

Effect of Dark Septate Endophytes on Plant Performance of *Artemisia Ordosica* and Associated Soil Microbial Community Under Salt Stress

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Abstract

Background: Fungal endophytes can improve plant tolerance to abiotic stress, however, the role of these plant–fungal interactions in desert species ecology and their management implications remain unclear. This study aimed to assess whether dark septate endophytes (DSE) can shift the performance of *Artemisia ordosica* and associated soil microbial community under salt stress.

Methods: We investigated the effects of three DSE (*Alternaria chlamydosporigena* [AC], *Paraphoma chrysanthemicola* [PC] and *Bipolaris sorokiniana* [BS]) isolated from desert habitats on plant morphology, physiology and rhizosphere soil microhabitat of *Artemisia ordosica* seedlings under different NaCl concentrations (0 %, 0.1 %, 0.2 %, and 0.3 %) in a growth chamber.

Results: Three DSE strains could colonize the roots of *A. ordosica*, and the symbiotic response with host plants depended on DSE species and NaCl concentration. The greatest benefits associated with DSE occurred under 0.1 % NaCl. Specifically, AC improved root morphology, and increased total biomass and superoxide dismutase (SOD) activity; PC increased root morphology, root biomass, and glutathione (GSH) and indoleacetic acid (IAA) contents; and BS promoted SOD activity and GSH and IAA contents. DSE reduced the root Na⁺ content. Interestingly, BS promoted gram-positive (G +) and gram-negative (G –) bacteria under 0.1 % NaCl and the abundance of AM fungi under 0.2 % and 0.3 % NaCl. PC positively affected fungi, AM fungi, G – bacteria and actinomycetes under 0.2 % and 0.3 % NaCl, while AC increased the abundance of all examined microbes under 0.3 % NaCl. A structural equation modeling (SEM) demonstrated that DSE not only positively affects *A. ordosica* performance but also directly or indirectly impacts soil microbes by regulating the soil organic carbon (SOC), available phosphorus (AP), and alkaline nitrogen (AN) content.

Conclusions: DSE isolated from *A. ordosica* enhanced the root development of host plants and altered the soil nutrient content and soil microbiota under different NaCl concentrations, possibly contributing to plant growth and ecological adaptability under saline environment. These results contribute to the understanding of the ecological function of DSE in desert ecosystems and may be used to promote vegetation restoration in salinized desert areas.

Background

Soil degradation is a severe environmental problem worldwide, especially in arid and semiarid regions. The evaporation in these regions greatly exceeds the precipitation, and salt dissolved in the groundwater may accumulate at the soil surface through capillary movement [1]. In addition to water stress, salt accumulation is becoming a severe current and future problem faced by plants [2, 3]. Salt stress normally causes ion toxicity, osmotic shock and oxidative stress, which disrupt plant metabolic processes and hinder plant growth [4–6]. Thus, plants have evolved a series of physiological and biochemical mechanisms to alleviate these negative effects [7–9]. Soil microbes largely inhabit plants in their natural habitat, and some soil microbes are stress tolerant and capable of stimulating plant growth [10]. Plants

not only provide important habitats for their associated microbiome but also provide photosynthates for microbial growth [11, 12]. Moreover, the soil-associated microbiome has an important function in ecological functions and nutrient availability, further affecting plant growth and productivity [13, 14]. Previous studies have shown that plant tolerance to stress, such as drought, salinity and high temperature, is closely related to the colonization of endophytic fungi [15, 16]. Hence, the resource excavation of these associated endophytic fungi containing dark septate endophytes (DSE) is vital for improving the health and productivity of plants, especially for plant growth and vegetation restoration in degraded soils [17, 18].

DSE represent a diverse group of endophytic fungi mainly involving ascomycetes, which propagate via conidia or asexual reproduction. These fungi inhabit the root tissues of healthy plants, and the dark septal hyphae and microsclerotia grown intracellularly and intercellularly are their main characteristics [19–21]. DSE have been shown to harbor an extensive range of hosts and have a broad ecological distribution; they are typically found in plants growing in stressful habitats, including those experiencing drought, salt, or heavy metal stress [22–28]. Previous studies have shown that host plant responses to DSE are variable, including positive, neutral and negative responses [29]. Indeed, DSE can alter plant growth, alter the accumulation of auxin and other chemical components, and prevent the development of abiotic resistance in host plants [30–32]. However, the effects of DSE on host plant performance and fitness remain unknown, especially under salt-stress conditions.

DSE isolated from wetlands or coastal areas have been found to alleviate the adverse effects of salt stress on plant growth [33–36]. Pan et al. [33] reported that *Curvularia* spp. isolated from *Suaeda salsa* could alleviate the adverse effects of salt stress on *Populus tomentosa* by increasing both antioxidant enzyme activity and the content of chlorophyll and proline in the leaves. Farias et al. [34] reported that inoculation with DSE improved the absorption of nitrogen and phosphorus in *Vigna unguiculata* under salt stress, which was beneficial for increasing plant growth and net photosynthesis. In addition, rhizosphere-associated microbes have important functions in the maintenance of soil nutrient availability, which have positive impacts on the tolerance of host plants to salt stress [37, 38]. Similar results have also been reported in other stressful environments. He et al. [15] reported that DSE improved the root development and antioxidant enzyme activity of *Glycyrrhiza uralensis* and altered the soil organic nutrition content and microbiota composition, which may also promote plant growth and survival under water deficit conditions. To date, the effects of DSE on the rhizospheric microbial community of host plants under salt stress have not been studied.

Artemisia ordosica (Asteraceae) is one of the dominant sand-fixing shrub species that is widely distributed in the arid and semiarid regions of northwestern China and is an important medicinal and feed plant species. This species is tolerant to drought, soil infertility, wind erosion and sand burial and has been widely used for phytoremediation as a wind break and to sand fix, maintaining biodiversity and ecosystem stability in northwestern China [39, 40]. Excessive amounts of Na⁺ in the soil have been found to be a major cause of plant damage and restrict vegetation restoration in arid and semiarid regions [1, 41]. Investigating the response of host plants and soil microbial communities to DSE under salt-stress

conditions is highly important to improve the bioremediation of saline soils in arid and semiarid regions. In this study, we conjecture that DSE fungi could provide more beneficial effects on promoting the growth of *A. ordosica* and altering the soil microbial community and physicochemical properties in the rhizosphere of *A. ordosica* under conditions of salt stress. Based on our conjecture, we researched the response of (1) the growth of plants, (2) the antioxidant system, (3) Na⁺ and K⁺ ions, (4) soil physical and chemical properties, and (5) the composition of soil microbes to DSE inoculation under conditions of salt stress. We expect that the results will reveal the impact mechanism of DSE inoculation resistance to salinization conditions on the growth and production of plants and further explore the potential of these fungi to enhance plant stress tolerance and symbiotic function during vegetation restoration in saline desert regions.

Results

DSE colonization analysis

After harvest, typical DSE structures including dark septate hyphae and microsclerotia in inoculated plants were observed (Fig. 1). The total colonization rate was 10%–60% (Fig. 2). The colonization rate of *Paraphoma chrysanthemicola* (PC) peaked under 0.1% NaCl. The maximum colonization rate of *Bipolaris sorokiniana* (BS) occurred under 0.3% NaCl, and no significant differences were found among the different NaCl treatments. However, the total colonization by *Alternaria chlamydosporigena* (AC) in the *A. ordosica* roots significantly decreased with increasing NaCl concentration.

Plant biomass production

The shoot, root and total biomass and root:shoot ratio of host plants were significantly influenced by the interactions between DSE species and NaCl stress treatment (Table 1). Inoculation with AC resulted in an increase in the shoot biomass and total biomass under 0.1% NaCl compared to the control plants. No significant differences in shoot and total biomass were found in the other treatments (Fig. 3a, c).

Table 1

Two-way analysis of variance of the effects of dark septate endophytes (DSE) inoculation and NaCl stress on the growth and morphology parameters of *Artemisia ordosica*.

	DSE			NaCl stress			DSE × NaCl stress		
	<i>F</i>	<i>P</i>	η_p^2	<i>F</i>	<i>P</i>	η_p^2	<i>F</i>	<i>P</i>	η_p^2
Shoot biomass	3.17	0.038	0.229	81.621	< 0.001	0.884	2.75	0.017	0.436
Root biomass	25.43	< 0.001	0.704	150.596	< 0.001	0.934	4.758	< 0.001	0.572
Total biomass	3.739	0.021	0.26	108.139	< 0.001	0.91	2.724	0.018	0.434
Root:shoot ratio	17.465	< 0.001	0.621	7.155	0.001	0.401	3.049	0.009	0.462
Plant height	11.449	< 0.001	0.518	71.54	< 0.001	0.87	1.404	0.228	0.283
Shoot branching	12.082	< 0.001	0.531	109.254	< 0.001	0.911	6.533	< 0.001	0.648
Root length	63.019	< 0.001	0.855	298.463	< 0.001	0.965	10.594	< 0.001	0.749
Root surface area	50.486	< 0.001	0.826	276.307	< 0.001	0.963	11.165	< 0.001	0.758
Root diameter	1.02	0.397	0.087	4.089	0.014	0.277	0.477	0.879	0.118
Root volume	15.764	< 0.001	0.596	95.558	< 0.001	0.9	5.313	< 0.001	0.599
The partial eta squared (η_p^2) presented the effect size of different factors. Significant <i>P</i> -values (<i>P</i> < 0.05) are in bold.									

DSE increased the root biomass under 0% NaCl compared with the control plants. With increasing NaCl concentration, compared with that of the control plants, only inoculation with PC increased the root biomass under 0.1% and 0.3% NaCl, whereas BS inoculation decreased the root biomass under 0.2% and 0.3% NaCl (Fig. 3b).

The root:shoot ratio was significantly enhanced by DSE under 0% NaCl compared to control plants. With increasing NaCl concentration, only inoculation with PC increased the root:shoot ratio under 0.3% NaCl. However, inoculation with AC and BS decreased the root:shoot ratio under 0.1% NaCl (Fig. 3d).

Plant morphological traits

DSE species and NaCl stress treatment significantly influenced plant height. Furthermore, the interaction between DSE species and NaCl stress treatment significantly affected shoot branching (Table 1). Inoculation with AC positively affected plant height and was significant under 0.2% and 0.3% NaCl (Fig. 4a). A significant increase in shoot branching occurred only under 0% NaCl. However, with increasing NaCl concentration, inoculation with AC reduced shoot branching compared with that of the control plants under 0.1% and 0.3% NaCl (Fig. 4b).

Interactions between DSE species and NaCl stress treatment significantly influenced the root length, root volume and root surface area of *A. ordosica* (Table 1). Inoculation with DSE promoted the root growth of *A. ordosica* under 0% NaCl. In addition, under 0.1% NaCl, AC and PC inoculation enhanced the root length, root volume and root surface area compared to control plants. As the NaCl concentration increased, only inoculation with PC significantly enhanced the root length, root volume and root surface area under 0.3% NaCl. Furthermore, no significant differences in root diameter were found under any treatment (Fig. 4c, d, e, f).

Antioxidant enzyme activities in the leaves

Interactions between DSE species and NaCl stress treatment on the superoxide dismutase (SOD) activity and glutathione (GSH) content in host plants were significant (Table 2). DSE inoculation resulted in increased SOD activity and GSH content of the leaves under NaCl stress. AC and BS inoculation increased SOD activity under NaCl stress compared with that of the control plants, while PC inoculation improved SOD activity only under 0.2% NaCl (Fig. 5a). BS inoculation increased the GSH content compared to the control plants under NaCl stress, PC inoculation increased the GSH content only under 0.1% NaCl, and AC inoculation increased the GSH content under 0.2% and 0.3% NaCl (Fig. 5b).

