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Inoculation with arbuscular mycorrhizal fungi improves salt tolerance in *C. glauca* (Sieb).

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Abstract

Monitoring land salinization is a major global problem. One of the recommended solutions is the use of halotolerant species. Tropical trees of the *Casuarinaceae* family have the property of being pioneer, salt-tolerant species and can be used in rehabilitation programs. The aim of this study is to investigate the effect of inoculation with AMF (*Rhizophagus aggregatum*, *Rhizophagus fasciculatus* and *Rhizophagus irregularis*) on the salt tolerance of *Casuarina glauca*. The plants were grown under greenhouse in nutrient-poor soil and sterilized. At the beginning of the experiment, the plants were watered with tap water up to 15 days after inoculation to allow the establishment of symbiosis. After 2 weeks of acclimation, plants were watered with gradually increasing concentrations of saline solution, 0 mM NaCl for the controls or with 150 mM or 300 mM. After 4 months of cultivation the plants were harvested and morphological and physiological parameters were determined. Compared to uninoculated controls, inoculation with R. *fasciculatus* significantly increased mean plant height, total biomass production, water content (RWC) and chlorophyll content. Higher phosphorus and nitrogen levels were observed in plants inoculated with *R. fasciculatus*. However, the inoculation did not increase the total carbon content. Higher K+ ion content was observed in the leaves of plants inoculated with *R. fasciculatus*. Inoculation with AMF (*R. fasciculatus*) improved tolerance to salt stress in *C. glauca*. Our results suggest that inoculation with the best performing AMF can help increase the growth of *C. glauca* in saline soil rehabilitation programs.

1. Introduction

Arid and semi-arid regions are characterized by low rainfall and the uncertainty of their occurrence which limits agricultural production [1]. In these regions, plants are confronted with several stresses, in particular hydric, thermal and salt stress. This is true in Senegal, where the drought of the 1970s strongly contributed to aggravate the phenomenon of land salinization. Thus, land salinization affects almost all regions, particularly the Casamance River basin, Sine Saloum and Senegal River Delta [2].

Soil salinity is a major abiotic stress that negatively affects plant growth and crop production. These soil types are dominated by Na+ ions with an electrical conductivity (EC) greater than 4 dS/m corresponding to the approximate concentration of 40 mM NaCl [3]. According to Qadir et *al* [4], salinization of land in irrigated areas causes losses of more than US$ 27.3 billion per year, significantly impacting the economy and potentially causing food insecurity. Finding solutions for the rehabilitation of these soils

is becoming a major challenge. Many strategies are used to combat this soil salinization. However, some methods such as leaching are very costly.

An alternative consists to explore the property of certain plant species to adapt to salty environments in order to colonize the soil and make it productive again. Trees of the *Casuarinaceae* family are widely used in these programs because of their ability to grow on very poor and salt soils, which explains their wide use in agroforestry, forestry and silviculture [5-6]. *Casuarinaceae* are classified among the most salt-tolerant species [5]. In the case of Casuarina species, it has been reported that *C. glauca* tolerates up to 600 mM NaCl, a concentration close to that of seawater [7]. However, better resistance to stress is obtained when the plant is associated with arbuscular mycorrhizal fungi AMF [8] and/or nitrogen-fixing bacteria [9]. Several study have demonstrated that AM fungi promote plant growth and tolerance to salinity [8-10]. AM fungi act as a barrier, preventing the entry of Na^+ in photosynthesis tissues and increasing the accumulation of osmotic ions (Ca^{2+}, Cl^-, K^+...) [11]. AMFs play an important role in osmotic adjustment, facilitating the maintenance of leaf turgidity pressure and effects on photosynthesis, transpiration, stomatal conductance and water utilization. The aim of this work is to improve salt tolerance in *C. glauca* by inoculation with efficient arbuscular mycorrhizal fungi.

2. Material and Methods

2.1. Plant material

C. glauca seeds used in this experiment are from (lot 15934, ref 086-5929) supplied by CSIRO (Australian Tree Seed Centre). They were collected from Myall Lakes National Park, Australia, at a latitude of 32°25' North and a longitude of 152°19' East. Approximately 6700 viable seeds were counted in a 10g lot. These seeds did not pre-treatment and were stored at 4°C in the laboratory.

