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RESEARCH PAPER

Selection of arbuscular mycorrhizal fungal strains to improve *Casuarina equisetifolia* L. and *Casuarina glauca* Sieb. tolerance to salinity

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Abstract

• ***Key message*** **Selection of the best salt-tolerant combination of *Casuarina* sp. and arbuscular mycorrhizal fungi (AMF) is one of the key criteria for successful setup of saline land rehabilitation program.**

• ***Context*** Land salinization is a serious problem worldwide that mainly leads to soil degradation and reduces crop productivity. These degraded areas could be rehabilitated by planting salt-tolerant species like *Casuarina glauca* Sieb. and *Casuarina equisetifolia* L. These are pioneer plants, able to form symbiotic associations with arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (EMF), and nitrogen-fixing bacteria.

• ***Aims*** The aim of this study was to select the highest salt-tolerant combination of *Casuarina*/AMF that can be used for the rehabilitation of lands degraded by salinity.

• ***Methods*** *C. equisetifolia* and *C. glauca* were grown in sandy sterile soil in the greenhouse and inoculated separately with *Rhizophagus fasciculatus* (Thaxt.) C. Walker & A. Schüßler, *Rhizophagus aggregatus* (N.C. Schenck & G.S. Sm.) C. Walker, and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler. After confirming the establishment of a symbiosis, the plants were watered with gradually increasing concentrations of saline solution. After harvest, size and biomass of the seedlings, root colonization by AMF, and AMF metabolic activities were evaluated.

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Contribution of the co-authors PD, MN, and ND carried out the experiments, analysis, and interpreting of the results and wrote the manuscript;
VH, DF, DD, SS, and LL contributed in designing and interpreting the results;
ND, DN, SS, VH, LL, and AC carried out the correction of the paper and supervising the work.

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• ***Results*** A larger growth was obtained in the two species when the individuals were inoculated with *R. fasciculatus*. Root colonization rates did not differ among fungal species, but fungal metabolic activities were higher in mycorrhizal roots of *C. glauca* plants inoculated with *R. fasciculatus*.
• ***Conclusion*** Among the three mycorrhizal fungi, *R. fasciculatus* was more efficient in association with *Casuarinaceae* species under salt stress. Our results suggest that selection of appropriate fungal strains is crucial to improve plant performance in saline soils.

Keywords Salinity · Arbuscular mycorrhizal fungi · *C. glauca* · *C. equisetifolia* · Rehabilitation · Salt-affected land

1 Introduction

Land salinization is among the major limiting factors for crop productivity as it leads to a dramatic loss of arable lands worldwide (Al-Karaki et al. 2001; Tester and Davenport 2003; Giri et al. 2003). This problem has become a global concern and is expected to increase due to the effects of climate change (Roy et al. 2014). Rehabilitation of salinized lands is a priority to improve food security in semi-arid countries like Senegal. One of the environmentally friendly strategies to tackle land salinization is revegetation using salt-tolerant species. *Casuarina equisetifolia* and *C. glauca* are able to grow in salty sandy soils, where they prevent the removal of the surface layer by reducing erosion and water runoff while promoting its infiltration (Ngom et al. 2016). These species originate in Australia and the Pacific Islands and are often used in agroforestry as windbreaks, to stabilize coastal dunes, and for the rehabilitation of degraded soils (National Research Council 1984). Their economic importance is mainly related to the density of their wood, which makes it useful as fuelwood, as well as to their ability to improve the amount of nitrogen in the soil. In Senegal, *C. equisetifolia* has been planted on 180 km of the northern Atlantic fringe between Dakar and St. Louis to stabilize sand dunes. This green barrier protects farming basins collectively called "Niayes" (Tamba 2000), where vegetables are cultivated.

Casuarinaceae plants are considered as pioneer species as their root system can adapt to several soil nutrient deficiencies. Indeed, in very poor soils lacking microorganisms, they can develop nonsymbiotic cluster roots which will help phosphorus uptake (Diem et al. 2000; Lambers et al. 2006). They can also form root nodules in association with the nitrogen-fixing actinobacteria *Frankia*, which helps them to survive even in nutrient-poor soils thanks to biological nitrogen fixation. They can also develop a symbiotic association with arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EM), which enhance plant water absorption and promote the uptake of nutrients such as phosphorus (Sayed 2011; Smith et al. 2011; Diagne et al. 2013) and nitrogen (Duro et al. 2015). Inoculation with salt-tolerant AMF can improve plant salinity tolerance (Evelin et al. 2009; Campanelli et al. 2012; Hanin et al. 2016) and enhance plant growth and yield (Kaya et al. 2009; Porras-Soriano et al. 2009; Wu et al. 2010; Alqarawi et al. 2014), nutrient acquisition (Giri and Mukerji 2004; Diouf et al. 2005; Porras-Soriano et al. 2009; Wu et al. 2010; Krishnamoorthy et al. 2016), chlorophyll content (Feng et al. 2002; Giri and Mukerji 2004; Kaya et al. 2009), and proline concentration (Diouf et al. 2005; Kaya et al. 2009). However, salt stress interferes with the germination and growth of fungal hyphae (Wu et al. 2010; Shekoofeh et al. 2012). Barrow et al. (2001) and Carvalho et al. (2001) also reported that the number of AMF spores decreases with an increase in soil salinity. Hence, selection of the appropriate AMF that increases plant performance in saline conditions is important for the success of rehabilitation of lands affected by salinity.

