Endophyte Colonization Enhances Tolerance of *Casuarina Equisetifolia* to NaCl Stress by Modifying Osmoregulation, Antioxidant Enzymes, and Phytohormones

Feng Long

Fujian Agriculture and Forestry University

Anjie Liang

Fujian Agriculture and Forestry University

Jun Su

Fujian Agriculture and Forestry University

Zhiwei Chen

Fujian Agriculture and Forestry University

Tao Hong

Fujian Agriculture and Forestry University

Chengzhen Wu

Fujian Agriculture and Forestry University

Jian Li (✉ jianli@fafu.edu.cn)

Fujian Agriculture and Forestry University <https://orcid.org/0000-0003-0747-7525>

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Abstract

Background: Saline soils severely affect plant growth. Associations between endophytes and plants are known to significantly alter plant metabolism. This study reports the effects of a fungal endophyte species (*Botryosphaeria ramosa* ssp.) on osmoregulation, antioxidant enzymes, and the regulation of endogenous plant hormones in *Casuarina equisetifolia* under NaCl stress. *C. equisetifolia* plants, with and without *B. ramosa* ssp. colonization, were subjected to different levels of NaCl stress (0%, 5%, 10%, and 15%) for different amounts of time (0 d, 20 d, 40 d, and 60 d).

Results: Antioxidant enzymes, phytohormones, and nutritive elements in the leaves and roots were determined. The results showed that colonization of the roots by *B. ramosa* ssp. improved the growth rate and dry weight of salt-stressed plants. Moreover, *B. ramosa* ssp. colonization increased the activities of superoxide dismutase, catalase, and peroxide but decreased the hydrogen peroxide content in the branches of *C. equisetifolia* under salt stress. Meanwhile, compared with non-colonized plants, endophyte colonization reduced the abscisic acid and proline contents but increased the contents of auxin, zeatin, and gibberellins. Importantly, the nutrient elements in the roots and branches of colonized plants were significantly different from those in the roots and branches of non-colonized plants under saline conditions.

Conclusions: The results of this study showed that *B. ramosa* ssp. colonization can enhance the salt tolerance of *C. equisetifolia* by improving the antioxidant enzyme content, regulating the phytohormones, and adjusting proline accumulation under NaCl stress.

1. Background

Casuarina equisetifolia is one of the most important tree species for coastal protection due to its tolerance to salt, alkali, severe wind damage, and sand burying [1]. The planting area of *C. equisetifolia* in China has now reached 300,000 hectares and continues to grow. However, the heavy saline-alkaline land along the coast still presents a huge challenge for *C. equisetifolia* planting and has thus limited the expansion of the planting area. The heavy salt stress mainly limits *C. equisetifolia* growth by lowering the germination ratio and the nutritive element contents of the plant [2, 3].

Endophytes are a set of microorganisms that live within a plant for at least part of their life cycle without causing apparent disease symptoms. Endophytes also comprise an important microbe resource for enhancing host tolerance to salt stress [4]. It remains unclear mechanism that how endophytes manipulate and interact with host defenses. However, some researchers have shown that endophyte symbiosis involves regulating the mineral nutrients uptake and defensive metabolites exudation from plant roots, thereby maintaining the balance of phytohormones [5]. For instance, ectomycorrhizal fungi can enhance plant salt resistance by eliminating sodium ion and increasing mineral nutrients uptake [6]. In addition, arbuscular mycorrhizal fungi (AMF) can improve the growth of citrus and maintain the ionic dynamic balance [7]. Endophytes can also prevent plants from salt stress by altering the levels of

antioxidant enzymes, including those involved in the reactive oxygen species (ROS) scavenging system[8]. They can also regulate the Na^+/K^+ of the host plant [9]. All of these previous studies indicate that endophytes have great potential to reduce the high salt sensitivity of *C. equisetifolia*.

Soil salinity is a crucial abiotic stress that affects the growth and development of plants, and challenges both agriculture and forestry worldwide[10]. Due to climate change, salinity is expected to disturb the main plant metabolic process, as well as plant water and nutrient balance[10]. In response to salt stress, plants activate several signaling pathways to change their metabolism to maintain a defensive regime [11–12]. The balance of endogenous hormones, including abscisic acid (ABA), indoleacetic acid (IAA), and gibberellins (GAs), also plays an important role in the response to salt stress[13]. The toxicity of sodium can damage cellular organelles and lead to the production of reactive oxygen species[9]. However, the deleterious influences are weakened by cellular antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), and catalases (CAT) [14].