2.2. Microbial material

Three arbuscular mycorrhizal fungi (AMF) were used in this study *R. aggregatum* (Schenke and Smith emendand. Koske) (DAOM2277128), isolated from Dindéresso in Burkina Faso, *R. fasciculatus* (Thaxter sensu Gerdemann Gerd.) (DAOM227130), isolated from Quebec (Canada) and *R. irregularis* (Schenke and Smith) (DAOM197198), isolated from Quebec (Canada). The production of these AMF inoculums was carried out in greenhouses. A mycotrophic plant such as maize (*Zea mays* L.) was grown in the presence of AMF in pots containing 1.5Kg of coarse beach sand sterilized at 120°C for 2 hours. For each fungus, the inoculum consisted of a mixture of spores and mycorrhizal fragments. The inoculum (20g) was placed in the culture substrate at a depth of 2-3cm in contact with the root system of plant. The pots were regularly watered to capacity in the field.

2.3. Experimentation design

To study the impact of inoculation with endomycorrhizal fungi on the salt tolerance of *C. glauca*, 21-day-old seedlings of *C. glauca* were transferred into black nursery pot (PM 25X12X50) filled with 2 mm sieved Sangalkam sandy soil. The soil was sterilized in an oven (120°C, 60 min) to prevent colonization by native microorganisms. The physical and chemical characteristics of this soil are as follows: clay 3.6%; fine silt 0.0%; coarse silt 0.8%; fine sand 55.5%; coarse sand 39.4%; total carbon 0.17%; total nitrogen 0.02%; C/N 8.5; total phosphorus 39 mg.kg-1; assimilable phosphorus 4.8 mg.kg-1; pH (H_2O) 5.3. Three fungal strains *R. irregularis*, *R. aggregatum* and *R. fasciculatus* (previously tested for their ability to tolerate and improve the salt tolerance of plants) were used to study the impact of inoculation with symbiotic microorganisms on the salt tolerance of *C. glauca*. Seedlings were arranged in a randomized completed block design including three factors: plant species (*C.glauca*), AMF

inoculation (control, Rf, Ri and Ra) and salinity (0, 150, 300 mM NaCl), 16 treatments (4 X 4) with twelve replications per treatment. At the beginning of the experiment, the plants were watered with tap water up to 45 days after inoculation to allow the establishment of symbiosis. After 45 days, the plants were watered either with 0 mM NaCl for the controls or with 150 mM or 300 mM NaCl. After 4 months, the plants were harvested and different parameters were evaluated: survival rate, height growth, total biomass, mycorrhization rate, chlorophyll content, relative water content (RWC), N_2, Pi and K^+, Na^+ ion content.

2.4. Measurement of height growth and total biomass of C. glauca plants

The height growth (cm) of the plants was measured weekly using the gradually ruler. After 4 months of greenhouse cultivation, the plants were harvested and the fresh weight of the biomasses was weighed. The aerial and root parts were dried in an oven at 65°C. Once the samples were completely dry, their dry weight was determined using an electronic precision balance (Sartorius AG Germany TE1245).

2.5. Evaluation of mycorrhization of C. glauca plants

To assess the frequency and intensity of mycorrhization of each plant, 100 fragments of about 1 cm each were collected per plant. After abundant rinsing with distilled water, the root fragments were discoloured after incubation in 10% KOH at 90°C. This incubation time can vary from a few hours after root lignification and often a replacement of the KOH solution during the discoloration process is necessary. Well discoloured root fragments have been rinsed in distilled water. To visualize the fungal structures, these root fragments were stained with Uvitex 2B to 0.05% (w/v) [12] for 30 min at 90°C. The roots were rinsed with water and incubated for 12 h in water to remove excess Uvitex 2B. The frequency of mycorrhization as well as the intensity of mycorrhization were assess according to the method of [13] using a range of colonization intensity noted from zero (0) to five (5). Observations were made with an optical microscope.