Table 2

Two-way analysis of variance of the effects of dark septate endophytes (DSE) inoculation and NaCl stress on the physiological parameters of *Artemisia ordosica*.

	DSE			NaCl stress			DSE × NaCl stress		
	<i>F</i>	<i>P</i>	η_p^2	<i>F</i>	<i>P</i>	η_p^2	<i>F</i>	<i>P</i>	η_p^2
SOD	23.231	< 0.001	0.685	62.16	< 0.001	0.854	7.423	< 0.001	0.676
GSH	18.026	< 0.001	0.628	26.687	< 0.001	0.714	6.089	< 0.001	0.631
IAA	22.357	< 0.001	0.677	0.578	0.633	0.051	1.129	0.372	0.241
Na ⁺ in shoots	9.261	< 0.001	0.465	54.387	< 0.001	0.836	2.064	0.064	0.367
K ⁺ in shoots	64.415	< 0.001	0.858	45.176	< 0.001	0.809	25.911	< 0.001	0.879
Na ⁺ :K ⁺ ratio in shoots	0.802	0.502	0.07	37.577	< 0.001	0.779	2.189	0.05	0.381
Na ⁺ in roots	72.138	< 0.001	0.871	9.341	< 0.001	0.467	3.878	0.002	0.522
K ⁺ in roots	59.74	< 0.001	0.848	53.407	< 0.001	0.834	25.466	< 0.001	0.877
Na ⁺ :K ⁺ ratio in roots	26.675	< 0.001	0.714	3.821	0.019	0.264	8.819	< 0.001	0.713

The partial eta squared (η_p^2) presented the effect size of different factors. Significant *P*-values (*P* < 0.05) are in bold. SOD indicates superoxide dismutase; GSH indicates glutathione; IAA indicates indoleacetic acid.

Indoleacetic acid (IAA) content in the roots

DSE species significantly influenced IAA production in *A. ordosica* roots regardless of the NaCl stress treatment (Table 2). Inoculation with PC and BS increased the IAA content compared to the control plants under all NaCl treatments. Moreover, AC inoculation had a positive effect on IAA content under 0.2% and 0.3% NaCl, but there were no significant differences (Fig. 5c).

Na⁺ and K⁺ content

DSE species and NaCl stress treatment had significant interaction effects on the K⁺ content in the shoots of *A. ordosica*. Moreover, the Na⁺ content in the shoots was remarkably influenced by DSE species and NaCl stress treatment (Table 2). BS inoculation increased the Na⁺ and K⁺ content under NaCl stress

compared with that in the control plants. No significant difference in the $\text{Na}^+:\text{K}^+$ ratio was found under NaCl stress treatment (Fig. 6a, c, e).

DSE species and NaCl stress treatment had significant interaction effects on the Na^+ and K^+ content and the $\text{Na}^+:\text{K}^+$ ratio in the roots of *A. ordosica* (Table 2). Compared to the controls, the Na^+ content in roots of DSE-inoculated plants decreased under NaCl stress treatments. Inoculation with AC increased the K^+ content in the roots under 0.2% and 0.3% NaCl compared to control plants. Furthermore, inoculation with AC and PC decreased the $\text{Na}^+:\text{K}^+$ ratio under 0.2% and 0.3% NaCl (Fig. 6b, d, f).

Soil physicochemical properties

Interactions between DSE species and NaCl stress treatment remarkably influenced the soil organic carbon (SOC) content, while the soil available phosphorus (AP) content was significantly affected by DSE species and NaCl stress treatment. Moreover, the soil alkaline nitrogen (AN) content was significantly affected only by DSE species (Table 3). PC inoculation reduced the SOC content under all NaCl treatments and reduced the AP content under 0.1% NaCl compared with that in the control plants. AC inoculation reduced the SOC content only under 0.3% NaCl. However, BS inoculation reduced the content of SOC and AP under both 0.1% and 0.3% NaCl and increased the AN content under both 0% and 0.3% NaCl (Fig. 7).

Table 3

Two-way analysis of variance of the effects of dark septate endophytes (DSE) inoculation and NaCl stress on soil factors and microbial composition in rhizosphere of *Artemisia ordosica*.

	DSE			NaCl stress			DSE × NaCl stress		
	<i>F</i>	<i>P</i>	η_p^2	<i>F</i>	<i>P</i>	η_p^2	<i>F</i>	<i>P</i>	η_p^2
SOC	22.29	< 0.001	0.676	12.885	< 0.001	0.547	4.376	0.001	0.552
AN	10.552	< 0.001	0.497	0.754	0.528	0.066	0.357	0.947	0.091
AP	10.126	< 0.001	0.487	37.508	< 0.001	0.779	0.82	0.602	0.187
Fungi	5.18	0.005	0.327	1.501	0.233	0.123	2.896	0.013	0.449
AM Fungi	4.525	0.009	0.298	2.62	0.068	0.197	3.48	0.004	0.495
G - bacteria	5.024	0.006	0.32	1.381	0.266	0.115	5.076	< 0.001	0.588
G + bacteria	10.511	< 0.001	0.496	2.756	0.058	0.205	7.577	< 0.001	0.681
Actinomycetes	3.099	0.04	0.225	1.934	0.144	0.154	3.976	0.002	0.528

The partial eta squared (η_p^2) presented the effect size of different factors. Significant *P*-values (*P* < 0.05) are in bold. SOC indicates soil organic carbon; AN indicates alkaline nitrogen; AP indicates available phosphorus; G - bacteria indicates gram-negative bacteria; G + bacteria indicates gram-positive bacteria.

Soil microbial community composition

Interactions between DSE species and NaCl stress treatment significantly affected the microbial community composition in the rhizospheric soil of *A. ordosica* (Table 3). Under 0.1% NaCl, BS inoculation enhanced gram-negative (G -) and gram-positive (G +) bacterial abundance compared with those of the control plants. Under 0.2% NaCl, PC inoculation increased the abundance of G - bacteria, actinomycetes, fungi and arbuscular mycorrhizal (AM) fungi compared to controls, whereas BS inoculation only enhanced the abundance of AM fungi. When the NaCl concentration reached 0.3%, compared with the control, the plants inoculated with AC presented an increased abundance of soil microbes; the plants inoculated with PC presented an increased abundance of G - bacteria, actinomycetes, fungi and AM fungi, whereas BS inoculated plants presented an enhanced abundance of fungi and AM fungi (Fig. 8).

Correlation analysis

Spearman's rank correlation test indicated that significant relationships existed among DSE species, NaCl stress, soil factors, soil microbial composition, and the growth parameters of *A. ordosica* (Table S1). On

the basis of the correlation coefficients (R-value), structural equation modeling (SEM) further revealed the relative effects of DSE species, NaCl stress and soil factors on the growth of *A. ordosica* and on the rhizospheric microbial composition ($\chi^2 = 78.527$, $df = 60$, $P = 0.055$, CFI = 0.969, TLI = 0.953, and RMSEA = 0.081; Fig. 9). The SEM indicated that DSE directly and significantly positively influenced the plant hormone (IAA) content and antioxidant enzyme (SOD) activity and directly negatively affected the root Na^+ content. Moreover, DSE significantly indirectly affected plant hormone (IAA) content, antioxidant enzyme (SOD) activity, and root growth (root length and biomass) by affecting soil factors (SOC, AP, and AN). In addition, DSE directly and significantly positively influenced the abundance of G + bacteria and fungi, whereas DSE had significant indirect effects on the abundance of G - bacteria by affecting soil factors (SOC). In addition, G - bacteria and root biomass directly negatively affected the Na^+ content in the roots.

Discussion

The current study reported for the first time the effects of DSE isolated from an arid desert environment on the growth of *A. ordosica* under NaCl stress. In all inoculated plants, typical DSE structures (dark septate hyphae and microsclerotia) in roots were observed, revealing that these DSE can be regarded as effective colonizers of the roots of *A. ordosica* under all NaCl treatments. DSE hyphae are important media for water transport and nutrient exchange between plants and DSE, while microsclerotia are also considered to be propagules or hypopus, which constitute important ecological strategies to plants for tolerating environmental stress [23, 42]. However, different DSE strains showed variable colonization patterns with increasing NaCl concentrations, and similar results of studies of DSE improving the heavy metal tolerance of plants have been reported [43–45]. The primary reason may be that the stress affected the growth and physiological metabolism of the DSE hyphae [46, 47], thus regulating the infection ability of the DSE on plant roots. In addition, the growth response of *A. ordosica* to DSE colonization was also strain dependent, which is consistent with previous findings [29, 46]. Specifically, AC resulted in a significant increase in *A. ordosica* biomass accumulation under 0.1% NaCl, while PC exerted beneficial effects under both 0.1% and 0.3% NaCl. However, BS inoculation decreased the root biomass under 0.2% and 0.3% NaCl. Therefore, the variable effects of different DSE on plants may be related to the different colonization rates and DSE species, which need further investigation.

The root system is the primary plant part that senses salt stress, and roots can respond rapidly through changes in elongation [48] and function [49]. In the present study, DSE promoted the growth of the root system under NaCl stress, although the effects depended on the DSE species. Under the low-stress treatment (0.1% NaCl), AC and PC inoculation enhanced the root length, root volume and root surface area compared to control plants. As the stress increased, only PC inoculation significantly promoted root growth under 0.3% NaCl. Pan et al. [33] also reported that DSE had positive influences on root morphological characteristics under salt-stress conditions. Moreover, studies have shown that DSE can promote plant root growth under water deficiency and ion stress [50, 51]. Extensive and deep roots are conducive to increasing the water and nutrient intake of plants, and eventually influence biomass [31].

Thus, the relatively large root length, root volume and root surface area of *A. ordosica* in this study may be beneficial to improve the adaptability of plants to NaCl stress.

Previous studies have shown that fungal endophytes can produce phytohormones, the process of which is beneficial to host plants to counteract the adverse effects of abiotic stress [52]. IAA, an important plant hormone, plays an important function in plant responses to salt stress [53]. In our study, the content of IAA in PC- and BS-inoculated plant roots was significantly greater than that in the control plants regardless of the NaCl treatment. The SEM also showed that DSE species was the dominant impact factor and had a significant direct effect on the IAA content in the roots of *A. ordosica*. In previous studies, several DSE species were reported to produce IAA, thereby promoting plant growth and increasing plant stress tolerance [54, 55]. Qiang et al. [56] reported that inoculation with *Alternaria alternata* (LQ1230) increased the IAA content, increased the growth of wheat and increased drought tolerance. These results indicated that DSE may also promote the growth of *A. ordosica* by regulating the plant root IAA content, thereby improving the salt stress tolerance of host plants.