In this study we aim (i) to analyze the effects of inoculation separately with three selected AMF strains *Rhizophagus fasciculatus*, *Rhizophagus aggregatus*, and *Rhizophagus intraradices* in *C. glauca* and *C. equisetifolia* on tolerance to salinity and (ii) to understand the mechanisms involved in this salt tolerance by assessing fungal metabolic activity, root colonization, and chlorophyll and proline concentrations under salt stress.

2 Material and methods

2.1 Plant material and AMF strains

C. glauca seeds (seed lot15, 934, ref. 086-5929) were collected at the Myall Lakes National Park in Australia and purchased from the Australian Tree Seed Centre (ATSC, CSIRO). *C. equisetifolia* seeds (seed lot SN/2011/0014/D) were collected in Louga in northwestern Senegal and were provided by the National Tree Seed Program (PRONASEF).

AMF strains tested for their ability to tolerate and improve the salt tolerance of plants were selected based on the results of previous studies conducted in our lab (Diagne et al. 2014). The following arbuscular mycorrhizal fungi were used to inoculate *C. glauca* and *C. equisetifolia*: *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler strain DAOM197198 isolated in Quebec, *Rhizophagus aggregatus* (N.C. Schenck & G.S. Sm.) C. Walker strain DAOM2277128 isolated in Burkina Faso, and

Rhizophagus fasciculatus (Thaxt.) C. Walker & A. Schüßler strain DAOM227130 isolated in Quebec (Schüßler and Walker 2010). AMF inoculum was generated in a greenhouse using *Zea mays* plants grown in the presence of AMF in pots containing 1.5 kg of sandy soil previously sterilized at 120 °C for 2 h. The soil used for the study was collected at Sangalkam in Senegal (14°46′52″ N, 17°13′40″ W) and the physicochemical characteristics were as follows: pH (H_2O) 6.5, clay 3.6%, fine silt 7.4%, fine sand 36.6%, coarse sand 21.55%, total carbon 0.54%, total nitrogen 0.06%, C/N 8.5, total phosphorus 39 mg kg^{-1}, and soluble phosphorus 4.8 mg kg^{-1} (Diouf et al. 2005). It was used in this experiment because of its nutrient poverty. For each AMF, the inoculum consisted of a mixture of spores and root fragments. The number of spores was evaluated according to the method of Gerdemann and Nicolson (1963).

2.2 Seedling growth, experimental design, and salt stress treatment

Seeds were fumigated with carbon disulfide by CSIRO/ATSC (Australian Tree Seed Centre) and kept in a cool, air-tight container before use. *C. equisetifolia* and *C. glauca* seeds were germinated in 23.5 cm × 9.5 cm polyethylene bags containing soil. Since *Casuarinaceae* can develop tripartite symbiosis, soils were previously sterilized by autoclave (120 °C for 20 min) to prevent colonization by native AM/ectomycorrhizal fungi and/or *Frankia* and thus separate the effects of the inoculated fungi. One-month-old seedlings were transferred into pots (25 cm × 12 cm × 50 cm) filled with sterilized soil and kept in a nethouse at ~30 °C at the LCM-Laboratoire Commun de Microbiologie IRD/ISRA/UCAD, Bel Air experimental station (certified ISO 9001: 2015) (14°44′ N–17°30′ W, Dakar, Senegal).

Seedlings were arranged in a randomized completed block design including three factors: plant species (*C. equisetifolia* and *C. glauca*), AMF inoculation (control, Rf, Ra, Ri), and salinity (0, 150, 300 mM NaCl), 24 treatments (2 × 4 × 3) with ten replications per treatment.

Seedlings were inoculated with 20 g of each AMF inoculum containing an average of 32.4 spores/g of soil. The most probable number (MPN) of *R. fasciculatus* (Rf), *R. aggregatus* (Ra) and *R. intraradices* (Ri) were respectively 1210 [565; 2580], 1635 [763; 3483], and 1347 [629; 2870]. Calculation of MPN values were made as described by Sieverding (1991) according to Fisher and Yates (1948) and expressed per 100 g of dried soil.

During the formation of mycorrhizae, *C. equisetifolia* and *C. glauca* plants were watered daily with pure water. Watering was done by hand using a graduated pot filled with the same amount of water for each plant. One month after AMF inoculation, salt stress was gradually applied, twice a week. Then, a weekly increase in NaCl concentration was made to avoid an osmotic shock. After 3 weeks of acclimation, control plants were watered with 0 mM, and stressed plants with 150 and 300 mM (n = 10 plants per treatment). Throughout the duration of the experiment, the salinity levels of soils were checked regularly using a salinometer (Extech portable salinity refractometer, sigma, ref. Z741839-1EA) to ascertain NaCl concentrations. Exceeded NaCl concentration was corrected by adding water up to the required one. After 4 months of salt stress, growth and biochemical parameters, i.e., plant shoot length, total biomass, chlorophyll, leaf proline concentrations, AMF colonization rate, and fungal metabolic activity were measured.

2.3 Shoot growth and total dry biomass measurement

Shoot length was measured first using a graduated scale (ruler graduate). The plants were then harvested and the shoot and root systems were separated with a chisel, washed in deionized water, and dried at 70 °C for 72 h. The dried biomass of each sample was evaluated separately.