2.6. Determination of chlorophyll content

Chlorophyll was determined using the method of Makeen et *al* [14]. For each sample 100 mg taken from the middle third of the youngest leaves were crushed in 10 ml of 80% acetone (Panreac Quimica SA 131007.1612). The crushing step was repeated several times to extract all the chlorophyll pigments. The extract obtained was centrifuged at 10,000 rpm at 4°C for 10 min, then incubated at 4°C in the dark for 24 hours. After incubation, the optical density (OD) of all the supernatants obtained was measured at 645 nm and 663 nm (spectrophotometer). The concentrations of total chlorophylls (Chlorophylls a and b), expressed in mg. g-1 MF using the formula 8.02 x OD (663) + 20.2 x OD (645) V / M; where V denotes the volume of the total extract in liters and M the mass of the fresh ground material in grams.

2.7. Relative water content (RWC) and chemical analysis of shoot and root parts

The relative water content of *C. glauca* plants subjected to different doses of NaCl was determine after 4 months under salt as described by González et *al* [15]. For each plant, a stem fragment was cut and weighed to obtain the weight of fresh material FW. This fragment was then immersed in a 15 ml falcon tube containing distilled water and kept in the dark at room temperature for 24 hours. It is then removed and weighed to obtain the turgidity weight or saturation weight TW. After weighing, the stem are dried in an oven until a constant weight corresponding to the dry weight DW is obtained. The relative water content was obtained according to the formula:

$$RWC = [(FW - DW) / (TW - DW)] \times 100$$

The contents of N, C, Pi and K+, Na+ ions in the shoots were determined at the LAMA (Laboratory of Analytical Means IRD/Dakar-Bel air certified ISO 9001).

2.8. Statistical analysis

The measured parameters (height, total biomass, chlorophyll content, RWC, N, P, C, Na^+, and K^+) of *C. glauca* plants were statistically processed using R 3.4.2 software. A two-factor analysis (inoculation and concentration) was performed using the Fisher's LSD test at $P < 0.05$.

3. Results and discussion

The analysis of variance (ANOVA) showed a significant effect of salt stress on all the parameters studied (height, total biomass, frequency and intensity of mycorrhization, relative water content and total chlorophyll content). The inoculation had an effect on all parameters except mycorrhization frequency and intensity. A significant effect of salt stress × inoculation interaction was observed on the total biomass of *C. glauca* (**Table 1**).

3.1 The impact of inoculation with AMF strains on height growth and total biomass of C glauca under salt stress conditions

The height and total dry biomass of *C. glauca* was determined after four months of salt stress in greenhouse. The results obtained indicate a significant decrease in height and total dry biomass at 150 and 300 mM NaCl (**Figure 1**). Inoculation with *R. fasciculatus* and *R. aggregatum* significantly increased height and total biomass at all NaCl concentrations. Inoculation with *R. fasciculatus* improved height by 67%, 66% and 46% at 0, 150 and 300 mM NaCl, respectively. At 300 mM NaCl, only *R. irregularis* did not significantly improve the height in *C. glauca*. Total biomass was significantly increased by inoculation at 150 and 300 mM.

Table 1. Interaction test between "Inoculation", "NaCl concentration" factors and measured parameters

	Height	Total Biomass	Total chlorophyll content	RWC	Frequency of mycorrhization	Intensity of mycorrhization
Inoculation	***	***	***	***	ns	ns
[NaCl]	***	***	**	**	***	***
Inoculation× [NaCl]	.	*	.	ns	ns	ns

. ,*, ** and *** indicate significant differences at p < 0.1, 0.05, 0.01 and 0.001 respectively; ns: no difference

The results show that *C. glauca* plants can grow in the presence of salt ranging from 0 to 300 mM NaCl. The work of Scotti-Campos et *al* [7] showed that *C. glauca* plants are very tolerant to salt. The difference in salt tolerance in this species could be related to the difference in ion absorption. Hydromineral uptake is considered an important factor associated with improved growth of different plant species [16]. In our study, inoculation with *R. fasciculatus* improved height and dry biomass of *C. glauca* in the presence and absence of NaCl. This improvement is explained by a better hydromineral nutrition of plants in presence of *R. fasciculatus* due to the development of extra-matrical hyphae [17-18]. The results obtained by [19] show that AMF improves the mineral nutrition of the plant under salt stress conditions and also attenuates the Mg2+, P and N deficiencies. This result was confirmed in this work by higher Pi, N and water contents in *C. glauca* plants inoculated with *R. fasciculatus*. Several studies have also shown that inoculation with AMF improves nutrient uptake under saline stress conditions, especially in pepper [20], banana [21] and olive [22].