An appropriate $\text{Na}^+:\text{K}^+$ ratio in plant tissues represents an important basis for the normal physiological metabolism of plants [8]. Under salt stress, the substitution of Na^+ for K^+ in plants often results in an increased Na^+ content, which in turn damages cells [57]. In the present study, with increasing NaCl stress, the Na^+ content and $\text{Na}^+:\text{K}^+$ ratio in the shoots of *A. ordosica* tended to increase. However, no significant changes were observed between inoculated *A. ordosica* (AC, PC) and control plants. These results indicate that DSE did not seem to limit the translocation of Na^+ to the aboveground plant parts, which agrees with the results of Farias et al. [34]. In addition, the DSE significantly directly affected the Na^+ content in the roots of *A. ordosica*. Some DSE promoted the absorption of K^+ in the roots of *A. ordosica* and decreased the root Na^+ content regardless of NaCl stress. The significantly decreased Na^+ content in the roots inoculated with DSE may be related to DSE promoting the upregulation of RpsSOS1 in the roots and increased Na^+ export to the soil [57, 58]. Moreover, the SEM analysis showed that root biomass had direct negative effects on the Na^+ content in the roots. DSE promoted the growth of plant roots and caused a dilution effect, which may be an important reason for the decrease in Na^+ in the roots [59]. In addition, G - bacteria had direct negative effects on the Na^+ content in roots. These findings are consistent with those of Mohamed et al. [33], in which G - bacteria decreased the Na^+ in the roots of plants under salt stress. Mendpara et al. [60] also reported that most salt-tolerant bacteria are G - bacteria. Studies have shown that bacteria can secrete exopolysaccharides (EPSs) to bind excess Na^+ and restrict the influx of Na^+ into the roots. Moreover, these bacteria can also increase the absorption of K^+ and the export of Na^+ by the roots, thus improving plant salt tolerance [61].

Generally, salt stress causes oxidative damage to plant cells, thereby negatively affecting plant growth [62]. In this study, DSE enhanced the antioxidant enzyme activity in the leaves to remove reactive oxygen species (ROS) under salt-stress conditions. SOD, an important antioxidant enzyme, can eliminate ROS generated from oxidative damage [63]. Compared with the control plants, the plants inoculated with AC and BS presented increased SOD activity under NaCl stress, and the plants inoculated with PC presented

increased SOD activity only under 0.2% NaCl. Similar results have been reported by Pan et al. [33], in which DSE increased SOD activity in the leaves of plants under salt stress. As an antioxidant and a ligand peptide, GSH plays an important role in mitigating salt-induced damage [64]. In our study, BS inoculation increased the GSH content of plants under NaCl stress, and PC inoculation increased the GSH content in plants under 0.1% NaCl; however, an increase in GSH content in the AC-inoculated plants was detected only under 0.2% and 0.3% NaCl. Similar results in which DSE could activate GSH metabolism in *Zea mays* under heavy metal stress have been reported [65]. These results indicated that, by regulating the accumulation of antioxidants and the Na⁺:K⁺ balance, DSE can alleviate the adverse effects of NaCl stress on plants.

Plant symbiotic fungi normally play important roles in the regulation of the soil microenvironment [12, 66]. Here, DSE had direct negative influences on the soil AP and SOC content but significant direct influences on the soil AN content. BS and PC inoculation reduced the SOC and AP content under 0.1% and 0.3% NaCl, and AC inoculation decreased the SOC content under 0.3% NaCl. These findings indicated that DSE may regulate the balance of soil nutrients under NaCl stress. As a bridge between plants and the soil, DSE have been determined to convert soil organic carbon and nitrogen and insoluble phosphorus mineralization into effective forms, which can greatly expand plant organic nutrient pools [67–70]. Moreover, DSE may increase the contact area between plants and the soil through the extension of hyphae, increasing the absorption of nutrients by host plants [22, 23]. In addition, PC inoculation promoted the abundance of G – bacteria, actinomycetes, fungi and AM fungi in the soil under 0.2% and 0.3% NaCl, and AC inoculation increased the abundance of soil microbes under 0.3% NaCl, while BS increased the abundance of soil bacteria and AM fungi under 0.1% and 0.2% NaCl, respectively. These findings indicated that DSE increased the abundance of soil microorganisms under salt stress. Similar results have been reported by He et al. [15], in which DSE increased the abundance of fungi, bacteria and actinomycetes under drought stress. Previous research has also shown that fungi and G – bacteria have more adaptive capacity to stress environments, and G + bacteria may positively influence AM fungi propagation [60, 71, 72]. Actinomycetes are also an important part of the rhizosphere microbial community, which can promote plant growth and soil nutrient cycling [73]. In our study, DSE not only significantly directly affected the abundance of fungi and G + bacteria but also indirectly affected the abundance of G – bacteria and fungi by influencing the SOC content. These results indicated that DSE may release increased amounts of organic nutrients into the soil, thereby promoting the growth of salt-tolerant microorganisms. In addition, the influence of DSE on the composition and quantity of host plant root exudates may be another factor influencing the microbial community composition. Further studies associated with the effects of DSE on plant growth and soil microflora under salt-stress conditions will help us better understand the ecological functions of DSE in stressful ecosystems.

Conclusion

In the present study, three DSE isolated from *A. ordosica* could effectively colonize the roots of host plants under different NaCl concentrations. Although derived from the identical environment, these DSE

strains exerted considerably different effects on the growth of plants. The effect of DSE inoculation on plant function ranges from beneficial to neutral with increasing NaCl concentrations and depends on the DSE species and NaCl treatment. Under NaCl stress, inoculation with DSE reduced the Na⁺ content and Na⁺:K⁺ ratio in plant roots and increased the SOD activity, GSH content and IAA content, as well as the root growth of *A. ordosica*, which alleviated the negative effects of NaCl stress on the host. DSE also affected the rhizospheric soil microbial community by influencing the status of the SOC, AP and AN contents. Further investigations of the relationship between DSE from different sources and different plants are still needed to confirm the detailed ecological functions under salt-stress conditions.

Methods

Fungus and plant materials

Three dark septate endophytes (DSE) isolates were obtained from the roots of *Artemisia ordosica* grown in the Mu Us Sandy Land of Northwestern China. These DSE were identified as *Alternaria chlamydosporigena* (AC; MK246176), *Paraphoma chrysanthemicola* (PC; MF966391), and *Bipolaris sorokiniana* (BS; MK246180) on the basis of their morphological characteristics and on the basis of sequence analysis of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) [74]. The DSE strains have been deposited in the Plant Ecology Laboratory of Hebei University, China. All DSE strains were maintained in the dark at 27 °C on potato dextrose agar (PDA) media via subculturing every two weeks.

The seeds of *A. ordosica* were supplied and identified by the Desert Forest Experimental Center, Chinese Academy of Forestry, China. The details of the deposited *A. ordosica* specimens (PE02235073) were provided by Herbarium, Institute of Botany, Chinese Academy of Sciences. All the seeds were stored at 4 °C until pregermination.

Plant growth promotion experiment

A potted plant experiment was conducted in accordance with a randomized complete design (4 × 4) containing two factors: (1) DSE inoculation treatments, which involved inoculation with *A. chlamydosporigena* (AC), *P. chrysanthemicola* (PC), and *B. sorokiniana* (BS) and a noninoculated control (C) treatment; (2) NaCl stress treatments, which involved concentrations of 0%, 0.1%, 0.2% and 0.3% NaCl in the soil. Each treatment was replicated five times (three plants/pot/replicate). So there were total 120 experimental pots.

The seeds of *A. ordosica* were sterilized in ethanol (70%) for 3 min followed by sodium hypochlorite (2.5%) for 10 min, after which they were rinsed three times in sterilized water. The sterilized seeds were germinated on 10 g/L agar-water media at 27 °C. After that, three seedlings of uniform size were selected and transplanted into a sterile pot (11.5 cm height, 13.6 cm diameter at the top and 9.5 cm diameter at the base) containing 1,000 g of growth media, which consisted of a 1:1 (v/v) mixture of river sand and soil (< 2 mm). The soil was collected from farmland in North China. The growth media included

9.94 mg/g soil organic carbon (SOC), 16.55 mg/kg alkaline nitrogen (AN), and 11.26 mg/kg available phosphorus (AP). With respect to the DSE inoculation treatments, two 9 mm diameter discs obtained from the margins of an active colony were inoculated within 1 cm from the roots of *A. ordosica* seedlings. With respect to the noninoculated treatments, two 9 mm discs were removed from PDA medium without any fungi. All plants were grown in a greenhouse at 27 °C/22 °C day/night temperature, 14 h/10 h photoperiod and 60% mean relative humidity.

Thirty days after inoculation, NaCl stress treatments were imposed by the application of NaCl solutions to the pots. To avoid osmotic shock, NaCl stress was applied in increments [75]; that is, each pot received 50 mL of 0.34 mol/L NaCl solutions at 5-day intervals successively. Fifty milliliters of sterile water without NaCl was applied to the controls each time to ensure consistent soil moisture. NaCl solutions were applied such that the desired 0%, 0.1%, 0.2% and 0.3% NaCl levels were reached [76]. Finally, the plants were harvested after 60 days of treatment.

Plant biomass and morphological parameters

Prior to harvest, the plant height and shoot branching in each replicate (three plants/pot) were recorded. The roots and shoots in each replicate were subsequently harvested separately. The cleaned roots were scanned utilizing an Epson Perfection V800 Photo scanner. The root morphological parameters, including root length, root volume, root diameter and root surface area, were measured using the WinRHIZO image analysis system (Regent Instruments, Quebec, Canada). Fresh roots were subsequently collected and used to estimate the DSE colonization levels and indoleacetic acid (IAA) concentration. Similarly, fresh leaves were used to measure physiological parameters, such as superoxide dismutase (SOD) activity and glutathione (GSH) content. The remaining fresh shoots and roots were dried to a constant weight at 70 °C to confirm the plant biomass and the Na⁺ and K⁺ contents. Soil samples from each pot were sieved (2-mm mesh size) and then split into two parts: one subsample was stored at -80 °C to analyze the community composition of soil microbes, and the other subsample was dried at room temperature to analyze soil physicochemical properties.

DSE colonization analyses

Fresh roots of *A. ordosica* were randomly sampled from each pot to determine DSE colonization levels. First, the roots were cleaned under running water. The root segments (0.5 cm) were clarified in potassium hydroxide (10%, w/v) and then dyed in acid fuchsin (0.5%, w/v) according to the methods of Phillips and Hayman [77]. Thirty root segments of each sample were randomly selected and pressed onto slides, and then observed under a light microscope (Olympus BX51) with 20 × and 40 × eyepieces [78]. The DSE total colonization rate (%) was expressed as the percentage of infected root segments per root sample.

Determination of SOD activity and GSH content

The activity of SOD in the leaves was assayed by measuring the diminution in the optical density of the nitro blue tetrazolium (NBT) complex according to Elavarthi and Martin [79]. Briefly, fresh leaves (0.2 g) were ground together with phosphate buffered saline (PBS) (50 mM, 5 mL; pH 7.8) in an ice bath and

then centrifuged for 10 min at $4,000 \times g$ and $4 \text{ }^\circ\text{C}$. The reaction solution (5 mL) contained 0.1 mL of enzyme extract, 30 mL of riboflavin ($33 \text{ } \mu\text{mol/L}$), 0.3 mL of NBT (1.25 mmol/L), 0.3 mL of methionine (220 mmol/L) and 4 mL of PBS (50 mM). One unit of SOD was defined as enzyme activity that inhibited NBT reduction by 50% at 560 nm.

The GSH content was assayed according to the dithiobis-nitrobenzoic acid (DTNB) method described by Anderson [80]. Briefly, fresh leaves (0.2 g) were ground together with sulfosalicylic acid (5%, w/v) at $4 \text{ }^\circ\text{C}$. The crude extract was subsequently centrifuged for 10 min at $10,000 \times g$. The reaction mixture consisted of PBS (100 mM , 0.6 mL), DTNB ($40 \text{ } \mu\text{L}$) and the supernatant (0.5 mL). The optical density of the supernatant was determined at 421 nm. The standard curve was used to calculate GSH content.