2.4 Chlorophyll and proline concentration determination

The chlorophyll concentration was evaluated using 100 mg of ground fresh shoots according to Makeen et al. (2007). Absorbance of chlorophyll a and b was measured using a UV-1800 spectrophotometer at $\lambda = 663$ and 645 nm, respectively. Total chlorophyll concentration was calculated using the following formula (μg/MF):

$$(\mathrm{Chl}) = 8.02 \times \mathrm{DO}\,(663) + 20.2 \times \mathrm{DO}\,(645)\; V/M$$

where V is the volume of the total extract, M the mass of the fresh material, and DO the optical density (nm).

To determine the concentration of proline in the plants, 100 mg of fresh shoots were ground and proline concentrations were quantified by spectrophotometry (520 nm) according to Monneveux and Nemmar (1986).

2.5 Root colonization measurement

To visualize fungal structures, root fragments were washed under tap water and cleared in 10% KOH for 1 h at 90 °C then stained with Uvitex2B, as described by Diagne et al. (2011). For each condition, 10 plants were considered and 100 root fragments of about 2 cm were observed for each plant. The intensity and frequency of mycorrhization (%) were recorded according to Trouvelot et al. (1986). Roots of control plants were checked to make sure that no contamination was present.

2.6 Fungal metabolic activity

To assess the fungal metabolism, we used nitro blue tetrazolium (NBT) to reveal metabolically active arbuscular mycorrhizal (AM) structures. NBT reacts with succinate dehydrogenase (SDH), a tricarboxylic acid cycle enzyme in AMF, resulting in insoluble formazan, which can be clearly distinguished in roots (Vierheilig et al. 2001, 2005). The presence of blue formazan in stained roots points to SDH activity in fungal mitochondria (Kough et al. 1987). Senescence of arbuscular mycorrhizal (AM) structures was evaluated using *diaminobenzidine coloration* (*DAB*) as described by Vierheilig et al. (2005). To analyze the metabolic activity of AMF strains, percentage of stained roots were recorded for the most salt-tolerant strain, *R. fasciculatus*, and compared with the percentage of stained roots observed for the most sensitive strain, *R. intraradices*. After staining, roots were visualized under a light microscope.

2.7 Statistical analysis

Statistical analysis was performed using the 2017 version of XLstat software. For the percentage values (i.e., DAB test, SDH test, frequency, and intensity of colonization), the data were arcsin square transformed before statistical analysis (Saint-Etienne et al. 2006). Normality of all data sets was assessed using the Shapiro–Wilk test. Variable following normal distribution (i.e., DAB test, SDH test, frequency, and intensity of colonization) were analyzed with ANOVA to compare accuracy in the different conditions, and Fisher's LSD test was used for post hoc comparisons with a significance threshold set at 0.05. For data sets which do not follow a normal distribution (i.e., height, biomass, chlorophyll and proline concentration), a non-parametric Kruskal–Wallis test followed by the Steel–Critchlow–Fligner multiple comparison test was used with a significance threshold set at 0.05.

2.7.1 Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

3 Results

As a first step for selecting performing AMF inoculants, we checked non-inoculated axenic soil on the two plant species: none of the four features, i.e., nodules, ectomycorrhizal, AMF, or clustered roots, were observed, and results obtained throughout the study are thus due to our selected AMF inoculants. According to statistical analyses, significant effects of AMF inoculation and salinity were recorded for height, biomass, metabolic activities, and chlorophyll and proline concentration studied, whereas marginal effects were observed between plant species and no significant interactions among factors were observed (Table 1).

3.1 Impact of each AMF strain on *Casuarina* growth and total biomass under saline conditions

After 4 months of growing in the nethouse, the growth performance of *C. glauca* was higher than those of *C. equisetifolia* plants whatever the culture condition (Table 2). Increasing NaCl concentrations decreased growth in both control and most of AMF-inoculated plants of both plant species. Only the Ra strain significantly improved *C. equisetifolia* shoot growth at 0 and 150 mM NaCl compared to non-inoculated plants (Table 2), while the biomass was however not significantly improved by AMF strains in the presence of NaCl according to the Steel–Critchlow–Fligner test ($P < 0.05$). For *C. equisetifolia* plants, the inoculation improved neither height nor biomass at 300 mM compared to control non-inoculated plants. In *C. glauca* plants, the Rf strain significantly improved both plant height and total biomass in saline conditions compared to controls (Table 2). Our study showed that in saline conditions, a better growth was observed in *C. glauca* plants inoculated with Rf strain.

3.2 Salt stress affects root colonization by AMF in *C. equisetifolia* and *C. glauca*

In both plant species, the intensity and frequency of mycorrhizal colonization decreased significantly with increasing salinity (Fig. 1). Between 0 and 300 mM NaCl, salt stress caused a significant decline (respectively, 10, 12.1, and 11.8%) in the intensity of Ra, Rf, and Ri colonization of *C. equisetifolia* roots. Similar observation was also recorded for *C. glauca* roots with a significant reduction in the intensity of Ra, Rf, and Ri colonization (respectively 13.2, 14.8, and 15.2%). In *C. glauca*, a decrease of 38.8, 45.8, and 57.3%, respectively, in the frequency of Ra, Rf, and Ri was observed at 300 mM compared to plants at 0 mM NaCl. However, at each salt concentration, no difference in intensity and frequency was observed whatever the AMF strains in either *C. equisetifolia* or *C. glauca* (Fig. 1). A comparison of the intensity and frequency of mycorrhizal colonization between both species was observed and showed no significant differences in terms of intensity. However, the frequency of mycorrhizal colonization was significantly higher in *C. glauca* plants inoculated with Rf and Ra at 150 mM NaCl compared with *C. equisetifolia*.