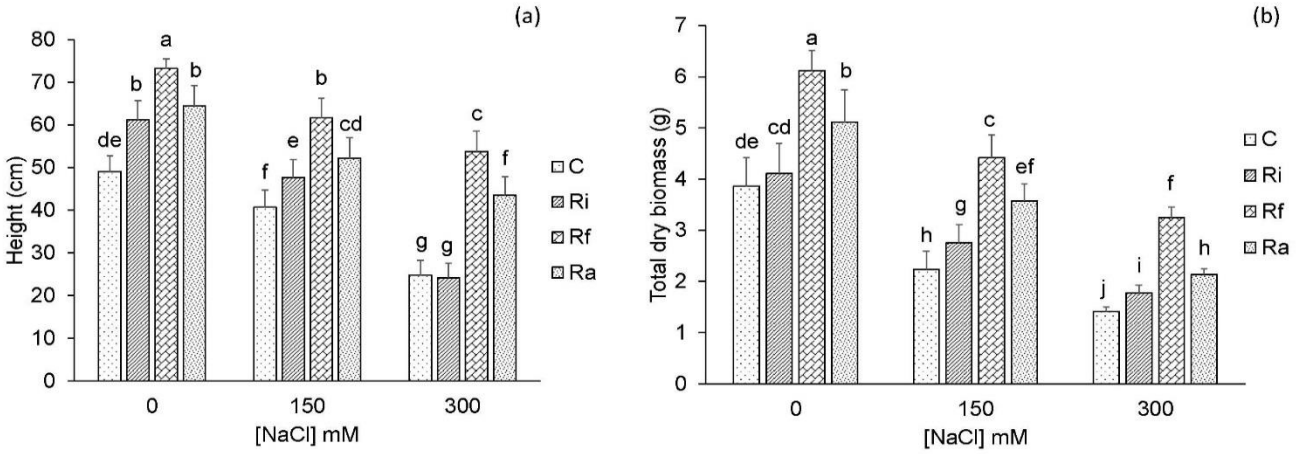

Figure 1: Height (a) and total dry biomass (b) of *C. glauca* after 4 months cultivation under salt stress. C: Control, Ri: *Rhizophagus irregularis*, Ra: *Rhizophagus aggregatum*, Rf: *Rhizophagus fasciculatus*. The letters indexing the histograms indicate the differences between treatments according to the Fisher's LSD test at P<0.05.

3.2. Colonization rate of C. glauca roots

Uvitex 2B staining for fungal structures allowed the determination of the frequency and intensity of colonization of *C. glauca* roots by the three strains *R. fasciculatum*, *R. aggregatum* and *R. irregularis*. These results show that colonization frequencies and intensities of AMF were not significantly different per NaCl concentration (**Figure 2**).

