuce) [Egamberdieva et al. 2013]. The stimulation of plant growth by PGPB might be a consequence of synthesis of phytohormones (such as auxins, cytokinins, and gibberellins) that promote shoots and entire plant growth [Gamalero and Glick 2014, Bona et al. 2015].

The co-inoculation of AM fungi and bacteria shows significantly higher DM_{root} and DM_{shoot} compared with Ctr plants, but interestingly, DM_{root} in AMF + Bc plants was significantly smaller than AMF+, or Bc+. While they are also characterized by significantly smaller DM_{root} : DM_{plant} ratio compa red with AMF+, or Bc+ plants, an enhancement of nutrient uptake capabilities of co-inoculated plants should be expected to explain significantly higher nutrient concentrations in AMF + Bc plants.

Effect of AM fungi and bacteria on root morphology of inoculated plants

The increase in salinity in the irrigation water from 0 mM (control) to 20 and 50 mM sodium chloride was followed by a steady, though not always significant, reduction of root length (RL) and root surface area (RSA). The results fit well with previous findings of Estañ et al. [2005], Aloni et al. [2011] and Meça et al. [2016], who have also reported severe inhibition of root development due to increased salinity.

Since specific characteristics of root system, such as root length and surface area, which play an active role in ion and water uptake, would enhance salt tolerance of cultivated plants [Himmelbauer et al. 2004], it would be of great interest to have increasing effects of symbiotic microorganisms on these root parameters. This was not the case in our experiment. With a single exception (AMF+ plants under mild salinity stress), no significant differences were found between AMF+ and Rhl+ plants with Ctr. Furthermore, in some cases, a significant reduction in root length (RL) and root surface area (RSA) was found in Brh+, AMF + RhL and AMF + Brh plants compared with the control plants. Therefore, it seems that rather than enlarging the root length and root surface area, AM fungi and PGPB contribute to the increase in the nutrient uptake efficiency of roots of colonized plants. The conclusion is well supported from the following discussion regarding the specific absorption rate (SAR) of main nutrient elements.

Effect of AM fungi and bacteria on nutrient concentration and uptake of inoculated plants

Despite the root growth promoting effects [Ruiz-Lozano and Azcón 2000], other mechanisms of salinity alleviation by AM fungi are reported: increased nutrient uptake, accumulation of osmo-regulatory compounds, increased photosynthetic rates [Abdel Latef and Chaoxing 2011], decrease in root respiration [Rewald et al. 2015], facilitation of water uptake by plants and mitigation of ionic imbalance [Porcel et al. 2016].

Interestingly, we found that AMF+ plants did have significantly smaller DM_{root} : DM_{plant} ratio compared with either Ctr or Bc+ plants. Some previous reports [Balliu et al. 2007] explain that with increased nutrients availability to the plant. Indeed, considering significantly higher N, P and K concentrations in AMF+ plants, while no significant increase in RL or RSA, makes it clear that AM fungi have significantly contributed to the enhancement in N, P and K uptake. Therefore, since reduced growth under salinity is caused by ion imbalances due to their competition with Na^+ and Cl^- in the soil [Gomes et al. 2011], the sustained growth of AMF+ plants under salinity should be, at least partially, a consequence of improved nutrient uptake and maintaining the favorable ionic ratios [Evelin et al. 2012]. According to Al-Karaki [2000], the extensive hyphal network and higher hyphae affinity to a lower threshold of nutrient concentration than plant' roots are the most important mechanism of higher nutrient uptake by mycorrhized plants. Indeed, this hypothesis is strongly supported by respective significantly higher SAR values of AMF+ plants compared with Ctr or Bc+ plants. Obviously, the corresponding SAR values of N, P and K of AMF+ plants were higher than Ctr, either in non-saline, or saline conditions.

Similarly to AMF+, Rhl+ and Brh+ plants have shown significantly higher SAR-N and SAR-K values compared with Ctr plants, but not SAR-P. As such, our results confirm previous reports that in addition to their N fixation ability [Egamberdieva et al. 2013, Peix et al. 2014], PGPBs are able to produce different enzymes that increase nutrient availability to the plants [Gamalero and Glick 2014, Bona et al. 2015]. The fact that the inoculation of *Rh. leguminosarum* and *Burkholderia* has significantly increased the tissue concentration of N and K compared to control plants, but not of P, can be explained with the fact that biological nitrogen fixation process in legume crops is highly P consuming activity [Liu et al. 2011]. Therefore, a high P use from bacteria at this stage of plant development (60 DAS) is reflected by lower SAR-P values in Bc+ plants.