According to our previous study, two endophytes (*Aspergillus* sp. and *Phyllosticta* sp.), which were isolated from *C. equisetifolia*, can reduce allelopathy effects on seedlings by regulating the osmotic substances and the ROS scavenging system[15–16]. Thus, we hypothesized that endophyte colonization can improve *C. equisetifolia* growth under salt stress. To further investigate whether endophyte colonization can enhance the salt tolerance of *C. equisetifolia*, the endophytic fungus, *Botryosphaeria ramosa* ssp., which was isolated from *C. equisetifolia*, was used to infect the host plant seedlings under different salt stress conditions. Furthermore, the plant growth characteristics and the physiological changes in antioxidant enzyme activities, marker osmolytes, and the balance of nutrient elements were used to explore the tolerance mechanisms induced by *B. ramosa* ssp.

2. Results

2.1 *B. ramosa* ssp. enhances *C. equisetifolia* growth under NaCl stress

Under normal conditions (no salt stress), the colonization of *C. equisetifolia* by *B. ramosa* ssp. partially enhanced the growth rate and fresh and dry weights of the shoots and roots after 60 d (Table 1). Under NaCl stress, all plants displayed immensely reduced saplings growth compared to control. Salt stress decreased the growth rates of all plants from 13.63–3.98% and 38.84–14.80%, respectively. However, the growth rates of the colonized plants were higher than those of the non-colonized plants under different stress conditions. Salt stress led to reduced shoot and root fresh and dry weights in E- plants, but increased weights in E+ plants. More importantly, all of the fresh and dry weights of E+ were higher than those of E- plants (Table 1).

Table 1

Effects of NaCl treatment on growth, and fresh and dry weights of endophyte-colonized (E+) and non-colonized (E-) *Casuarina equisetifolia*

Treatments		Plant height (cm)		Growth rate	Fresh weight of shoots (g)	Dry weight of shoots (g)	Fresh weight of roots (g)	Dry weight of roots (g)
		0 d	60 d					
0%	E-	49.43 ± 9.31a	56.03 ± 7.35b	13.63%	13.33 ± 5.69b	5.00 ± 2.00a	8.33 ± 1.53a	2.33 ± 0.58b
	E+	51.57 ± 8.06a	71.60 ± 9.87a	38.84%	19.00 ± 2.00a	7.33 ± 0.58a	11.67 ± 3.21a	5.00 ± 2.00a
5%	E-	53.57 ± 15.96a	57.90 ± 15.86a	8.00%	13.67 ± 4.73b	4.33 ± 2.31b	9.33 ± 2.31a	2.00 ± 1.00b
	E+	45.03 ± 4.02a	53.97 ± 2.91a	19.85%	21.00 ± 7.21a	8.00 ± 3.61a	11.67 ± 3.51b	3.33 ± 1.53a
10%	E-	49.00 ± 5.43a	53.03 ± 4.31a	8.22%	11.00 ± 2.00b	3.67 ± 1.15b	7.33 ± 1.15a	1.00 ± 0.00b
	E+	48.67 ± 6.49a	55.13 ± 6.55a	13.27%	20.33 ± 3.21a	7.00 ± 1.73a	11.00 ± 1.00a	3.33 ± 0.58a
15%	E-	55.90 ± 4.58a	58.13 ± 2.65a	3.98%	9.33 ± 3.06b	2.33 ± 0.58b	7.33 ± 2.31b	1.67 ± 1.15b
	E+	49.13 ± 4.21a	56.40 ± 5.30a	14.80%	25.33 ± 3.51a	8.67 ± 1.53a	10.00 ± 4.00a	3.33 ± 1.53a

Note: Values are shown as the means ± SD of three replicates. Different lowercase letters between E- and E+ in the same salt treatment indicate a significant difference ($p < 0.05$).