Table 1 Statistical analyses showing the effect of three factors, i.e., plant species, arbuscular mycorhizal fungi (AMF) inoculation, and salinity and their interactions with morphological (height, biomass), physiological (intensity and frequency of root colonization by AMF), and biochemical (fungal metabolic activity, chlorophyll and proline content) data collected on plants sampled 4 months after growth in saline stress conditions. SDH and DAB, intensity, and frequency of AMF colonization data were first transformed with an arcsin square function as described (Saint-Etienne et al. 2006), and then, an ANOVA and a post hoc Fisher's LSD test were performed. Height, biomass, and chlorophyll and proline concentrations were analyzed using the Kruskal–Wallis test followed by a Steel–Critchlow–Fligner test. Values were considered to be statistically significant at $p < 0.05$. Fungal metabolic activity was determined using the succinate dehydrogenase activity test (SDH) to reveal active AMF and the diaminobenzidine test (DAB) to reveal senescent AMF structures (Vierheilig et al. 2005)

	Growth		AMF colonization		Fungal metabolic activity[1]			
Factor tested	Height	Biomass	Intensity	Frequency	SDH	DAB	Chlorophyll	Proline
AMF	***	***	ns	ns	***	*	***	***
Salinity	***	***	***	***	*	*	***	***
Species	.	.	ns	.	ns	ns	.	ns
AMF × salinity	ns	ns	ns	ns	ns	ns	ns	.
AMF × species	ns	ns	ns	ns	ns	ns	ns	ns
Salinity × species	ns	ns	.	ns	ns	ns	ns	.
AMF × salinity × species	ns	ns	ns	ns	ns	ns	ns	ns

ns not significant

. ≤ 0.1, ∗$p \leq 0.05$, ∗∗$p \leq 0.01$, ∗∗∗$p \leq 0.001$

3.3 Metabolic activity of *R. fasciculatus* and *R. intraradices* strains in saline conditions

As the intensity of colonization did not explain the growth differences observed in the various plant/AMF combinations tested, we analyzed the metabolic activity of the most contrasted AMF strains regarding salt sensitivity, i.e., Rf and Ri, to assess the effectiveness of the symbiosis under saline conditions. The raise in salinity increased the number of senescent structures and decreased the number of active fungal structures for both Rf- and Ri-inoculated plants whatever the plant species as revealed by succinate dehydrogenase (SDH) activity and diaminobenzidine (DAB) coloration test (Fig. 2). In both plant species, more active fungal and fewer senescent structures were observed in plants inoculated with Rf than in plants inoculated with Ri in saline conditions (Fig. 2). At 150 mM NaCl, *C. equisetifolia* and *C. glauca* plants inoculated with Rf showed 38 and 53% respectively

Table 2 Changes in mean (± SD) values ($n = 10$) of height and biomass of *Casuarina equisetifolia* and *Casuarina glauca* plants inoculated with three different AMF strains and cultivated 4 months with 0, 150, or 300 mM NaCl. Values followed by the same letter are not significantly different at $p < 0.05$ according to the Steel–Critchlow–Fligner test ("a" = lowest values and "b" = highest values). Data presented are representative of two independent experiments. Ra *Rhizophagus aggregatus*, Rf *Rhizophagus fasciculatus*, Ri *Rhizophagus intraradices*. Lowercase letters (a-b) indicate significant differences regarding AMF inoculation. Uppercase letters (A-B) indicate significant differences regarding NaCl concentrations. Uppercase letters in parentheses (A)-(B) indicate significant differences regarding species