Measurement of IAA content

The IAA content in the roots was estimated by an IAA ELISA Kit (MLBIO, Shanghai, China). Fresh roots (approximately 100 mg) were homogenized in a precooled mortar ($4 \text{ }^\circ\text{C}$) containing PBS (0.01 mol/L , 1 mL; $\text{pH} = 7.2\text{--}7.4$). The supernatant was collected after centrifugation at $3,000 \times g$ for 20 min at $4 \text{ }^\circ\text{C}$. The supernatant ($10 \text{ } \mu\text{L}$) was used to determine the IAA content, and the procedures were conducted in accordance with the instructions provided by the manufacturer. The absorbance was detected by the use of an Epoch 2 microplate reader (BioTek, Winooski, USA) at a wavelength of 450 nm. The IAA content in the roots was determined in terms of the standard curve.

Determination of Na^+ and K^+ contents

Dried powdery samples (approximately 0.1 g) of shoots and roots were immersed in $\text{HNO}_3\text{:HClO}_4$ (3:1, v/v) solution and heated to $220 \text{ }^\circ\text{C}$ for digestion to extract Na^+ and K^+ ions. The cooled digestion solution was diluted to 25 mL by adding deionized water, after which the Na^+ and K^+ contents were measured by inductively coupled plasma optical emission spectrometry (ICP-OES; Beijing Ruiguang Technology Co., Ltd., China).

Soil physicochemical properties analysis

The SOC content was measured according to the potassium dichromate oxidation method [81, 82]. A dried soil sample (approximately 1 g) was placed in a tube containing $\text{K}_2\text{Cr}_2\text{O}_7$ solution (0.8 mol/L , 5 mL) and H_2SO_4 (98%, 5 mL) and then boiled for 5 min at $170 \text{ }^\circ\text{C}$. After cooling, the solution was transferred to a conical flask, and the volume was adjusted to 60 mL by dilution with distilled deionized water. The excess dichromate was subsequently titrated with 0.2 mol/L FeSO_4 with diphenylamine used as an indicator.

The AN content was determined according to the alkaline hydrolysis diffusion method [82]. Approximately 2 g of dried soil sample was uniformly distributed in the outer chamber of the diffusion dish. H_3BO_3 indicator (20 g/L , 2 mL) and NaOH (1 mol/L , 10 mL) were added to the inner and outer chambers, respectively. The diffusion dishes were then sealed and incubated at $40 \text{ }^\circ\text{C}$ for 24 h. Titration was subsequently performed via 0.01 mol/L H_2SO_4 solution.

The AP content was measured by the chlorostannus-reduced molybdophosphoric blue color method according to the methods of Olsen et al. [83]. Briefly, dried soil samples (approximately 1 g) were placed into a conical flask containing both NaHCO₃ solution (0.5 mol/L, 50 mL) and phosphor-free activated carbon (0.1 g). After the mixture had been shaken for 30 min, the filtrate (10 mL) and molybdenum vanadate solution (5 mL) were mixed together, followed by dilution with distilled deionized water to 50 mL. The mixed reagent was then nurtured for 30 min, after which the absorption values were determined at 700 nm via a spectrometer.

Soil microbial community composition

The composition of the rhizosphere soil microbial community was determined by phospholipid fatty acid (PLFA) analysis. Briefly, soil subsamples (approximately 8.0 g, frozen) were placed into a tube containing 23 mL of chloroform:methanol:phosphate buffer (1:2:0.8 v/v/v) solution to extract lipids according to the method of Bossio and Scow [84]. The extracts were separated by continuous elution with chloroform (5 mL), acetone (20 mL) and methanol (5 mL) on silica acid columns. Then, the organic solvent was evaporated using a nitrogen blowing instrument. The phospholipids were sequentially saponified and methylated to form fatty acid methyl esters (FAMES).

The gas chromatograph (Agilent 6890 N) in conjunction with MIDI software (MIDI, Newark, Delaware, USA) was used to identify and quantify the extracted FAMES. The software package Sherlock MIS 4.5 could automatically control the gas chromatograph for calibration, subsequent sample sequencing, naming, and peak integration operations. The mixture of straight-chain saturated and hydroxy FAMES with a length of 10–20 carbons (MIDI Part No. 1208) was used as the calibration standard. Gram-positive (G+) bacterial biomarkers included 14:1 iso w7c, 14:0 iso, 14:0 anteiso, 15:1 iso w9c, and 15:1 iso w6c, and gram-negative (G-) bacterial biomarkers included 14:1 w9c, 14:1 w8c, 14:1 w7c, 14:1 w5c, 15:1 w9c, 15:1 w8c, 15:1 w7c, and 15:1 w6c. The actinomycete biomarkers included 16:0 10-methyl, 17:1 w7c 10-methyl, 17:0 10-methyl, and 18:1 w7c 10-methyl. The fungal biomarkers contained 18:2 w6c and 18:1 w9c. The fatty acid 16:1 w5c was determined to be a biomarker for AM fungi [15].

Statistical analysis

Two-way ANOVA was applied to evaluate the influences of DSE inoculation, NaCl stress and interactions on biomass production, morphological characteristics, physiological parameters, soil factors and microbial community composition in the rhizospheric soil of each plant. The partial eta squared (η_p^2) value represented the effect size of the different factors. Duncan's multivariate range test was used to assess the differences in means between the different treatments ($P < 0.05$). Structural equation modeling (SEM) was constructed to determine the causal relationships between DSE species, NaCl stress and soil factors on the growth of *A. ordosica* and the rhizospheric microbial composition via AMOS 21.0. Before constructing the SEM, Spearman's correlation analysis was conducted to evaluate the interrelationships among the variables via SPSS 19.0 software. The statistical analysis and figure plotting were performed by SPSS 19.0, AMOS 21.0 and Kaleida Graph 4.5.

Abbreviations

DSE: Dark septate endophytes; PC: *Paraphoma chrysanthemicola*; BS: *Bipolaris sorokiniana*; AC: *Alternaria chlamydosporigena*; C: Noninoculated control; SOD: Superoxide dismutase; GSH: Glutathione; IAA: Indoleacetic acid; SOC: Soil organic carbon; AP: Available phosphorus; AN: Alkaline nitrogen; AM: Arbuscular mycorrhizal; G – bacteria: Gram-negative bacteria; G + bacteria: Gram- positive bacteria; SEM: Structural equation modeling; TLI: Tacker-Lewis index; CFI: Comparative fit index; RMSEA: Root mean square error of approximation; EPSs: Exopolysaccharides; ROS: Reactive oxygen species; ITS: Internal transcribed spacer; PDA: Potato dextrose agar; NBT: Nitro blue tetrazolium; PBS: Phosphate buffered saline; DTNB: Dithiobis-nitrobenzoic acid; PLFA: Phospholipid fatty acid; NLFAs: Neutral lipid fatty acids; FAMES: Fatty acid methyl esters; η_p^2 : The partial eta squared

Declarations

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Authors' Contributions

LH, LZ and XH conceived and designed the experiments. LH, YZ and DZ performed the experiments. LH analyzed the data. XH, XL and LH wrote the manuscript. LH and XL contributed equally to this manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

A potted plant experiment in this study was performed in a greenhouse. We confirmed that the sampling did not involve any endangered or protected plant species and declare that the work reported here complies with the current laws of China and the IUCN Policy Statement on Research Involving Species at Risk of Extinction.

Consent to publish

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

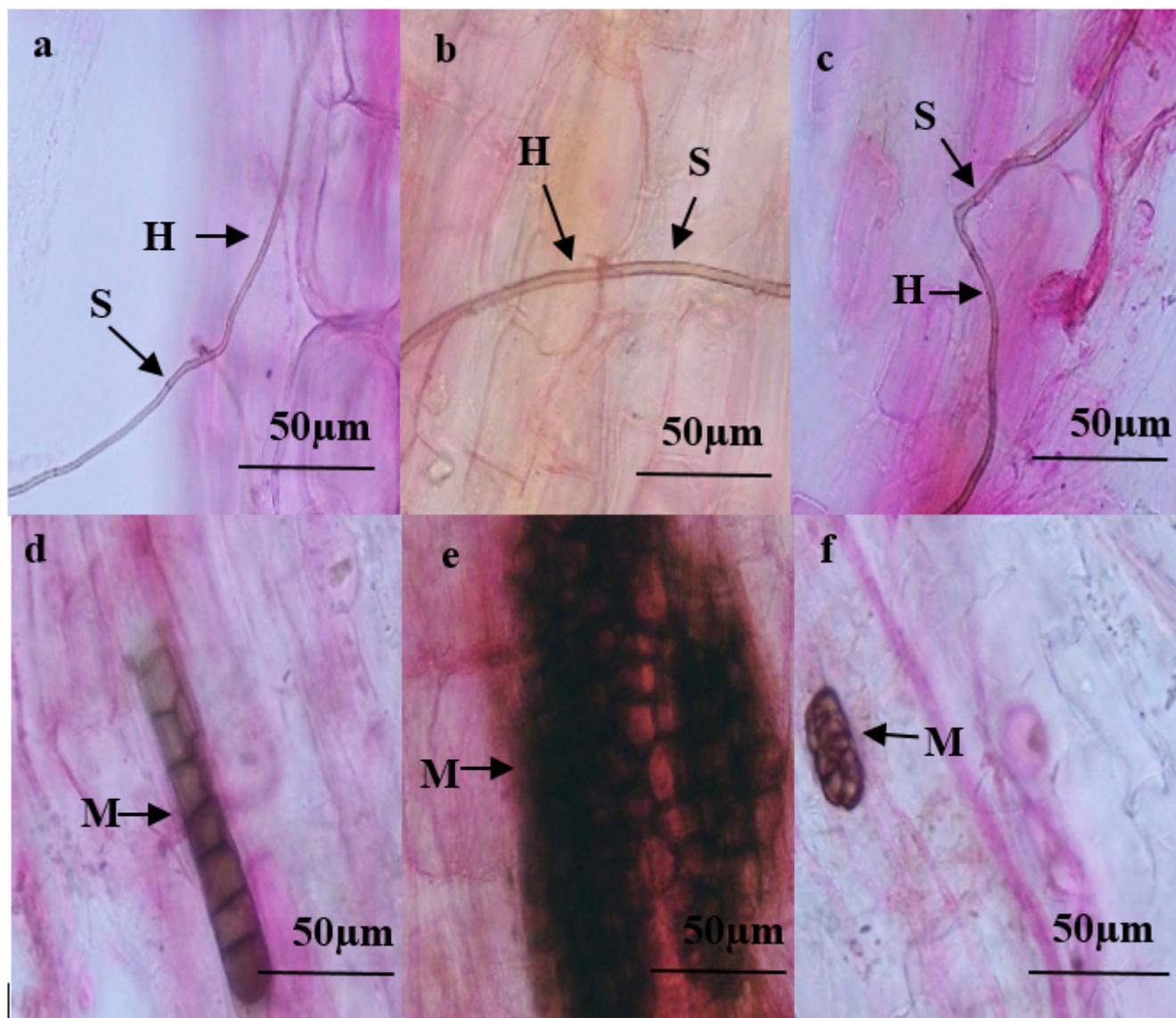


Figure 1

Colonization of dark septate endophytes (DSE) in the roots of inoculated *Artemisia ordosica*. Arrows indicate: H=DSE hyphae, M=DSE microsclerotia, S= DSE hyphal septa. Scale bars=50 μm.