2.2 B. ramosa ssp. colonization affects ROS scavenging system

Among the ROS scavenging and detoxification enzymes, the activity levels of SOD, CAT, and POD were lower in E+ saplings than in E- saplings under normal conditions and when subjected to 5% NaCl stress for 60 d (Fig. 1A, B, C). In contrast, the activity of SOD, CAT and POD were significantly higher in E+ than in E- saplings in 10% and 15% salt treatments (Fig. 1; $p < 0.05$). The activities of the three enzymes increased when the NaCl concentration increased from 0–10% but decreased at 15%, except SOD. Furthermore, the SOD, CAT, and POD activities increased in E+ plants from 0 d to 40 d but then decreased (Fig. S1A–L). Moreover, the SOD and CAT activities were higher than the POD activity under all conditions. The H_2O_2 content in E+ was significantly higher than in E- saplings at 10% and 15% NaCl concentrations (Fig. 1D; $p < 0.05$). The H_2O_2 of both E+ and E- plants increased during the first 20 days of NaCl stress but then decreased with time (Fig. S1M–P).

2.3 Plant hormones and osmotic adjustment

Plant growth and stress tolerance are regulated by several phytohormones. Auxin (IAA) plays a key role during the growth stage and ABA is important for abiotic stress adaptation. *B. ramosa* ssp. invasion upregulated the IAA content in *C. equisetifolia*; the IAA content in E + was significantly higher than in E– plants. However, as the NaCl concentration increased, the IAA contents of E + and E– plants decreased the difference (Fig. 2A). On the contrary, the ABA contents in all plants increased the difference with NaCl concentration increasing, as the ABA content in E + plants was down regulated (Fig. 2B). The dynamic changes in IAA and ABA contents in both the E + and E– plants were opposing (Fig. S2A–H). Specifically, IAA content decreased at the beginning of the salt stress treatments (0 – 20 d) but then increased. However, ABA content increased from 0 to 20 d and then declined gradually. The contents of ZT and GA₃ in E– were lower than in the E + plants under normal conditions and 10%, 15% salt conditions, but their contents in E– were higher than in the E + plants under 5% NaCl stress (Fig. 2C, D). Both ZT and GA₃ contents in E + and E– plants decreased from 0 d to 40 d but increased from 40 d to 60 d (Fig. S2I–P).

In both E + and E– plants, electrical conductivity increased with NaCl concentration from 0–10% but decreased from 10–15%. Colonized plants had a higher electrical conductivity than non-colonized plants under NaCl stress (Fig. 2E). Under salt stress, the electrical conductivity in E + plants increased from 0 d to 40 d and decreased from 40 d to 60 d, but that of E – plants increased from 0 d to 20 d and decreased from 20 d to 60 d (Fig. S3A–D). *C. equisetifolia* saplings were also found a large accumulation of proline in the leaves under NaCl stress, and the accumulation was enhanced when the NaCl concentration increased from 5–10%, but decreased at 15% (Fig. 2F). Notably, *B. ramosa* ssp. infection increased the accumulation of proline under normal conditions. Both in E + and E– plants, the accumulation of proline increased early in the treatment (20 d) but decreased from 20 d to 60 d (Fig. S3E–H).

2.4 Nutrients in branches and roots

In all leaves sample, P content significantly increased and the K content significantly decreased ($p < 0.05$) as with the NaCl concentration increased. Meanwhile, the C, N, and Mg contents did not change significantly ($p > 0.05$; Table 2). *B. ramosa* ssp. colonization led to reduced P and K contents, but elevated Ca content, under NaCl stress. In all plants, the Na content increased significantly as the NaCl concentration increased from 0–10%, but decreased as the concentration increased from 10–15%. *B. ramosa* ssp. colonization led to reduced leaf Na content under 5% and 10% NaCl stress conditions, but increased the leaf Na content at 15% NaCl.

In the E– plant roots, C, N, P, and K content increased as the concentration of NaCl increased from 0–5%, but these contents decreased as NaCl increased from 5–15% (Table 2). Meanwhile, the Ca and Mg contents significantly increased and decreased, respectively, as the NaCl concentration increased ($p < 0.05$). In E + plant roots, the C and K contents decreased significantly as the NaCl concentration increased. Meanwhile, the N content displayed significant fluctuation with increasing NaCl concentration. However, the contents of P, Ca, and Mg did not change ($p > 0.05$; Table 2). In both E– and E + plants, C, N, P, K, Na, and Mg contents were greater in the leaves than in roots, while Ca content was similar in leaves and roots.