	Height (cm)			Biomass (g)		
[NaCl]	0 mM	150 mM	300 mM	0 mM	150 mM	300 mM
Casuarina equisetifolia						
Control	$42.08 \pm 12.25^{a\text{-}B\ (A)}$	$36.51 \pm 5.37^{a\text{-}AB\ (A)}$	$35.94 \pm 6.31^{a\text{-}A\ (A)}$	$3.62 \pm 0.76^{a\text{-}B\ (A)}$	$1.88 \pm 0.68^{a\text{-}A\ (A)}$	$1.58 \pm 0.22^{a\text{-}A\ (A)}$
Ra	$57.92 \pm 9.92^{b\text{-}B\ (A)}$	$46.99 \pm 5.36^{b\text{-}A\ (A)}$	$40.17 \pm 9.61^{a\text{-}A\ (A)}$	$4.48 \pm 1.24^{ab\text{-}B\ (A)}$	$2.62 \pm 0.65^{a\text{-}AB\ (A)}$	$1.66 \pm 0.69^{a\text{-}A\ (A)}$
Rf	$59.83 \pm 4.39^{b\text{-}B\ (A)}$	$43.73 \pm 7.02^{b\text{-}A\ (A)}$	$41.17 \pm 7.17^{a\text{-}A\ (A)}$	$5.08 \pm 1.03^{b\text{-}B\ (A)}$	$2.73 \pm 0.68^{a\text{-}A\ (A)}$	$1.78 \pm 0.47^{a\text{-}A\ (A)}$
Ri	$52.81 \pm 15.96^{b\text{-}B\ (A)}$	$37.79 \pm 5.07^{a\text{-}A\ (A)}$	$36.80 \pm 5.89^{a\text{-}A\ (A)}$	$4.38 \pm 1.39^{ab\text{-}B\ (A)}$	$2.31 \pm 0.55^{a\text{-}A\ (A)}$	$1.61 \pm 0.87^{a\text{-}A\ (A)}$
Casuarina glauca						
Control	$57.62 \pm 7.13^{a\text{-}B\ (B)}$	$53.88 \pm 8.32^{a\text{-}AB\ (B)}$	$48.50 \pm 7.50^{a\text{-}A\ (B)}$	$3.75 \pm 0.70^{b\text{-}B\ (A)}$	$2.81 \pm 0.37^{a\text{-}A\ (B)}$	$1.82 \pm 0.62^{a\text{-}A\ (A)}$
Ra	$64.54 \pm 7.40^{ab\text{-}B\ (A)}$	$59.18 \pm 7.53^{ab\text{-}B\ (B)}$	$51.11 \pm 5.60^{ab\text{-}A\ (B)}$	$4.53 \pm 1.15^{ab\text{-}B\ (A)}$	$2.91 \pm 0.69^{a\text{-}A\ (A)}$	$2.42 \pm 0.58^{a\text{-}A\ (A)}$
Rf	$66.52 \pm 7.35^{b\text{-}B\ (B)}$	$63.82 \pm 6.55^{b\text{-}B\ (B)}$	$55.93 \pm 9.64^{b\text{-}A\ (B)}$	$4.86 \pm 1.15^{a\text{-}B\ (A)}$	$4.01 \pm 0.89^{b\text{-}AB\ (B)}$	$3.21 \pm 1.19^{b\text{-}A\ (B)}$
Ri	$63.69 \pm 7.29^{ab\text{-}B\ (A)}$	$53.66 \pm 7.41^{a\text{-}A\ (B)}$	$52.26 \pm 4.50^{ab\text{-}A\ (B)}$	$4.65 \pm 0.90^{ab\text{-}B\ (A)}$	$2.93 \pm 0.90^{a\text{-}A\ (A)}$	$2.29 \pm 0.74^{a\text{-}A\ (A)}$

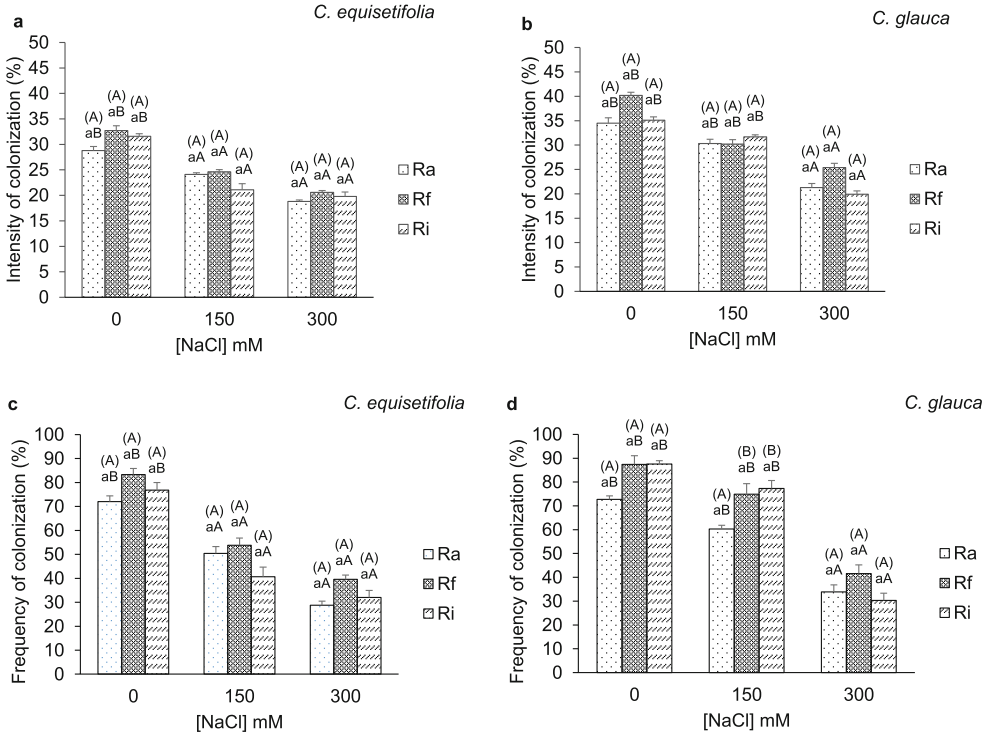

Fig. 1 Intensity (**a**, **b**) and frequency (**c**, **d**) of root colonization of *Casuarina equisetifolia* and *Casuarina glauca* plants inoculated with *Rhizophagus aggregatus* (Ra), *Rhizophagus fasciculatus* (Rf), or *Rhizophagus intraradices* (Ri) in the presence of 0, 150, or 300 mM of NaCl. Values are the mean of 10 plants. Standard deviations are displayed as bars, and bars sharing the same letter are not significantly different at $p < 0.05$ according to Fisher's LSD test ("a" = lowest values and "b" = highest values). Data presented are representative of two independent experiments. Lowercase letters (a-b) indicate significant differences regarding AMF inoculation. Uppercase letters (A-B) indicate significant differences regarding NaCl concentrations. Uppercase letters in parentheses (A)-(B) indicate significant differences regarding species

of active structures while plants inoculated with Ri showed only 21 and 29% respectively (Fig. 2). At 300 mM NaCl, a significant difference in senescent structures was observed in *C. equisetifolia* compared to *C. glauca.* More active structures were observed in *C. glauca* plants inoculated with Rf compared to *C. equisetifolia*.