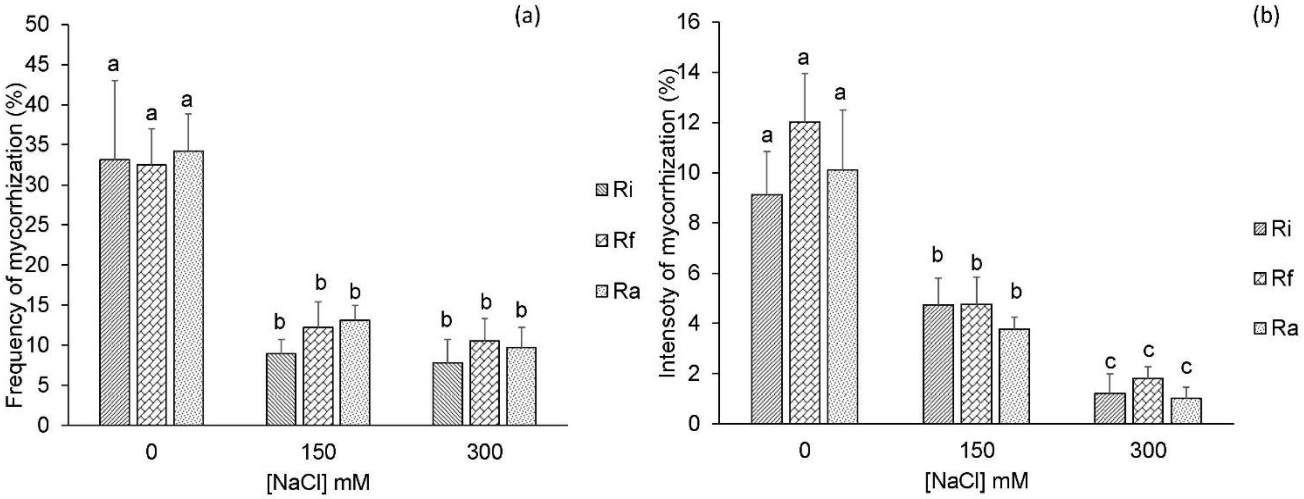

Figure 2: Frequency (a) and intensity of mycorrhization (b) of *C. glauca* after 4 months of greenhouse cultivation under salt stress. Ri: *Rhizophagus irregularis*, Ra: *Rhizophagus aggregatum*, Rf: *Rhizophagus fasciculatus*. The letters indexing the histograms indicate the differences between treatments according to the Fisher's LSD test at P<0.05.

However, a decrease in frequency and intensity was observed with increasing salinity levels (**Figure 3**). Thus, non-stressed plants had a higher colonization frequency than salt-stressed plants. These results could be explained by an inhibition of germination and development of propagules such as spores and hyphae [23]. This could be due to the negative effects of NaCl on plant tissues and AMF [24] and / or on the establishment of symbiotic associations [25]. These results show that the different response of the plant to inoculation with AMF could be related to host specificity.

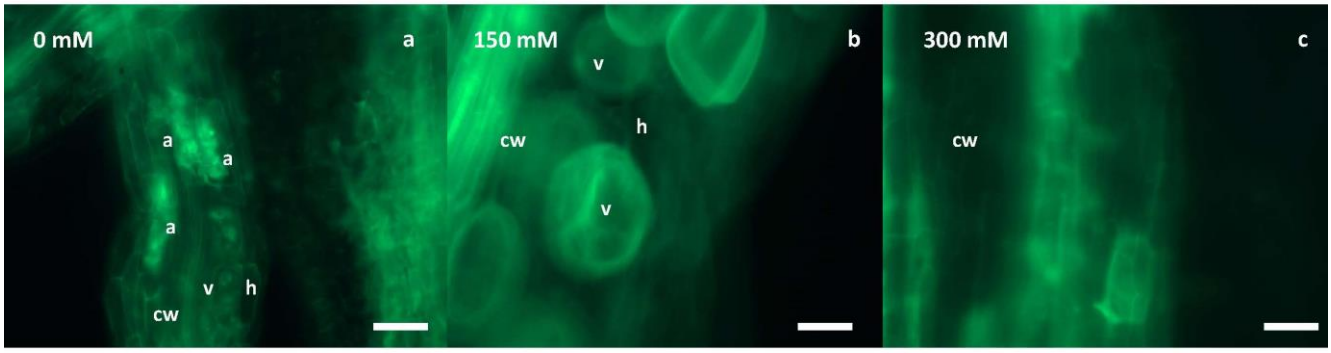

Figure 3: Effect of NaCl on mycorrhization of *C. glauca* (a) 0, (b) 150, (c) 300 mM. The notations in figures represent a: arbuscles, h: intra-root hyphae, v: vesicles, cw: cell wall bars = 100 µM.

3.3. The impact of inoculation with AMF on chlorophyll content and relative water content in C. glauca plants under salt stress conditions

The results show a decrease in chlorophyll content with the salinity level increases. The total chlorophyll content of plants inoculated with *R. fasciculatus* and *R. aggregatum* was significantly higher compared to control plants at all concentrations tested (**Figure 4**).

