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

Research article

**Keywords:** Casuarina equisetifolia, fungal endophyte (Botryosphaeria ramosa ssp.), Salt stress, Antioxidant enzymes, Phytohormones

**Posted Date:** October 8th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-76071/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
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 deletion 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           |
| E+         | 51.57 ± 8.06a    | 71.60 ± 9.87a | 38.84%                    | 19.00 ± 2.00a           | 7.33 ± 0.58a              | 11.67 ± 3.21a          |
| 5% E–      | 53.57 ± 15.96a   | 57.90 ± 15.86a | 8.00%                     | 13.67 ± 4.73b           | 4.33 ± 2.31b              | 9.33 ± 2.31a           |
| E+         | 45.03 ± 4.02a    | 53.97 ± 2.91a | 19.85%                    | 21.00 ± 7.21a           | 8.00 ± 3.61a              | 11.67 ± 3.51b          |
| 10% E–     | 49.00 ± 5.43a    | 53.03 ± 4.31a | 8.22%                     | 11.00 ± 2.00b           | 3.67 ± 1.15b              | 7.33 ± 1.15a           |
| E+         | 48.67 ± 6.49a    | 55.13 ± 6.55a | 13.27%                    | 20.33 ± 3.21a           | 7.00 ± 1.73a              | 11.00 ± 1.00a          |
| 15% E–     | 55.90 ± 4.58a    | 58.13 ± 2.65a | 3.98%                     | 9.33 ± 3.06b            | 2.33 ± 0.58b              | 7.33 ± 2.31b           |
| E+         | 49.13 ± 4.21a    | 56.40 ± 5.30a | 14.80%                    | 25.33 ± 3.51a           | 8.67 ± 1.53a              | 10.00 ± 4.00a          |

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) 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 | 2.97 ± 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).
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$_2$O$_2$ 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$_2$O$_2$ 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 GA$_3$ 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-dimethylthyl)-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 absorbence 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**

**Acknowledgments**

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

**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.

**Funding**

This work was supported by the National Natural Science Foundation of China [grant number 31400533]; the Special Fund for Science and Technology Innovation of Fujian Agriculture and Forestry University [grant numbers CXZX2016055, CXZX2018122]; and the Outstanding Doctoral and Master Breeding Program of Forestry College of Fujian Agriculture and Forestry University [grant number 71201800781].

**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.

References

1. Zhong CL, Bai JY, Zhang Y. Introduction and conservation of Casurina trees in China. Forest Res. 2005; 18:345-350.
2. Wu C, Zhang Y, Tang SM, Zhong CL. Effect of NaCl stress on Casuarina seed germination. Seed 2010; 29: 30-33.
3. Yang T, Yan CL, Liang J, Li YH, Tang HH. The nutrient elements distribution in Casuarina equisetifolia seedlings under salt stress. Subtropical Plant Science 2003;32:1-4
4. Arnold AE, Maynard Z, Gilbert GS. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. Mycol Res. 2001; 105: 1502-1507.
5. Bashan Y, de-Bashan L, Prabhu S, Hernandez JP. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant Soil. 2014; 378:1–33.
6. Li XM, Ma LJ, Bu N, Li YY, Zhang LY. Endophytic infection modifies organic acid and mineral element accumulation by rice under Na2CO3 stress. Plant Soil. 2017; 420: 93-103.
7. Chen SL, Hawighorst P, Sun J, Polle A. Salt tolerance in Populus: Significance of stress signaling networks, mycorrhization, and soil amendments for cellular and whole-plant nutrition. Environ Exp Bot. 2014; 107: 113–124.
8. Baltruschat H, Fodor J, Harrach BD, Niemczyk E, Barna B, Gullner G, Janeczko K, Karl-heinz,S P, Schwarzcinger I, Zuccaro A, Skoczowski A. Salt tolerance of barley induced by the root endophyte piriformospora indica is associated with a strong increase in antioxidants. New Phytol. 2008; 180: 501-510.
9. Abdelaziz EM, Kim DJ, Ali S, Fedoroff VN, Al-Babili S. The endophytic fungus Piriformospora indica enhances Arabidopsis thaliana growth and modulates Na+/K+ homeostasis under salt stress conditions. Plant Sci. 2017; 263:107-115.
10. Guerrero-Galán C, Calvo-Polanco M, Zimmermann SD. Ectomycorrhizal symbiosis helps plants to challenge salt stress conditions. Mycorrhiza, 2019; 29:291-301
11. Khan AL, Hussain J, Al-Harras IA, Al-Rawahi A, Lee IJ. Endophytic fungi: resource for gibberellins and crop abiotic stress resistance. Crit Rev Biotechnol. 2013; 35: 62-74.
12. Khan AL, Waqas M, Hussain A, Al-Harrasi A, Hamayun M, Lee IJ (2015) Phytohormones enabled endophytic fungal symbiosis improve aluminum phytoextraction in tolerant Solanum lycopersicum: an examples of Penicillium janthinellum LK5 and comparison with exogenous GA3. J Hazard Mater. 295: 70-78.

13. Caarls L, Pieterse CMJ, Van Wees SCM. How salicylic acid takes transcriptional control over jasmonic acid signaling. Front Plant Sci. 2015; 6: 170.

14. Li L, Li L, Wang XY, Zhu PY, Wu HQ, Qi ST. Plant growth-promoting endophyte Piriformospora indica alleviates salinity stress in Medicago truncatula. Plant Physiol Bioch. 2017; 119: 211-223.