3.4 Impact of AMF on chlorophyll and proline concentrations in salt stress conditions

Total chlorophyll concentrations decreased with increasing salt concentration in both plant species (Fig. 3). Whatever NaCl concentrations, inoculation with AMF Rf significantly increased the total chlorophyll concentrations in both species compared to those in non-inoculated plants (Fig. 3).

In parallel, the salt treatment resulted in an increase in the proline concentration in the shoots of both species (Fig. 4). In the presence of NaCl, plants inoculated with Rf showed higher significant concentration of proline than control plants (Fig. 4), and a significant difference was also observed between both 150 and 300 mM salt concentrations. Inoculation with Rf increased the proline concentration by 5 and 4.2% at 150 and 300 mM, respectively, in *C. equisetifolia* and by 6 and 7.2% in *C. glauca*. However, there was no significant difference in proline concentration between AMF treatments at 0 mM and controls in *C. equisetifolia* and *C. glauca* plants. A comparison between the proline and chlorophyll concentrations between the two species showed no significant differences with or without NaCl.

4 Discussion

In this study, we analyzed the impact of AMF inoculation on the salt tolerance of two *Casuarina* species. First, our study showed that *C. glauca* and *C. equisetifolia* plants were in general bigger and developed more biomass when AMF-inoculated. This confirmed previous results obtained by Duponnois et al. (2003). This improvement in growth could be explained by an increasing mineral nutrient content especially P and N favored by the fungus (Talaat and Shawky 2011). Previous results showed that *C. equisetifolia* and *C. glauca* can tolerate salt concentrations ranging from 0 up to 300 mM NaCl (Diagne et al. 2014; Ngom et al. 2016), even if salt stress affects their growth. Here we demonstrated that the inoculation of *C. glauca* and *C. equisetifolia* with Ra and Rf generally reduced the salt impact. Moreover, according to the plant species, AMF strains had a different effect. Indeed, only

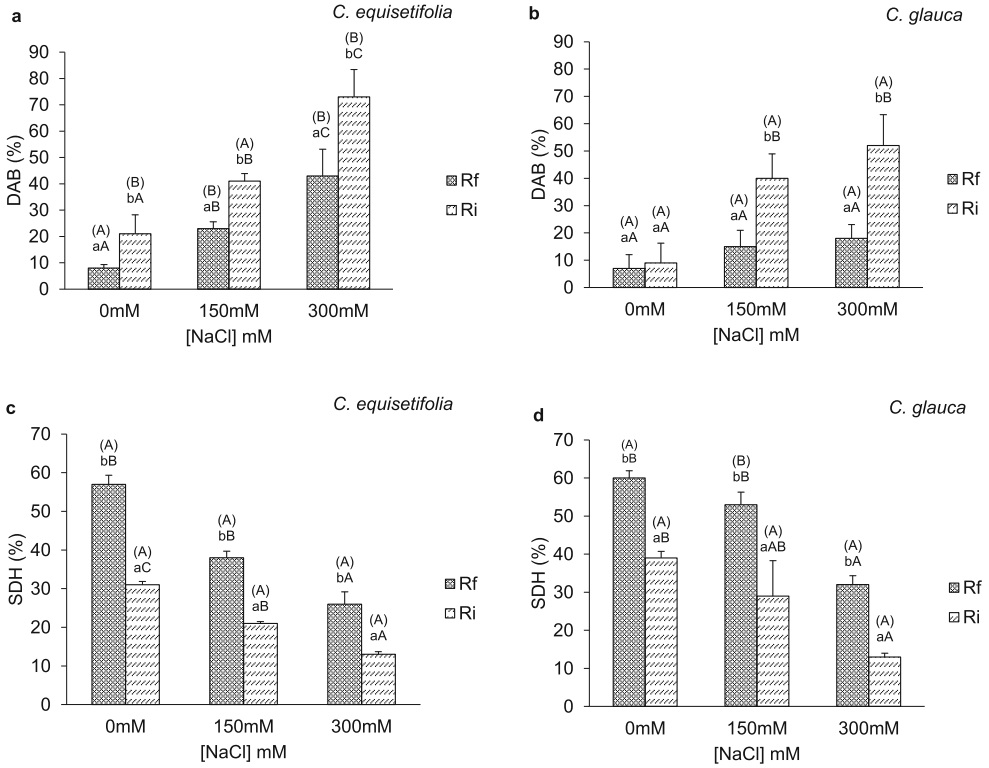

Fig. 2 Mean comparison of metabolic activity of arbuscular mycorrhizal fungi *C. equisetifolia* (**a**, **c**) and *C. glauca* (**b**, **d**) roots inoculated with *Rhizophagus fasciculatus* (Rf) or *Rhizophagus intraradices* (Ri) in the presence of 0, 150, or 300 mM of NaCl. Fungal metabolic activity was determined using the succinate dehydrogenase test (SDH) to reveal active AMF structures and the diaminobenzidine test (DAB) to reveal senescent AMF structures. Values are the mean of five plants. Standard deviations are displayed as bars, and bars sharing the same letter are not significantly different at $p < 0.05$ according to Fisher's LSD test ("a" = lowest values and "b" = highest values). Data presented are representative of two independent experiments. Lowercase letters (a-b) indicate significant differences regarding AMF inoculation. Uppercase letters (A-C) indicate significant differences regarding NaCl concentrations. Uppercase letters in parentheses (A)-(B) indicate significant differences regarding species