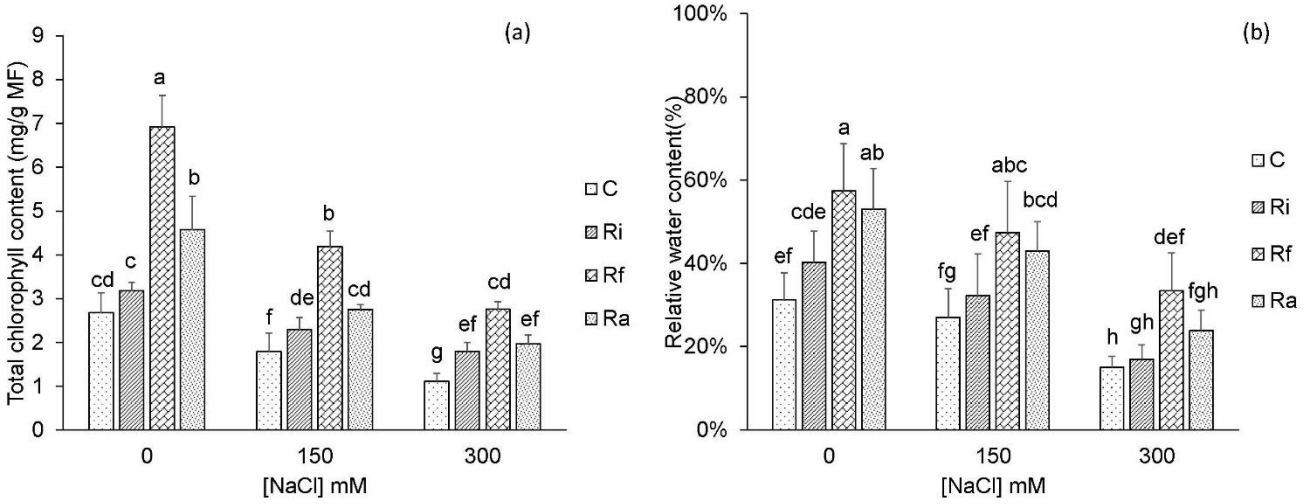

Figure 4: Chlorophyll content (a) and relative moisture content (b) of *C. glauca* after 4 months of greenhouse cultivation under salt stress. C: Control, Ri*: Rhizophagus irregularis*, Ra: *Rhizophagus aggregatum*, Rf: *Rhizophagus fasciculatus*. The letters indexing the histograms indicate the differences between treatments based on Fisher's LSD test at P<0.05.

Inoculation with *R. irregularis* did not improve chlorophyll content in the absence of NaCl. However, it is significant in the presence of NaCl. The relative content also decreased with increasing salinity levels. Inoculation with *R. fasciculatus* and *R. aggregatum* significantly increased the water content compared to the control plants. However, inoculation with *R. irregularis* did not improve the relative water content at all concentrations tested. The increase in chlorophyll content through inoculation with AMF under salt stress conditions has been described by several authors [26-10-27]. However, the presence of salt had a negative effect on the chlorophyll content and relative water content of the plants. This could be explained by the fact that Na^+ leads to the suppression of enzymes involved in the synthesis of photosynthetic pigments and the reduction of nutrient uptake such as Mg^{2+} necessary for chlorophyll biosynthesis [20-28] but also that the presence of salt reduces the water potential of the plant. The work of Jorge et al. [29] revealed that *C. glauca* plants can tolerate high levels of salinity by altering the levels of certain neutral sugars, proline and ornithine. Osmoprotective molecules such as L-Proline play an

important role in the selection of Casuarina / Frankia adapted to the saline zone [30]. Jorge et *al* [31] and Graça et *al* [32] suggest that tolerance to saline stress in Casuarina is related to maintaining proteome stability and triggering antioxidant defense mechanisms.

3.4. Impact of inoculation on N, C, Pi, Na^+ and K^+ ion content in C. glauca under saline stress conditions

Inoculation with *R. fasciculatus* significantly increased the total nitrogen content of *C. glauca* plants at all concentrations compared to control plants. Total carbon content was not improved by inoculation with AMF in the presence and absence of NaCl. The total phosphorus content of the aerial parts was significantly increased by inoculation with *R. fasciculatus* and *R. aggregatum* in the presence of NaCl. The plants generally have similar Na^+ contents. However, high levels of K^+ ion were found in plants inoculated with the fungus *R. fasciculaus* (Table 2).