Table 2
Effects of NaCl stress on nutrient contents in endophyte-colonized (E+) and non-colonized (E-) *Casuarina equisetifolia*

Treatment			C (g/kg)	N (g/kg)	P (g/kg)	K (g/kg)	Na (g/kg)	Ca (g/kg)	Mg (g/kg)
Leaves	E-	0%	412.90 ± 34.60a	11.02 ± 2.34a	0.64 ± 0.08 b	14.80 ± 0.83a	13.03 ± 12.01c	16.16 ± 0.09abc	2.98 ± 0.09a
		5%	398.56 ± 6.00a	10.87 ± 4.73a	0.86 ± 0.33ab	12.93 ± 2.74ab	32.07 ± 12.43ab	16.39 ± 0.16a	2.97 ± 0.11a
		10%	407.08 ± 33.64a	10.16 ± 5.37a	0.86 ± 0.13ab	12.31 ± 1.55ab	32.05 ± 3.71ab	15.65 ± 0.28b	2.98 ± 0.59a
		15%	400.01 ± 12.86a	11.51 ± 5.11a	0.93 ± 0.14a	10.48 ± 1.06b	26.52 ± 20.83a	15.83 ± 0.53bc	2.62 ± 0.80a
	E+	0%	391.23 ± 29.19a	14.85 ± 0.26a	0.60 ± 0.05b	14.84 ± 2.20a	10.86 ± 11.10c	15.73 ± 0.14bc	4.01 ± 1.15a
		5%	399.47 ± 48.24a	14.61 ± 2.69a	0.58 ± 0.03c	10.13 ± 1.34b	29.85 ± 6.89ab	16.04 ± 0.08abc	2.79 ± 0.3b
		10%	410.37 ± 15.49a	15.96 ± 2.23a	0.61 ± 0.07b	10.84 ± 1.50b	31.43 ± 6.12a	16.05 ± 0.13abc	2.97 ± 0.64a
		15%	422.20 ± 24.38a	15.59 ± 3.09a	0.66 ± 0.06ab	11.28 ± 1.03b	28.86 ± 16.57b	16.22 ± 0.40ab	3.33 ± 0.60a
Roots	E-	0%	213.06 ± 36.20b	4.25 ± 0.31ab	0.36 ± 0.05b	2.84 ± 0.86a	6.60 ± 8.22c	15.22 ± 0.09b	1.95 ± 0.21a
		5%	325.02 ± 27.23a	6.54 ± 1.93a	0.62 ± 0.27a	3.14 ± 0.05a	17.64 ± 3.31b	15.48 ± 0.50a	1.95 ± 0.20a
		10%	304.49 ± 70.81a	5.20 ± 2.13ab	0.45 ± 0.05ab	1.07 ± 0.63b	19.24 ± 3.57a	15.84 ± 0.46a	1.70 ± 0.24a
		15%	308.53 ± 26.68a	4.74 ± 1.82ab	0.43 ± 0.10ab	0.12 ± 0.48c	17.33 ± 9.15b	15.34 ± 0.13ab	1.54 ± 0.25b
	E+	0%	319.74 ± 44.72a	4.57 ± 0.55a	0.40 ± 0.05a	3.22 ± 0.51a	11.31 ± 1.31b	15.22 ± 0.03a	1.53 ± 0.13a

Note: Values are show as the mean ± SD of three replicates. Different lowercase letters between different salt concentrations in the same endophyte treatment indicate a significant difference ($p < 0.05$).

Treatment		C (g/kg)	N (g/kg)	P (g/kg)	K (g/kg)	Na (g/kg)	Ca (g/kg)	Mg (g/kg)
	5%	298.22 ± 35.27a	3.40 ± 1.05b	0.39 ± 0.02a	2.76 ± 0.33a	26.13 ± 2.22a	15.10 ± 0.08a	1.37 ± 0.31a
	10%	280.05 ± 30.30ab	4.89 ± 0.71a	0.40 ± 0.04a	2.37 ± 0.66a	25.84 ± 0.14a	15.07 ± 0.03a	1.39 ± 0.16a
	15%	258.09 ± 28.84b	3.04 ± 0.40b	0.41 ± 0.04a	2.10 ± 1.25b	24.79 ± 1.49a	15.08 ± 0.06a	1.47 ± 0.26a
Note: Values are show as the mean ± SD of three replicates. Different lowercase letters between different salt concentrations in the same endophyte treatment indicate a significant difference ($p < 0.05$).								