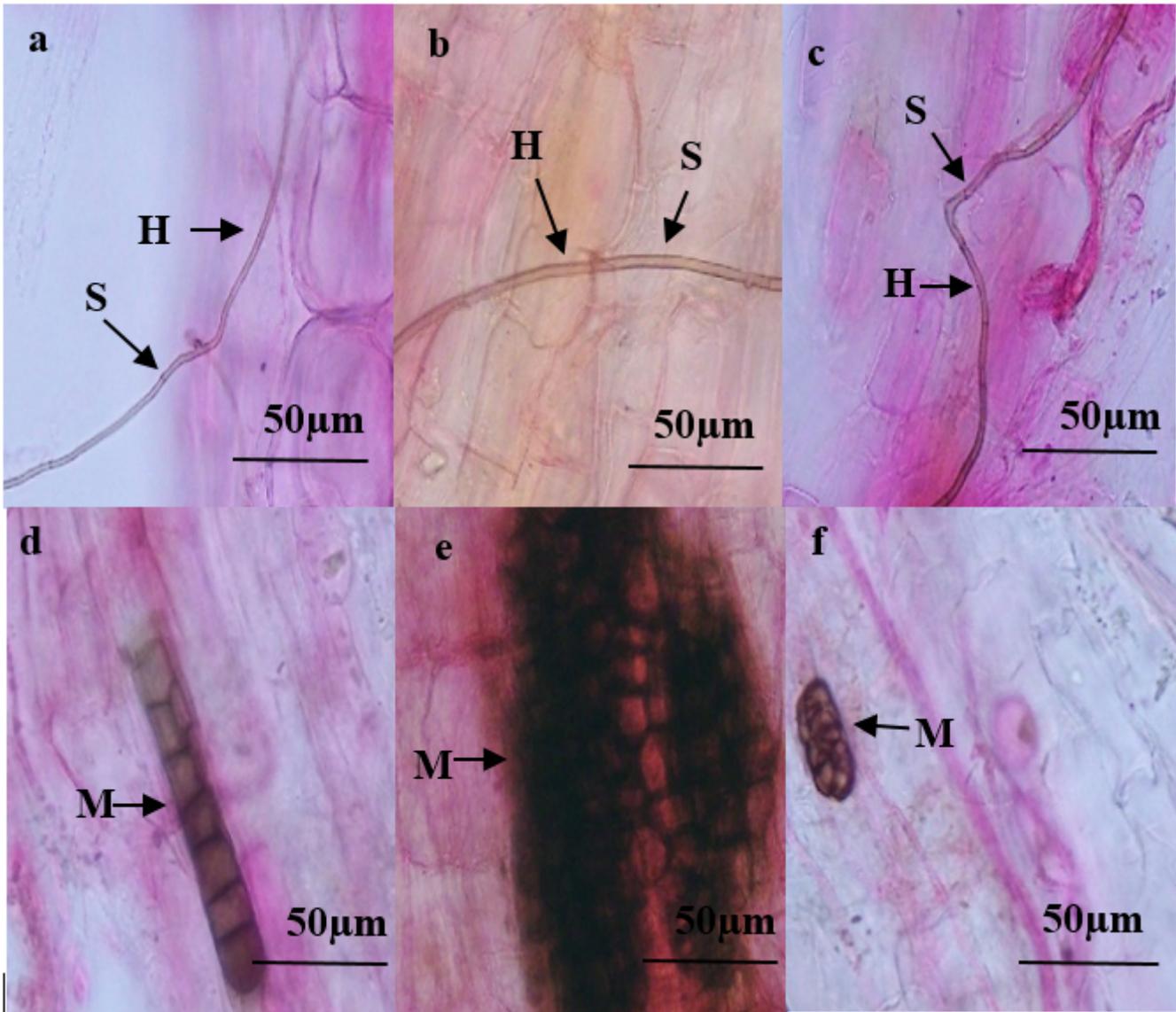


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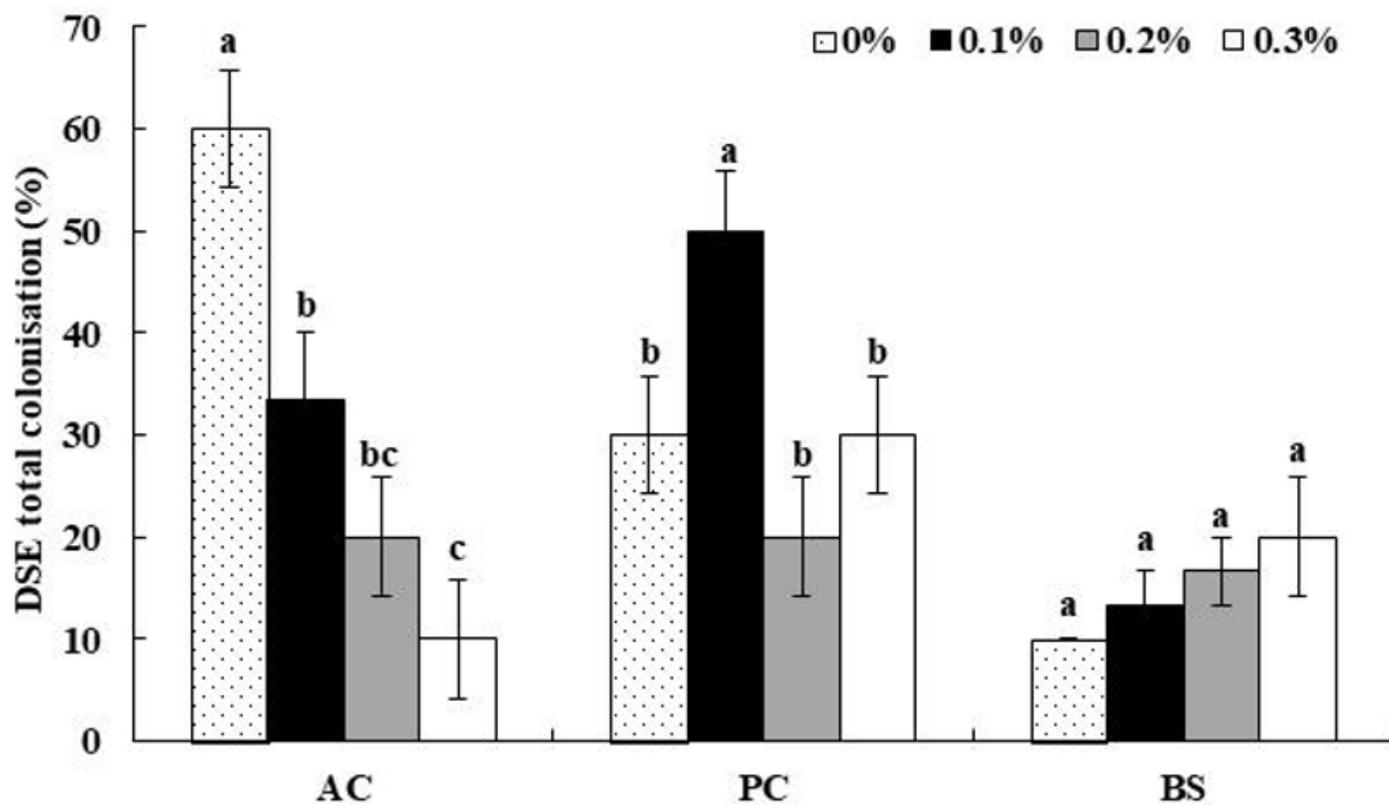


Figure 2

Total colonization rate of dark septate endophytes (DSE) in the roots of inoculated *Artemisia ordosica* under NaCl stress. The error bars represent the standard errors (SE) of the means. The different letters above the error bars indicate significant differences at $P < 0.05$ according to Duncan's multiple-range test. 'AC' indicates plants inoculated with *Alternaria chlamydosporigena*. 'PC' indicates plants inoculated with *Paraphoma chrysanthemicola*. 'BS' indicates plants inoculated with *Bipolaris sorokiniana*.