Table 2. Effect of AMF inoculation on the chemical content of aerial parts in *C. glauca* under salt stress conditions.

Concentration	Treatments	Total Nitrogen	Total Carbon	Total phosphorus	Na^+	K^+
		%	%	g/Kg	g/Kg	g/Kg
0 Mm	C	1.18 bcd	45.33 bcd	1.27 def	3.07 f	9.91 d
	Ri	1.03 d	44.79 cd	1.40 bcd	4.87 cdef	9.62 d
	Rf	1.43 ab	46.30 ab	1.71 a	3.43 ef	10.95 bcd
	Ra	1.20 bcd	45.69 abc	1.36 cde	4.28 def	12.53 a
150 Mm	C	1.08 cd	45.70 abc	1.25 def	7.49 ab	10.80 bcd
	Ri	1.09 cd	44.53 cd	1.51 bc	5.23 cde	10.11 cd
	Rf	1.39 ab	45.36 abcd	1.50 bc	5.91 bcd	12.70 a
	Ra	1.27 abcd	46.13 ab	1.19 f	6.83 abc	11.58 abc
300 Mm	C	1.21 bcd	46.58 a	1.22 ef	7.47 ab	10.21 cd
	Ri	1.36 abc	44.84 cd	0.99 g	6.43 abc	9.89 d
	Rf	1.52 a	45.33 bcd	1.52 bc	8.46 a	12.08 ab
	Ra	1.17 bcd	44.35 d	1.54 b	6.46 abc	10.10 cd

C: Control, Ri*: Rhizophagus irregularis*, Ra: *Rhizophagus aggregatum*, Rf: *Rhizophagus fasciculatus*. The letters indicate the differences between treatments based on Fisher's LSD test at P<0.05

Inoculation with AMF did not increase the carbon content in *C. glauca* plants compared to control plants. This could be explained by the fact that symbiosis requires energy and elements from the plant's metabolism such as C that the fungus cannot produce on its own and which is necessary for the proper functioning of the fungus [33]. The inoculation did not reduce Na^+ levels compared to the control plants. This could be due to a mechanism of plant adaptation through compartmentalization of Na^+ ions within vacuoles. Thus *C. glauca* stocks a part of the NaCl in the aerial parts for order to increase its water potential. The selective absorption of Na^+ and Cl^- ions allows the protection of photosynthetic tissues [34-35]. This could increase relative water content and metabolic activities in host plants. This could increase relative water content and metabolic activities in host plants. Fan et *al* [36] attributed tolerance to saline stress in *C. equisetifolia* to an increase in the Na^+/K^+ ratio, upregulation of the Na^+/H^+ (NHX) anti-carrier genes in the roots and increased antioxidant activity.

In addition, higher K^+ contents were noted in plants inoculated with *R. fasciculatus* compared to control plants. The K^+ ion plays an osmoregulatory role in order to balance the concentration of the cytoplasm

and vacuole [37]. Under saline stress conditions, Na^+ moves through plant cells and reaches toxic levels, disrupting enzymes and cell function [38]. To avoid negative effects on plant cells, excess Na^+ must be moved out of the cytoplasm and compartmentalized in the vacuole. Thus, during saline stress, variations in proton gradients are generated by H^+ membrane pumps, which are crucial for the maintenance of cytoplasmic homeostasis [37].

Conclusion

In the presence of high concentrations of NaCl, improved growth was noted in plants inoculated with *R. fasciculatus*. Higher P, N and relative water contents were noted in plants inoculated with *R. fasciculatus*. These plants also have higher chlorophyll levels and absorb more K^+ ions in order to maintain their water potential. These results suggest the integration of AMF in the improvement of plant tolerance in saline soil rehabilitation programs. However, our study was performed under relatively controlled (net shaded greenhouse) and it would be important now to repeat the experiment under natural (uncontrolled) environmental conditions to confirm these results.

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