Correlation analysis showed that the non-metallic elements, C, N, and P, had a highly significant correlation with the metallic elements, K, Na, Ca, and Mg ($p < 0.01$). The correlation between N and Na was also significant ($p < 0.05$; Table 3). In addition, there were highly significant correlations among the C, N, and P ($p < 0.01$). There were also highly significant correlations among the metallic elements, excluding Na.

Table 3
The correlations among different nutrient contents in *Casuarina equisetifolia* plants

	Mg	Ca	Na	K	P	N
C	0.660**	0.708**	0.165	0.729**	0.505**	0.727**
N	0.790**	0.742**	0.235*	0.779**	0.497**	
P	0.543**	0.581**	0.350**	0.607**		
K	0.871**	0.738**	0.139			
Na	0.059	0.142				
Ca	0.738**					
Note: **indicates $p < 0.01$, * indicates $p < 0.05$						

3. Discussion

Biomass directly indicates plant adaptation to salt stress. Researches have showed that salt stress can lead to the decrease of dry weight for many plants [17–18]. Endophyte colonization has been shown plant biomass accumulation and the increase resistance to salt stress [19]. In our study, it was shown that colonization of *C. equisetifolia* plants by *B. ramosa* ssp. increased the dry weight of shoots and roots, but salt stress led to decreases in the plant dry weight. Furthermore, it was found that *B. ramosa*

ssp. colonization accelerated the growth of plants under NaCl stress. This indicated that *B. ramosa* ssp. colonization could help maintain normal growth of aerial plant parts by promoting plant height and growth.

Plants trigger multiple defense mechanisms to regulate increased stress tolerance to reactive oxygen species. Therefore, the antioxidative defense systems can be analyzed to evaluate the stress tolerance of plants [20–21]. In this study, NaCl stress promoted H_2O_2 accumulation, which severely damaged to cell structures. Meanwhile, the defense system, which consisting of SOD, CAT, and POD [22], was activated under stress conditions. These enzymes have the potential to maintain the ROS balance within plants, thus preventing serious damage under abiotic stress [23]. In the present study, *C. equisetifolia* plants colonized with *B. ramosa* ssp. exhibited significantly higher ROS scavenging enzymes(SOD, CAT, and POD) under NaCl stress than the control saplings. This result indicates that plants colonized by *B. ramosa* ssp. resisted salt conditions by decrease ROS through increases in the activities of SOD, CAT, and POD. These results are similar with the findings of Yasmeen and Siddiqui [24], who reported that endophytes colonization diminished H_2O_2 production by the appearance of antioxidant enzymes under salt stress. Additionally, endophyte colonization has previously been shown to increase the levels of ROS-scavenging enzymes activity, which promote plants tolerance of salt stress[25–26].

Salt stress also can change membrane permeability, which affects substance exchange within the cell. The presence study showed that leaf conductivity increased with the concentration of NaCl. However, the leaves of plants colonized by *B. ramosa* ssp. showed lower conductivity than the leaves of control plants under all NaCl concentrations. It could thus be concluded that *B. ramosa* ssp. colonization prevented change or loss of membrane permeability. Plants can also enhance cellular osmotic regulation to adapt to environmental changes by accumulating organic matter under salt stress [27–28]. Previous studies have shown that endophytes can regulate proline accumulation for osmotic modulation, thus improving the salt tolerance of the host plants [29]. In the current study, *C. equisetifolia* plants inoculated with *B. ramosa* ssp. showed higher proline concentrations at 10% and 15% NaCl than control plants. This result is consistent with that of research by [30], who showed that proline content increased when arbuscular fungi are backgrafted under NaCl stress. These increases in proline upon the infection of *B. ramosa* ssp. could be caused by the decrease of ABA.