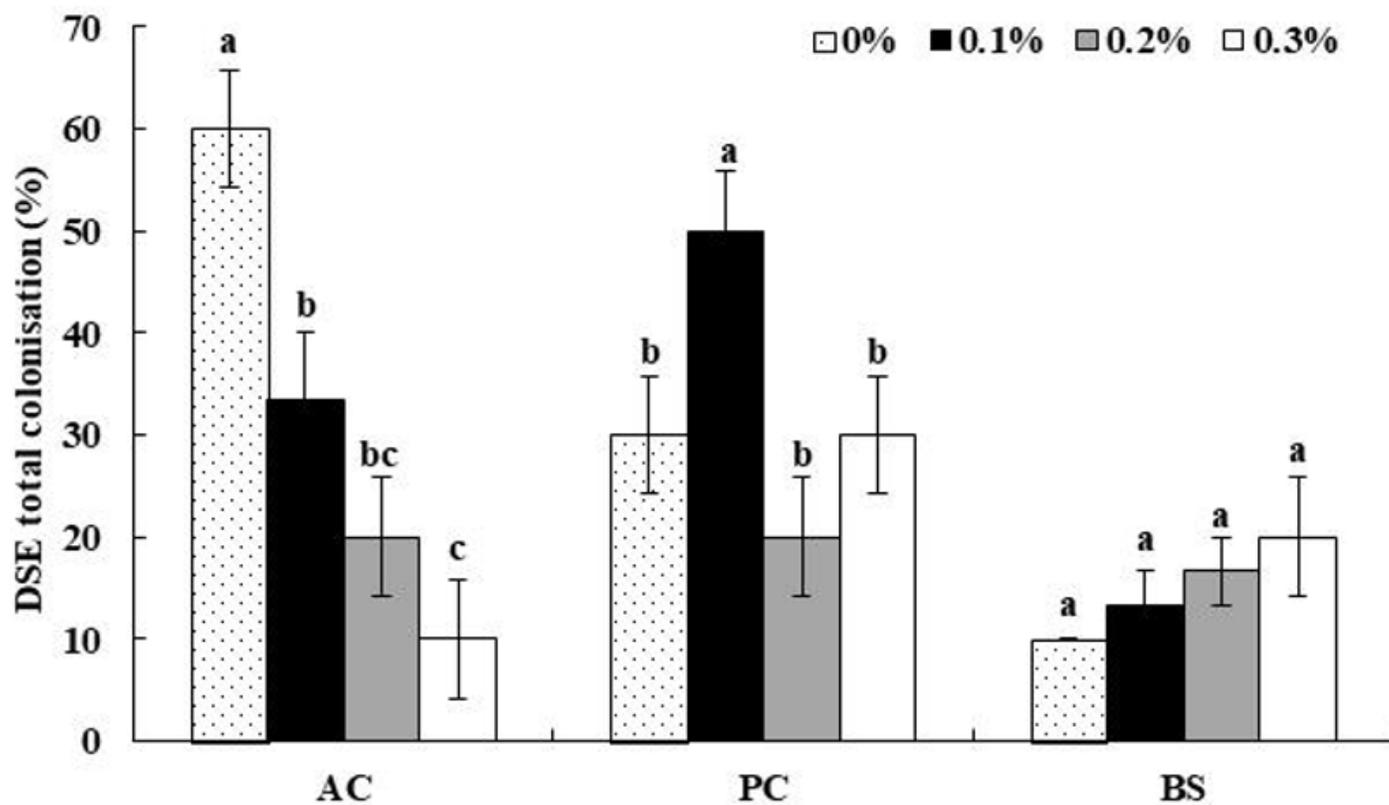


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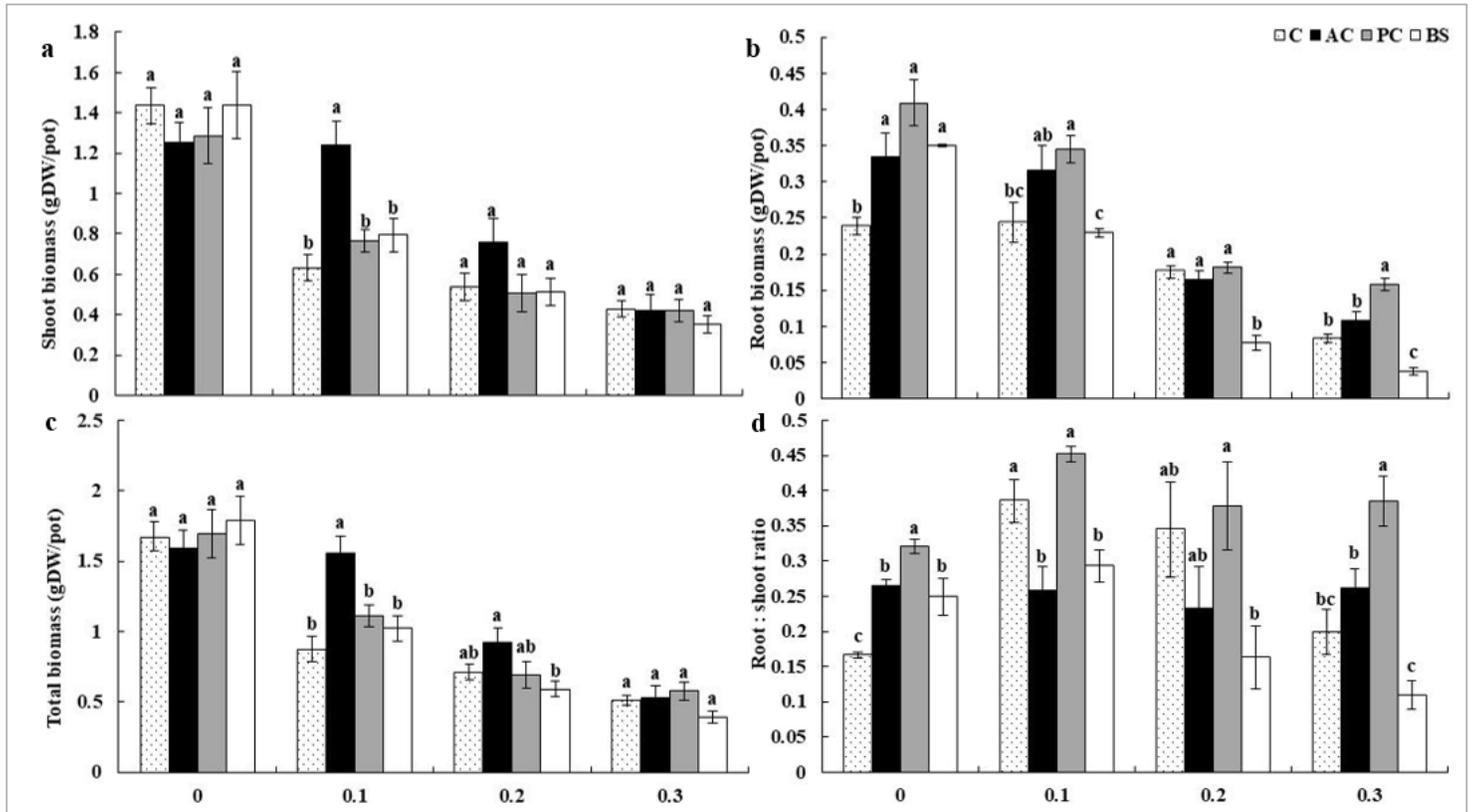


Figure 3

The effects of dark septate endophytes (DSE) inoculation and NaCl stress on the biomass production and root:shoot ratio of *Artemisia ordosica*. The error bars represent the standard errors (SE) of the means. The different letters above the error bars indicate significant differences at $P < 0.05$ according to Duncan's multiple-range test. 'C' indicates noninoculated plants. 'AC' indicates plants inoculated with *Alternaria chlamyosporigena*. 'PC' indicates plants inoculated with *Paraphoma chrysanthemicola*. 'BS' indicates plants inoculated with *Bipolaris sorokiniana*. DW indicates dry weight.

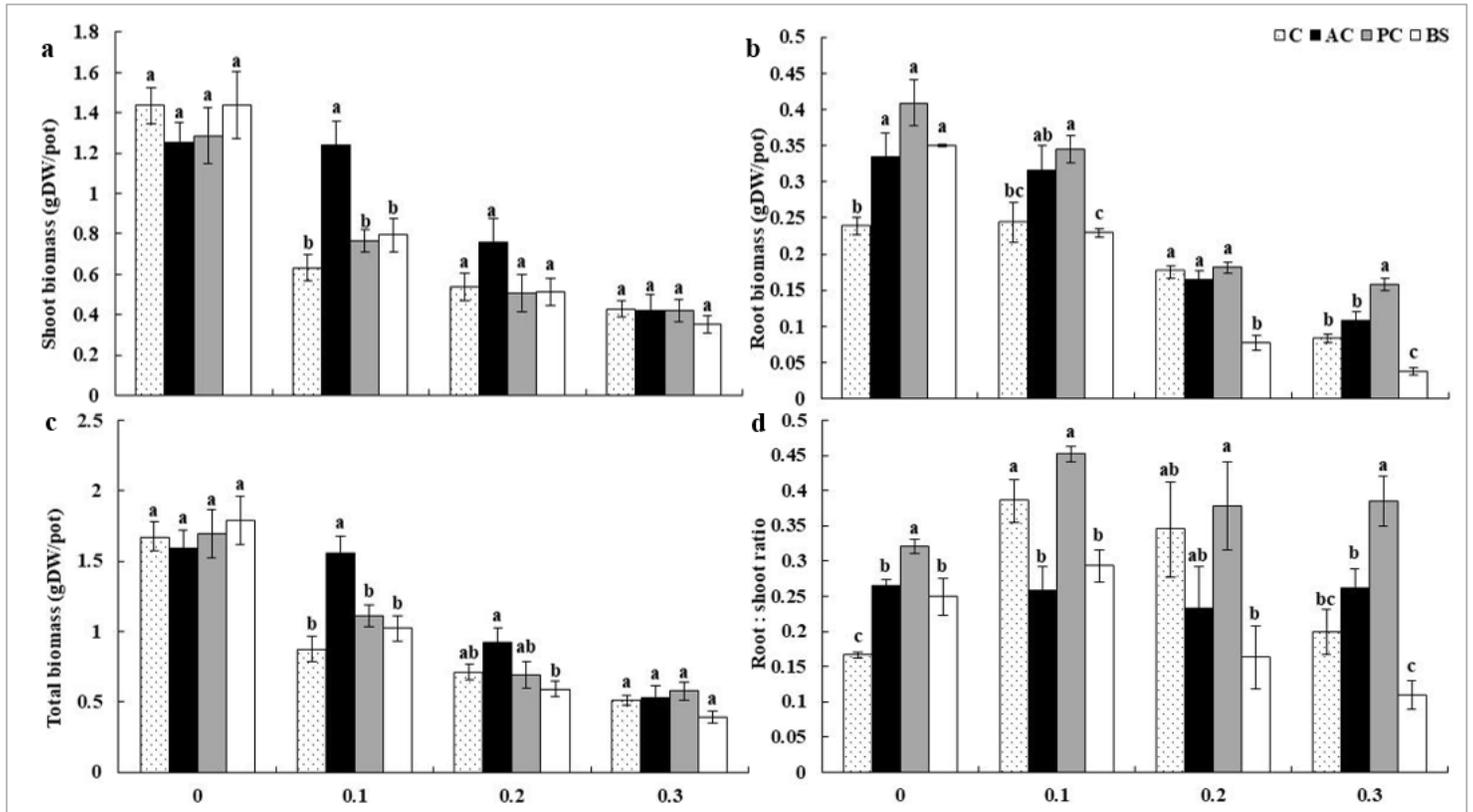


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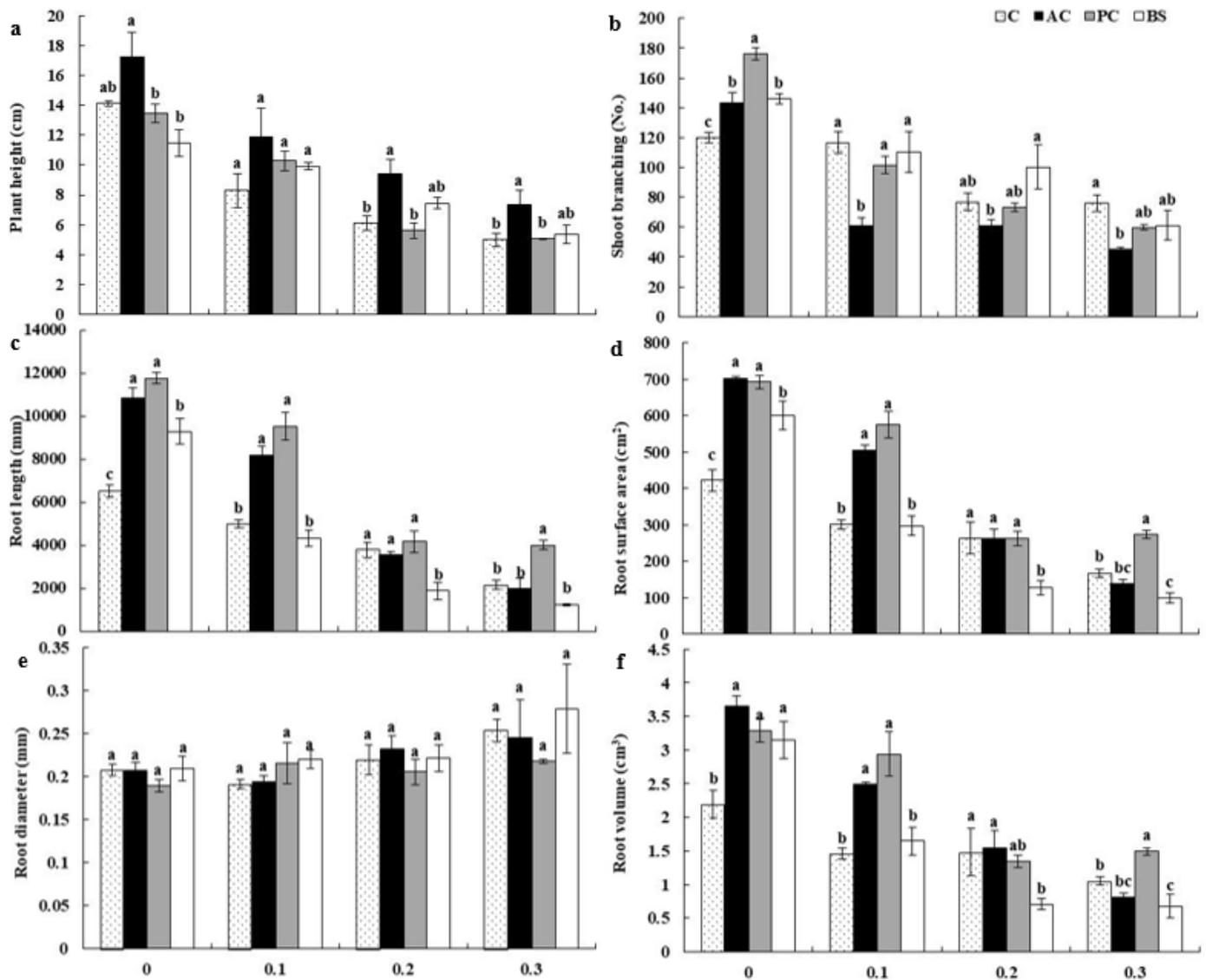


Figure 4

The effects of dark septate endophytes (DSE) inoculation and NaCl stress on the morphology of *Artemisia ordosica*. The error bars represent the standard errors (SE) of the means. The different letters above the error bars indicate significant differences at P < 0.05 according to Duncan's multiple-range test. 'C' indicates noninoculated plants. 'AC' indicates plants inoculated with *Alternaria chlamydosporigena*. 'PC' indicates plants inoculated with *Paraphoma chrysanthemicola*. 'BS' indicates plants inoculated with *Bipolaris sorokiniana*.