Rf was able to significantly improve both plant height and biomass in *C. glauca* in saline conditions (150 and 300 mM NaCl.), while in *C. equisetifolia*, Ra strain had a significant effect on the plant height at 150 mM. These results suggest that the effectiveness of the symbiosis in saline conditions depends on AMF associations. This was also observed by

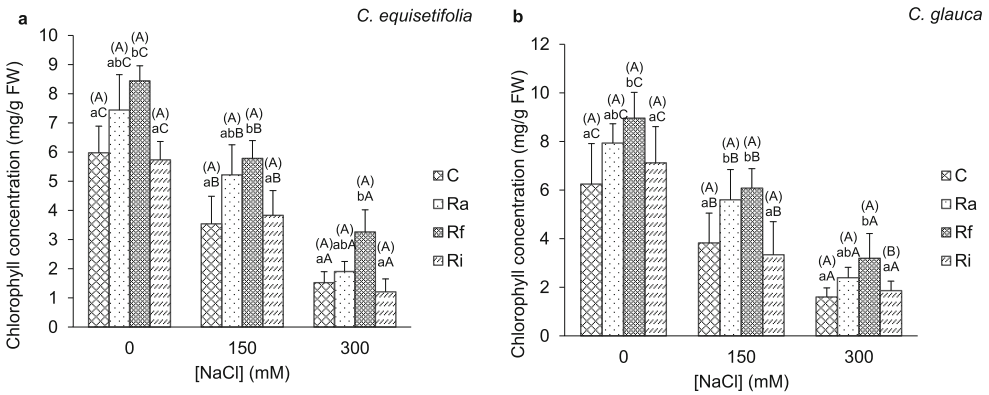

Fig. 3 Mean comparison of chlorophyll concentration in *C. equisetifolia* (**a**) and *C. glauca* (**b**) plants inoculated with *Rhizophagus aggregatus* (Ra), *Rhizophagus fasciculatus* (Rf), or *Rhizophagus intraradices* (Ri) in the presence of 0, 150, or 300 mM of NaCl. Values are the mean of five plants. Standard deviations are displayed as bars, and bars sharing the same letter are not significantly different at $p < 0.05$ according to the Steel–Critchlow–Fligner test ("a" = lowest values and "b" = highest values). Data presented are representative of two independent experiments. Lowercase letters (a-b) indicate significant differences regarding AMF inoculation. Uppercase letters (A-C) indicate significant differences regarding NaCl concentrations. Uppercase letters in parentheses (A)-(B) indicate significant differences regarding species

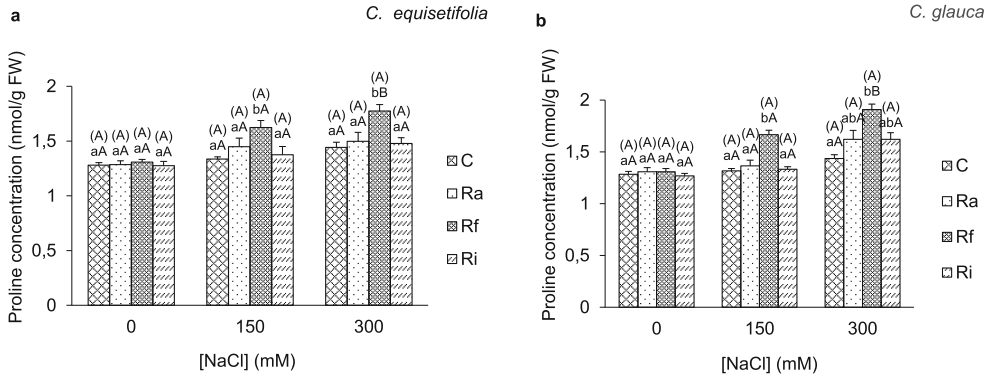

Fig. 4 Mean comparison of proline concentration in *C. equisetifolia* (**a**) and *C. glauca* (**b**) plants inoculated with *Rhizophagus aggregatus* (Ra), *Rhizophagus fasciculatus* (Rf), or *Rhizophagus intraradices* (Ri) in the presence of 0, 150, or 300 mM of NaCl. Values are the mean of five plants. Standard deviations are displayed as bars. Bars sharing the same letter are not significantly different at $p < 0.05$ according to the Steel–Critchlow–Fligner test ("a" = lowest values and "b" = highest values). Data presented are representative of two independent experiments. Lowercase letters (a-b) indicate significant differences regarding AMF inoculation. Uppercase letters (A-C) indicate significant differences regarding NaCl concentrations. Uppercase letters in parentheses (A)-(B) indicate significant differences regarding species

Zou and Wu (2011) who showed that *Glomus mosseae* has more potential to enhance salt tolerance of *Citrus* seedlings than *Glomus versiforme*. Positive effects of AMF inoculation on plant growth under salt stress have previously been described in several species (reviewed in Hanin et al. 2016). The benefits of microsymbionts such as *Glomus* or *Gigaspora* in saline conditions vary depending on the host species (Diouf et al. 2005; Hanin et al. 2016).