ABA regulates proline biosynthetic to prevent potential cytoplasmic osmotic stress caused by increased NaCl content in underground regions of plants [31]. Usually, as a stress phytohormone, ABA content will increase when plants are subjected to osmotic stress by salt or drought [32]. In the current study, ABA content in non-colonized plants was significantly higher than in plants colonized by *B. ramosa* ssp. under salt stress. This suggests that *B. ramosa* ssp. may alleviate the toxic effects of salinity. The results also showed that the promoted growth of *B. ramosa*-colonized seedlings under control or low NaCl concentration was correlated with higher IAA content in the *C. equisetifolia* leaves. This suggested that *B. ramosa* ssp. improved plant salt stress tolerance by increasing IAA accumulation. Similarly to IAA, GA_3 can promote plant growth. Meanwhile, ZR usually promotes the burgeon of branches and prevents leaves

from turning yellow. In this study, both GA₃ and ZT contents decreased under salt stress, but endophyte colonization increased their contents under low salt stress conditions (5% concentration).

Salt stress reduces the uptake or transport of plant nutrients, which results in an imbalance of nutrients within the plant [2]. However, endophyte colonization is beneficial for the uptake of water and mineral elements [9, 33]. In this study, the leaves of colonized plants had higher C, N, Ca, and Mg contents, but lower P, K, and Na contents, than non-colonized plants under NaCl stress. However, the roots of colonized plants had lower C, N, P, Ca, and Mg contents, but higher K and Na contents, than non-colonized plant roots under NaCl stress. This result indicates that *B. ramosa* ssp. Colonization accelerated the transport and uptake of nutrients to increase the nutrient contents of the aboveground plant portion. Plant nutrients need to be maintained in a relative balance by reaching certain contents and proportions [34]. Thus, there is certain correlation among these nutrient elements. In this study, there was a positive correlation between NaCl concentration and C and P content, but a negative correlation between NaCl concentration and N, K, Ca, and Mg content in the leaves. In overall consideration of the nutrient content results, it could conclude that this endophyte infection might facilitate nutrients uptake to maintain nutrient balances in plants under salt stress conditions [35].

4. Conclusion

This study represents a first attempt to investigate the effects of *B. ramosa* ssp. on the NaCl tolerance of *C. equisetifolia* in terms of osmotic adjustment, ROS, the ROS-scavenging system, phytohormones, and nutrient elements. The results indicate that NaCl stress impacted proline accumulation and nutrient uptake. NaCl stress also led to changes in phytohormone contents and ROS generation, which could trigger defense responses by increasing antioxidant enzyme activity. These responses influence plant growth and tolerance. However, *B. ramosa* ssp. colonization of the plants under stress reduced the accumulation of proline and ABA but enhanced the antioxidant enzyme activity and auxin content to alleviate the impact of NaCl stress on *C. equisetifolia*.

5. Methods

5.1 Plants and treatments

We choose the *C. equisetifolia* cultivar “Huian-1” plants for these studies because of its high survival rate and being important variety in forest plantations in southeast coastal China. Clonal saplings generated from the same mother *C. equisetifolia* cultivar, which were provided by the Chihu Protection Forest, located in Huian county of Fujian province (24°54'42" N, 118°54'43" E). Saplings were planted in pots filled with yellow-heart loam and sand at a ratio of 3:1. Before planting, the soil had been sterilized for 24 h using methanol. *C. equisetifolia* saplings were grown in a greenhouse (dark:light = 8 h:16 h) for 30 d before further treatment. Endophyte colonization was established by evenly irrigating fermentation liquid around the rhizosphere of the seedlings for three consecutive days. In detail, the endophytic fungi were isolated from a *C. equisetifolia* branch and were found to be congeneric to *Botryosphaeria ramosa* ssp.

(100% similarity). The endophytic fungi were cultured in 60 mL potato dextrose broth (PDB) medium in a 100 mL flask, which was incubated for 3 d at 120 rpm and 24 °C. The fermentation broth produced from the incubation was used for infection. ddH₂O was used as the negative control.

For the salt stress treatments, all the plant materials were divided into two groups: endophyte-colonized (E+) and non-colonized saplings (E-). Both groups were treated with 0%, 5%, 10%, and 15% NaCl solutions (w/v). All of the plant leaves were sampled on day 0, 20, 40, and 60 after salt treatment for further analyses. The plant height, fresh weight, and dry weight of treated *C. equisetifolia* were determined after 60 days.