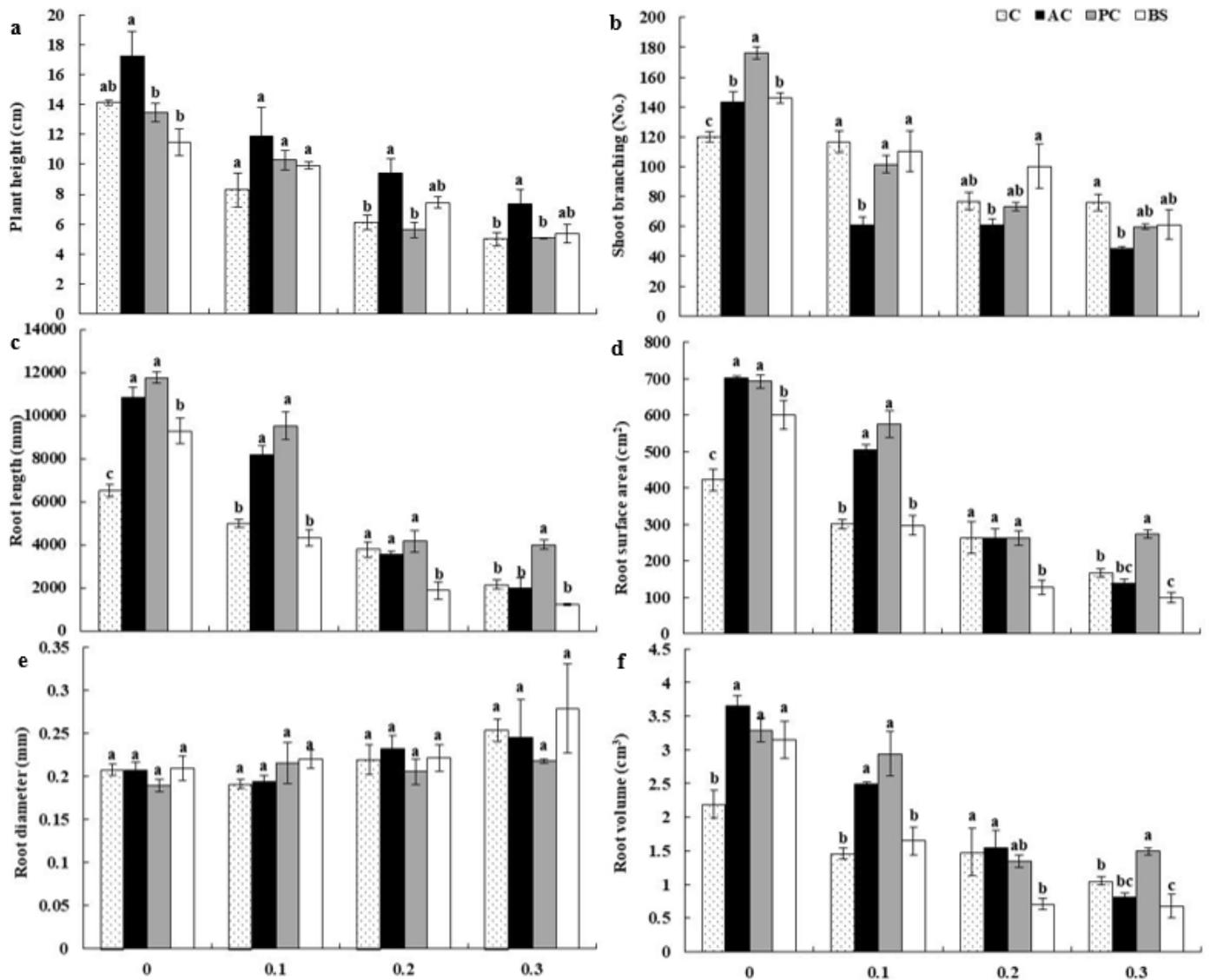


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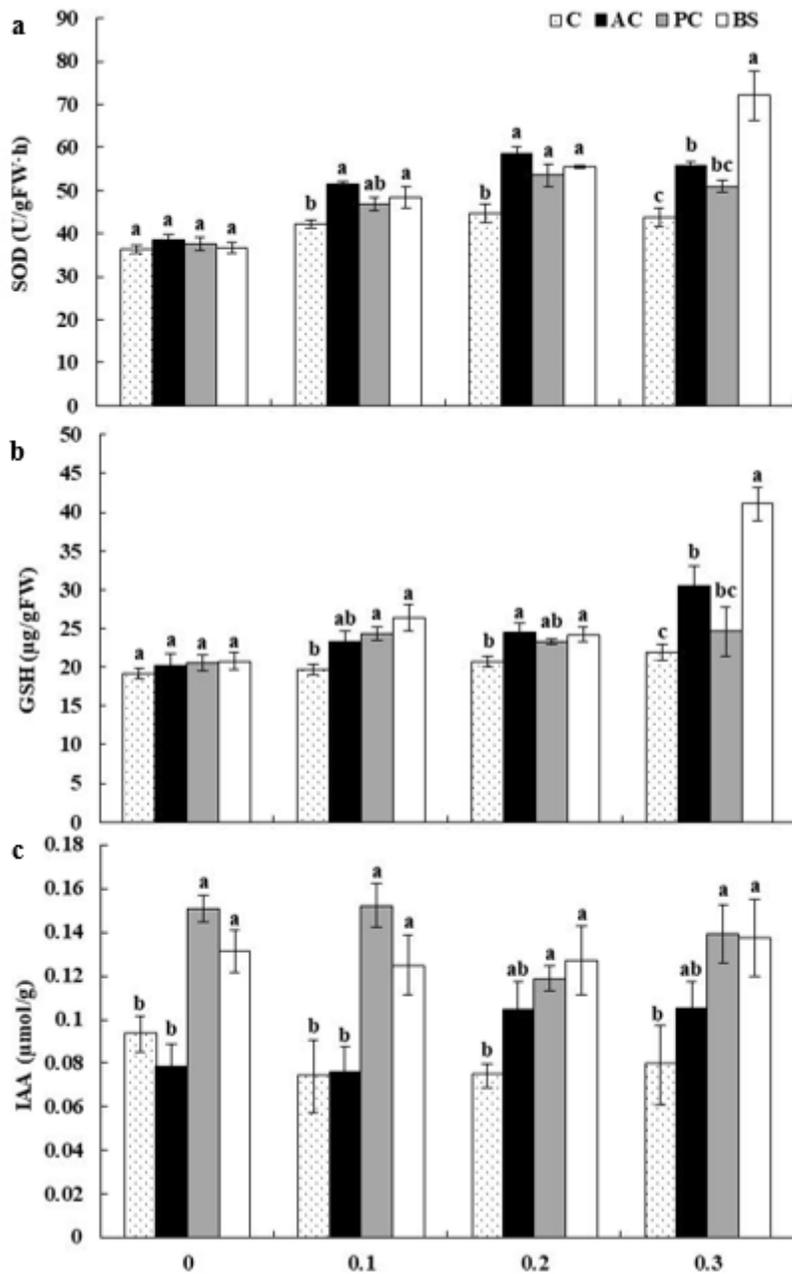


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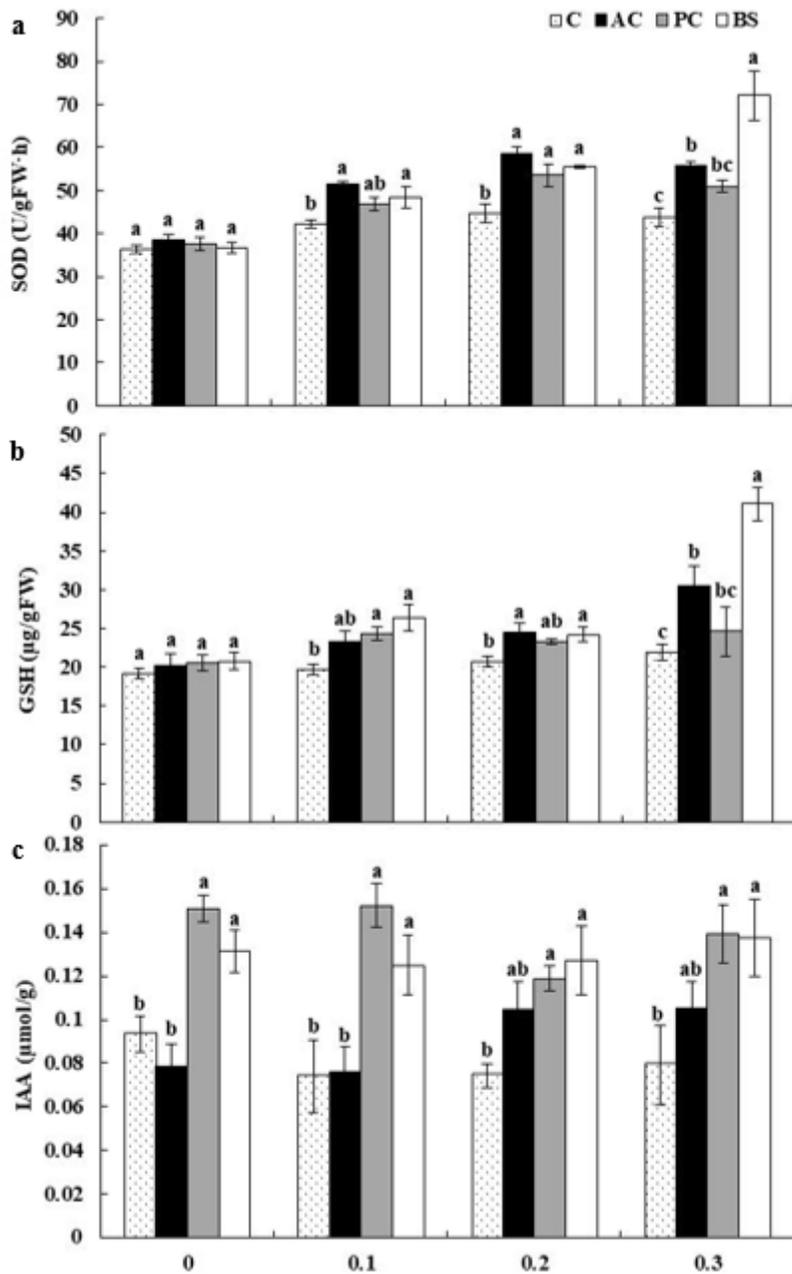