15. Liang AJ, Long F, Hong T, Lin YM, Chen C, Xie AQ, Wu CZ, Hong W, Li J. Effects of endophyte fungi on the morphological and physiological characteristics of Casuarina equisetifolia seedling roots under quercetin-3-α-arabinoside stress. Chin J Appl. Environ Biol. 2018; 24: 0797-0804.

16. Long F, Hong T, Lin YM, Xie AQ, Wu CZ, Hong W, Li J. Effects of endophyte infection on reactive oxygen content and protective Effects of endophyte infection on reactive oxygen content and protective enzyme activity in branchlet of enzyme activity in branchlet of Casuarina equisetifolia seedling under stress of two allelochemicals two allelochemicals. Chin J Appl Environ Biol 2016;22: 0462-0472.

17. Zhao CQ, Li JW, Fan XF, Hou XC, Wu JY, Hun YG, Liu JL. Effects of salt stress on biomass, quality, and photosynthetic physiology in switchgrass. Acta Ecol Sin 2015; 35: 6489-6495.

18. Wang SF, Hu YX, Sun HJ, Shi X, Pan HW, Chen YT. Effects of salt stress on growth and root development of two oak seedlings. Acta Ecol Sin 2014; 34: 1021-1029.

19. Filek M, Walas S, Mrowiec H, Rudolphy-Skorska E, Sieprawska A. Membrane permeability and micro-and macroelement accumulation in spring wheat cultivars during the short-term effect of salinity-and PEG-induced water stress. Acta Physiol Plant 2012; 34:985–995

20. Hernández Jose A, Aguilar AB, Portillo B, López-Gómez E, Beneyto JM, García-Legaz MF. The effect of calcium on the antioxidant enzymes from salt-treated loquat and anger plants. Funct Plant Biol. 2003; 30: 1127-1137.

21. Radhakrishnan R, Kumari BR. Protective role of pulsed magnetic field against salt stress effects in soybean organ culture. Plant Biosyst. 2013; 147: 135–140.

22. Lubna, Sajjad A, Hamayun M, Abdul LK, Waqas M, Aaqil Khan M, Jan R, Lee I-J, Hussain A. Salt tolerance of Glycine max .L induced by endophytic fungus Aspergillus flavus CSH1, via regulating its endogenous hormones and antioxidative system. Plant Physi. &Biochem. 2018;128:13-23.

23. Abogadallah MG. Differential regulation of photorespiratory gene expression by moderate and severe salt and drought stress in relation to oxidative stress. Plant Sci. 2011; 180:540–547.

24. Yasmeen R, Siddiqui ZS. Physiological responses of crop plants against Trichoderma Harzianum in saline environment. Acta Bot Croat 2017; 76: 154-162.

25. Ahmad P, Hashem A, Abd-Allah EF, Alqarawi AA, John R, Egamberdieva D, Gucel, S. Role of Trichoderma harzianum in mitigating NaCl stress in Indian mustard (Brassica juncea L) through antioxidative defense system. Front Plant Sci. 2015; 6:868.
26. Hashem A, Abd_Allah EF, Alqarawi AA, Huqai AAA, Egamberdieva D. Alleviation of abiotic salt stress in Ochradenus baccatus (Del.) by Trichoderma hamatum (bonord.) bainier. J Plant Interact. 2014; 9: 857-868.

27. Hoekstra FA, Golovina EA, Buitink J. Mechanisms of plant desiccation tolerance. Trends Plant Sci. 2001; 6: 431-438.

28. Morgan JM. Osmoregulation and water stress in higher plants. Ann Rev Plant Physiol. 1984; 35: 299-319.

29. Li E, Hu HR, Li JN, Du GH, Liu FH. Research progress on endophytic fungi improving plant resistance to salt stress. Biotech Bull. 2019; 35: 169-178.

30. Jindal V, Atwal A, Sekhon BS, Singh R. Effect of vesicular-arbuscular mycorrhizae on metabolism of moong plants under NaCl salinity. Plant Physiol Bioch. 1993; 3: 475-481.

31. Cao MJ, Wang Z, Zhao Q, Mao JL, Speiser A, Wirtz M, Hell R, Zhu JK, Xiang CB. Sulphate availability affects ABA levels and germination response to ABA and salt stress in Arabidopsis thaliana. Plant J. 2014; 77: 604-615.

32. Zhou X, Xia K. Biosynthesis, metabolism and mechanism of abscisic acid. Plant physiology and molecular biology.( Second Edition). Beijing, Science Press, 1998; 476-492.

33. Alikhani M, Khatabi B, Sepehri M, Nekouei MK., Mardi M, Salekdeh GH. A proteomics approach to study the molecular basis of enhanced salt tolerance in barley (Hordeum vulgare L.) conferred by the root mutualistic fungus Piriformospora indica. Mol Biosyst. 2013;9:1498-1510.

34. Mo JM, Zhang DQ, Huang ZL, Yu QF, Kong GH. Distribution pattern of nutrient elements in plants of Dinghushan lower subtropical evergreen broad-leaved forest. J Trop Subtrop Bot. 2000; 8: 198-206.

35. Song ML, Chai Q, Li XZ, Yao X, Li CJ, Christensen MJ, Nan ZB. An asexual Epichloë endophyte modifies the nutrient stoichiometry of wild barley (Hordeum brevisubulatum) under salt stress. Plant Soil 2014; 387: 153-165.

36. Claussen W. Proline as a measure of stress in tomato plants. Plant Sci. 2005; 168: 241-248.

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).
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation.doc
- FigS1.JPG
- FigS3.JPG
- FigS2.JPG