However, very interestingly, the co-inoculation of AM fungi and bacteria revealed significantly higher SAR-P values compared with Ctr and Bc+ plants. AMF+Brh plants did have significantly higher SAR-P values vs. Ctr and Bc+ plants at any level of sodium salinity and AMF + Rhl plants showed significantly higher SAR-P values than Ctr plants under

saline conditions. Similarly, the co-inoculated plants (AMF + Rhl, AMF + Brh) have shown the highest SAR-N and SAR-K, indicating significant synergistic effects of AM fungi with PGPB in pea plants. As such, our results fit well with a previous publication by Havugimana et al. [2016], who have also reported that the association of mycorrhizae fungi and Rhizobium, with pulse crops, increase the beneficial aspects compared with their single associations with the host plants.

Although, the exposure of plants to salinity in the period of DAS 35–60 has significantly reduced the SAR of main nutrient elements, AMF+, AMF + Rhl and AMF + Brh plants were able to retain significantly higher SAR-N, SAR-P and SAR-K than Ctr plants under both 20 mM and 50 mM sodium salinity levels. Hence, since improved N nutrition helps to reduce the toxic effects of Na ions by reducing its uptake and maintaining the chlorophyll content of the plant [Evelin et al. 2009], it is expected that high N absorption rate should induce higher growth rate in plants [Peng et al. 2011] and improve plant performance under stress conditions. Because the growth rate is principally determined by protein synthesis rates, the growth rate and biomass P concentration are as well closely and positively correlated [Peng et al. 2011]. Phosphorus (P) is a critical macro-nutrient required for numerous functions within plant, including energy generation, nucleic acid synthesis, photosynthesis, glycolysis, respiration, membrane synthesis and stability, enzyme activation/inactivation, redox reactions, signaling, carbohydrate metabolism and nitrogen fixation [Niu et al. 2013], and therefore it is determinant to plant reaction against the salinity stress. In addition, since K^+ ions play a critical role in enzyme activation, water use efficiency, photosynthesis, transport of sugars and protein synthesis [Mmbaga et al. 2014], an optimal potassium K^+ : Na^+ ratio is vital to activate cytoplasm enzymatic reactions [Wakeel 2013]. Therefore, by increasing SAR-K, AMF+, and co-inoculated AMF + Rhl and AMF + Brh plants would better resist the adverse effects of gradual Na accumulation in the plant.

As it was expected, sodium (Na) concentration in plant tissues was significantly influenced by the level of sodium chloride in the irrigation water. As a common rule, the differences regarding Na concentration among different variants were very small and mostly insignificant under non-saline and 20 mM sodium salinity conditions. On the contrary, under severe salinity conditions, the N concentration in AMF+ and Bc+ plants was significantly smaller than Ctr plants. AMF + Rhl and AMF + Brh+ plants did have an even smaller Na concentration. Similarly, no differences were found between Ctr and inoculated plants regarding SAR-Na under non-saline and 20 mM sodium salinity conditions, but interestingly, Rhl+ and Brh+ plants have shown significantly smaller SAR-Na value compared with Ctr plants and other variants under 50 mM salinity condition.

To the best of out knowledge, there is no evidence of any mechanism of Na exclusion or sequestration induced by PGPB. Therefore, considering that Rhl+ and Brh+ plants have also revealed the highest DM_{plant} values, we assume that smaller Na concentrations and significantly lower SAR-Na values in Rhl+ and Brh+ plants are a consequence of a dilution effect due to growth promotion by the bacteria capable to induce in colonized plants.

CONCLUSIONS

Saline irrigation water strongly diminishes the growth of pea plants; it reduces weight, length, and surface area of root system, and deteriorates its nutrient capabilities. The inoculation of either AM fungi or PGPB in the growing substrate could help to alleviate the salinity stress effects through promoting the growth and enhancing the nutrient uptake capabilities of the root system. The combined application of AM fungi and PGPB could further enhance the nutrient uptake capabilities of pea plants under adverse salinity conditions.

ACKNOWLEDGMENTS

These results are part of the work conducted in the framework of EUROLEGUME Project (Enhancing of legumes growing in Europe through sustainable cropping for protein supply for food and feed), founded by 7th Research Framework Programme of the European Union (FP7 Research Project No. 61378).

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