The salinity tolerance of many plants can be improved by symbiotic association with AMF by improving nutrient acquisition (especially phosphorus), plant growth, chlorophyll concentrations, and yield (for a review, see Hanin et al. 2016). This is in agreement with our results obtained for chlorophyll concentrations. Under saline conditions, chlorophyll concentrations were significantly increased in both *C. glauca* and *C. equisetifolia* plants inoculated with Rf, as compared to controls. This result suggests that under saline conditions, endomycorrhization increased photosynthetic potential and allows *Casuarina* inoculated plants to grow better than control plants. Higher proline accumulation under salt stress has previously been described in legumes such as *Acacia auriculiformis* and *Acacia mangium* inoculated with mycorrhizal fungi (Diouf et al. 2005) and in non-mycorrhizal *C. equisetifolia* seedlings (Tani and Sasakawa 2006). Our results indicated that inoculation with Rf increased proline concentration in *C. glauca* and *C. equisetifolia*. These findings indicate that, in addition to a better potential for photosynthesis, AMF inoculation also improved proline accumulation. Proline is the most common osmolyte accumulated in response to salt and drought stress in plants and allows the adjustment of the osmotic pressure to maintain cell homeostasis (Watanabe et al. 2000; Tani and Sasakawa 2003). Moreover, proline accumulation provides protection against stress by contributing to ROS detoxification, protection of membrane integrity, and enzyme/protein stabilization (Hayat et al. 2012). In the presence of NaCl, the AMF inoculation could set up mechanisms that trigger a better proline synthesis and thus a better tolerance of the plant to salt stress (Abeer et al. 2016).

These positive effects of AMF inoculation (i.e., chlorophyll concentration, proline accumulation) were more pronounced in plants inoculated with Rf, suggesting a more efficient symbiosis with this AMF strain under salt stress compared to the other two fungi species.

Protection against salt stress is one of several factors that can contribute to increased growth and chlorophyll and proline concentrations in inoculated plants. Studies have reported that mycorrhizal colonization can improve water absorption capacity of plants and reduce the uptake of Cl^- ions while preventing Na^+ translocation to shoot tissues under salinity (Hanin et al. 2016). This may lead to increased plant growth and subsequent dilution of toxic ion effect in mycorrhizal inoculated plants, in comparison to non-inoculated plants.

In the present study, salt stress significantly affected mycorrhizal colonization of *C. glauca* and *C. equisetifolia* plants by Ra, Rf, and Ri. These results are confirmed by our optical microscope images (supplemental fig. 1). This might be due to the negative effects of salinity on both the host plant and fungal growth (Giri et al. 2003; Evelin et al. 2009; Hanin et al. 2016) and/or on the establishment of arbuscular mycorrhiza (Sheng et al. 2008). Reduction of mycorrhizal root colonization under salt-stressed conditions has been observed in *Lycopersicon esculentum* (Al-Karaki 2000, 2006; Al-Karaki et al. 2001; Latef and Chaoxing 2011), *Capsicum annum* (Kaya et al. 2009), *Zea mays* (Sheng et al. 2008), *Olea europaea* (Porras-Soriano et al. 2009), *Citrus tangerine* (Wu et al. 2010), *Acacia auriculiformis*, and *A. mangium* (Diouf et al. 2005).

The impact of salinity on mycorrhizal root colonization varies with the AM fungus involved (Diouf et al. 2005; Porras-Soriano et al. 2009; Wu et al. 2010), suggesting that some strains are able to grow and therefore colonize the host plant in these stressful conditions.

This conclusion is supported by our results showing better metabolic activity of Rf in saline conditions compared to Ri. Indeed, for both *C. glauca* and *C. equisetifolia*, more active fungal (SDH test) and fewer senescent structures (DAB test) were observed in plants inoculated with Rf compared to those inoculated with Ri in all saline conditions. Similar observations were reported by Abdel-Fattah and Asrar (2012) on *Triticum aestivum* L. grown under saline conditions.

5 Conclusion

Our results indicate that inoculation with selected AMF Rf can improve the performance of *C. glauca* and *C. equisetifolia* under salt stress conditions. These results indicate a more efficient mycorrhizal symbiosis with Rf fungal strain that could improve the absorption of nutrients such as N and P and water absorption in salt-stressed conditions (Evelin et al. 2009; Beltrano et al. 2013).

Rf strain was the most efficient AMF in increasing the growth of *C. glauca* plants even at higher salt concentrations while no effect of inoculation was observed in *C. equisetifolia*. Our results thus suggest that *C. glauca* is more tolerant to salt stress than *C. equisetifolia* and that the selection of an appropriate AMF is an important criterion for improving salt stress tolerance. As the more performant combination regarding salt stress, *C. glauca* plants inoculated with Rf could be tested for their ability to improve the rehabilitation of saline soils especially in Senegal. However, in natural conditions, *Casuarina* roots exhibit an extraordinary developmental plasticity when nutrients are scarce: they are able to form mycorrhizae not only with AMF but also with ECM, they are able to develop nitrogen-fixing root nodules in symbiosis with the soil actinomycete *Frankia* (Péret et al. 2009), and they can even develop nonsymbiotic structures called cluster roots in soils severely deprived of phosphorus and phosphate (Diem et al. 2000). Interactions between these adaptations could strongly influence performance of AMF inoculation and also response to salt stress. Field experiments are therefore underway to confirm the effect of *R. fasciculatus* for salt tolerance of *C. glauca* in natural conditions. Moreover, according to Diagne et al. 2013, it would also be important to develop further studies aiming to consider the effect of the tripartite association between *Casuarina*, nitrogen-fixing bacteria, and mycorrhizal fungi (AM, EM) that could help to better understanding the behavior of *Casuarinaceae* in response to saline soils.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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