5.2 Hydrogen peroxide (H₂O₂) content and antioxidant enzyme activity assays

Approximately 2 g of fresh leaf was crushed to powder in liquid nitrogen and homogenized in 9 times tris buffered saline for 10% tissue homogenates. The tissue homogenates were then centrifuged at 2500 rpm for 10 min at 4 °C. The supernatants were then harvested to measure SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), and POD (EC 1.11.1.7) activity, and the H₂O₂ content.

5.3 Phytohormone contents, proline content, and electrical conductivity analysis

The contents of phytohormones, including IAA (auxin), ABA, zeatin (ZT) and gibberellin A₃ (GA₃), were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Chinese Agricultural University, NO.). Of the freeze-dried *C. equisetifolia* leaves, 1 g was homogenized with 80% methanol (v/v) and 2,6-bis(1,1-dimethylethyl)-4-methylphenol at 4 °C in the dark [9]. The extract was centrifuged at 2504 g for 15 min, and the supernatant was decanted. After removing the lipophilic pigments, the final solution was evaporated to dryness in vacuum at 35 °C. The product was redissolved in 1 mL methanol for IAA and GA₃ determination, and Tris-buffered saline (TBS; pH = 7.4) was used for ABA and ZT determination.

Proline content was measured by following a previously described protocol with little change [36]. Approximately 1 g fresh tissue was homogenized in 10 mL of 3% (w/v) sulfosalicylic acid solution, and extract for 10 min in a boiling water to get proline extract by filter with 2% (v/v) ninhydrin reagent with acetic acid (6 mol/L) and phosphoric acid (6 mol/L). Samples proline content was determined using a luminometer based on the absorbance at 520 nm (Tecan Infinite 200 Pro, Austria).

The relative conductivity was measured using a conductivity meter. In detail, the leaves were washed with distilled water and water was removed using filter paper. The samples were cut into 1–2 cm pieces and put into a tube with 20 mL distilled water. After 20 min at 25 °C, the conductivity of the samples was determined with an electric conductometer (Ohaus ST3100C-F, USA).

5.4 Determination of nutrients

Oven-dried of shoot and root materials were powdered to pass through a 100 mesh sieve. For the determination of C and N content, an elementary analyzer (Flash EA 1112, Thermo Scientific, West Palm Beach, USA) that uses the combustion method was adopted. To elucidate the contents of other elements, 500 mg of each sample was added to 10 mL mixed acid ($\text{H}_2\text{SO}_4:\text{HClO}_3 = 10:1$) and left overnight. Next, the samples were digested in a stove for 20 min at 250 °C, and then for 1 hour at 450 °C. Each sample was made up to a final volume of 50 mL with deionized water (Li et al. 2017). The K, Ca, Mg, and Na concentration of samples were measured using an Inductively Coupled Plasma Emission Spectrometer (ICP model Liberty 200, Varian Australia Pty. Ltd., Mulgrave Victoria, Australia). P content was determined using the molybdenum-antimony colorimetry method (GB7888-87).

Abbreviations

AMF

arbuscular mycorrhizal fungi; ROS:reactive oxygen species; ABA:abscisic acid; IAA:indoleacetic acid
GAs:gibberellins; ZT:zeatin; SOD:superoxide dismutase; POD:peroxidase; CAT:catalases; E + plants
endophyte-colonized; E- plants:non-colonized saplings

Declarations

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Authors' contribution

FL and AL conceived, designed research and analysed the data. ZC and TH cultivated the materials and measured the enzymes.. CW and FL did the physiological experiment and made Tables. JL and JS discussed the results and revised the manuscript

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Availability of data and materials

All of the datasets supporting the results of this article are included within the article and its supplementary information.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors have no conflict of interest to declare.

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Figures

Figure 1

Effects of endophyte colonization on the activities of (A) superoxide dismutase (SOD), (B) peroxidase (POD), and (C) catalase (CAT), and (D) the H₂O₂ content under NaCl stress. Different small letters indicate a significant difference between colonized and non-colonized plants (p < 0.05).

Figure 2

Effects of endophyte colonization on the content of endogenous hormones under NaCl stress. The contents of (A) indole acetic acid (IAA), (B) abscisic acid (ABA), (C) gibberellin A3 (GA₃), and (D) zeatin (ZT) are shown. The (E) electrical conductivity and (F) proline content are also shown. Different small letters indicate a significant difference between colonized and non-colonized plants ($p < 0.05$).

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