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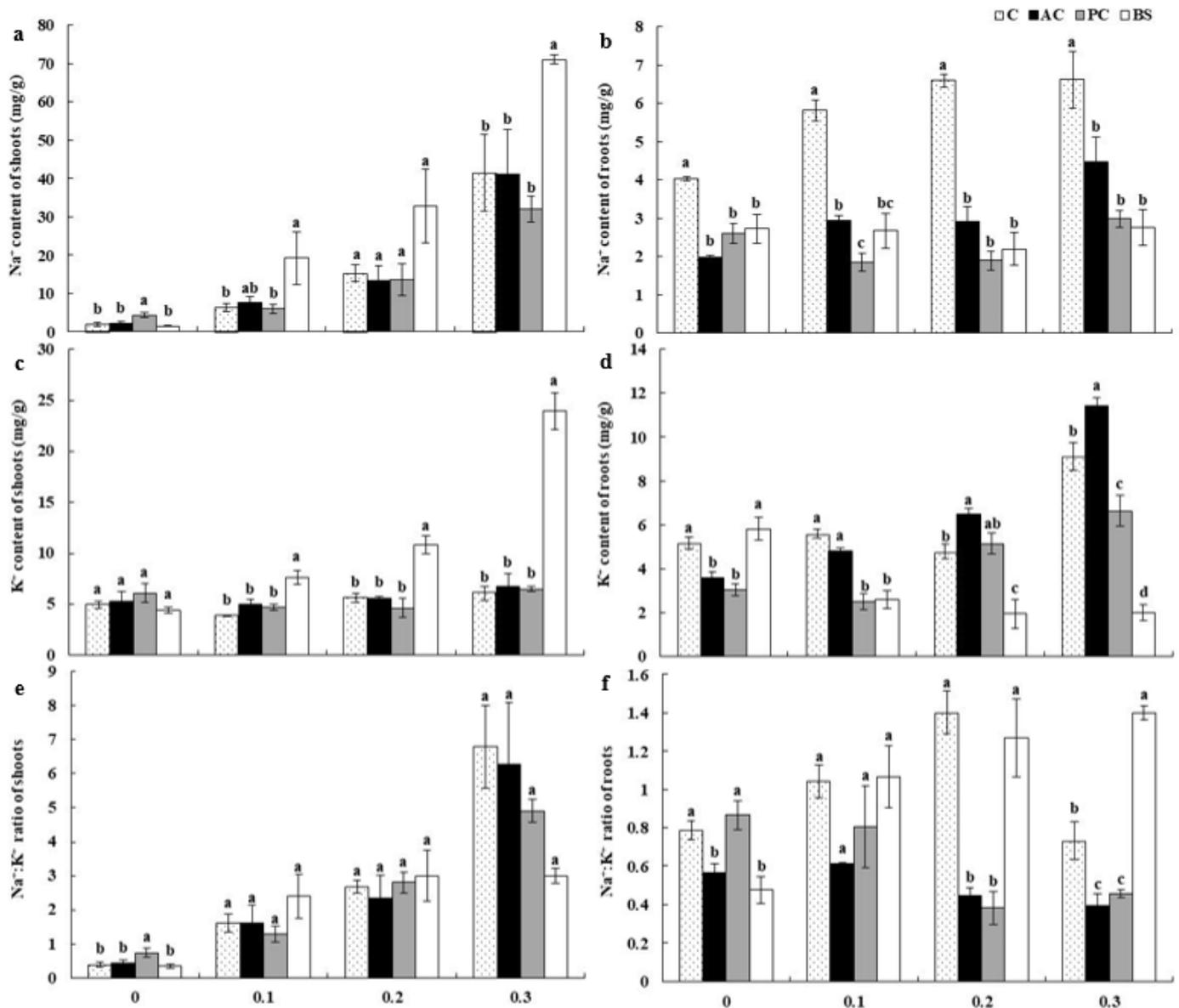


Figure 6

The effects of dark septate endophytes (DSE) inoculation and NaCl stress on the Na⁺ and K⁺ contents and Na⁺:K⁺ ratio of *Artemisia ordosica*. The error bars represent the standard errors (SE) of the means. The different letters above the error bars indicate significant differences at $P < 0.05$ according to Duncan's multiple-range test. 'C' indicates noninoculated plants. 'AC' indicates plants inoculated with *Alternaria chlamydosporigena*. 'PC' indicates plants inoculated with *Paraphoma chrysanthemicola*. 'BS' indicates plants inoculated with *Bipolaris sorokiniana*.

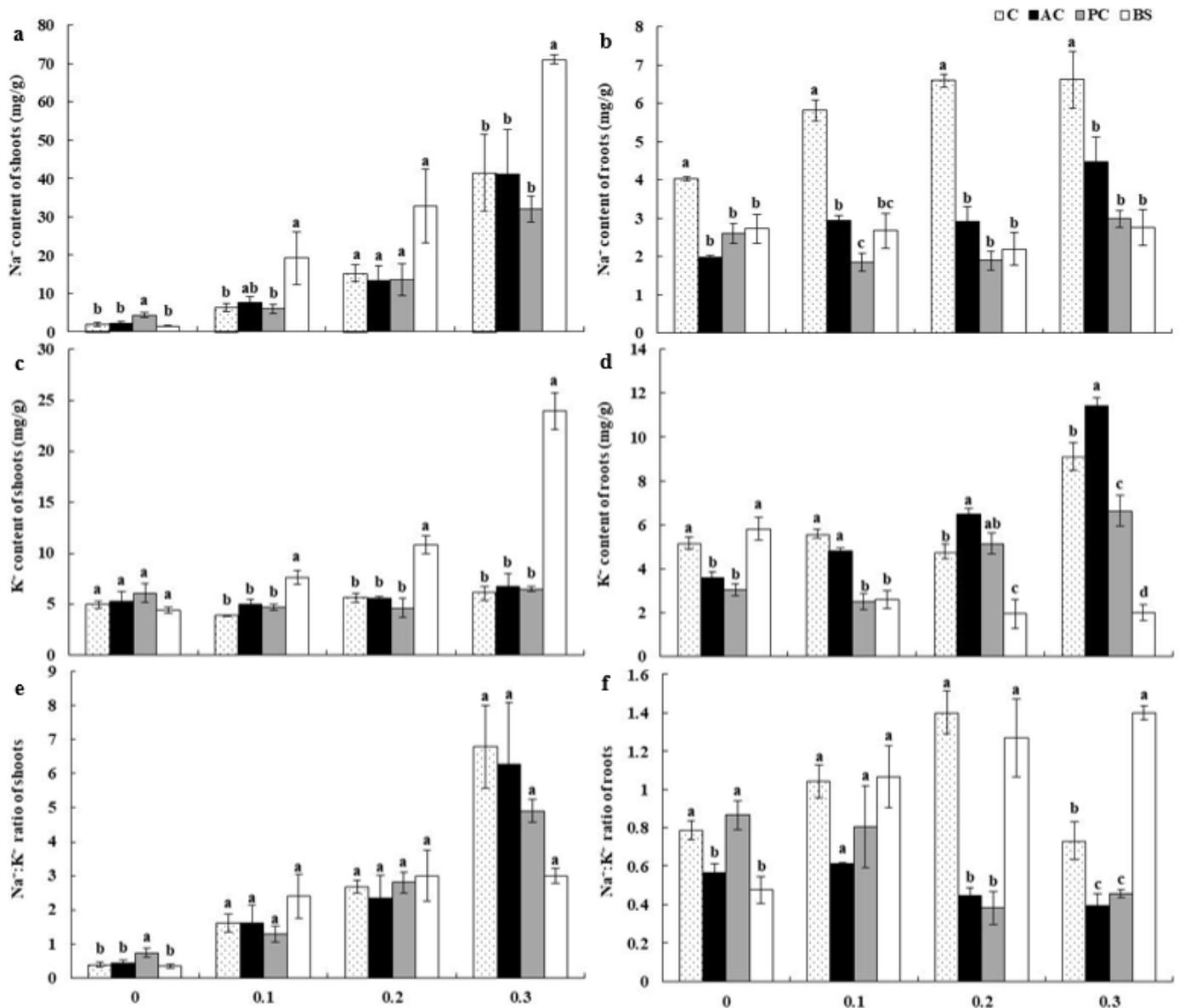


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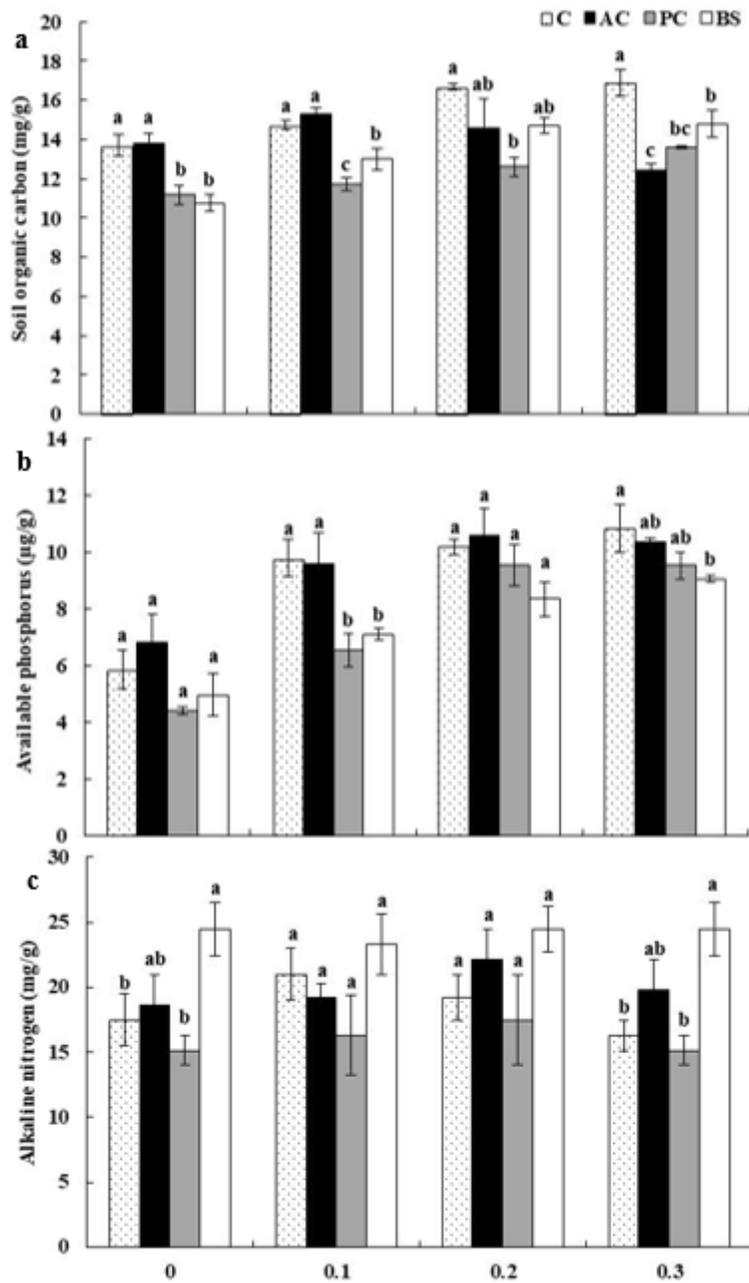


Figure 7

Effects of dark septate endophytes (DSE) inoculation and NaCl stress on the soil physicochemical properties in rhizosphere of *Artemisia ordosica*. The error bars represent the standard errors (SE) of the means. The different letters above the error bars indicate significant differences at $P < 0.05$ according to Duncan's multiple-range test. 'C' indicates noninoculated plants. 'AC' indicates plants inoculated with *Alternaria chlamyosporigena*. 'PC' indicates plants inoculated with *Paraphoma chrysanthemicola*. 'BS' indicates plants inoculated with *Bipolaris sorokiniana*.

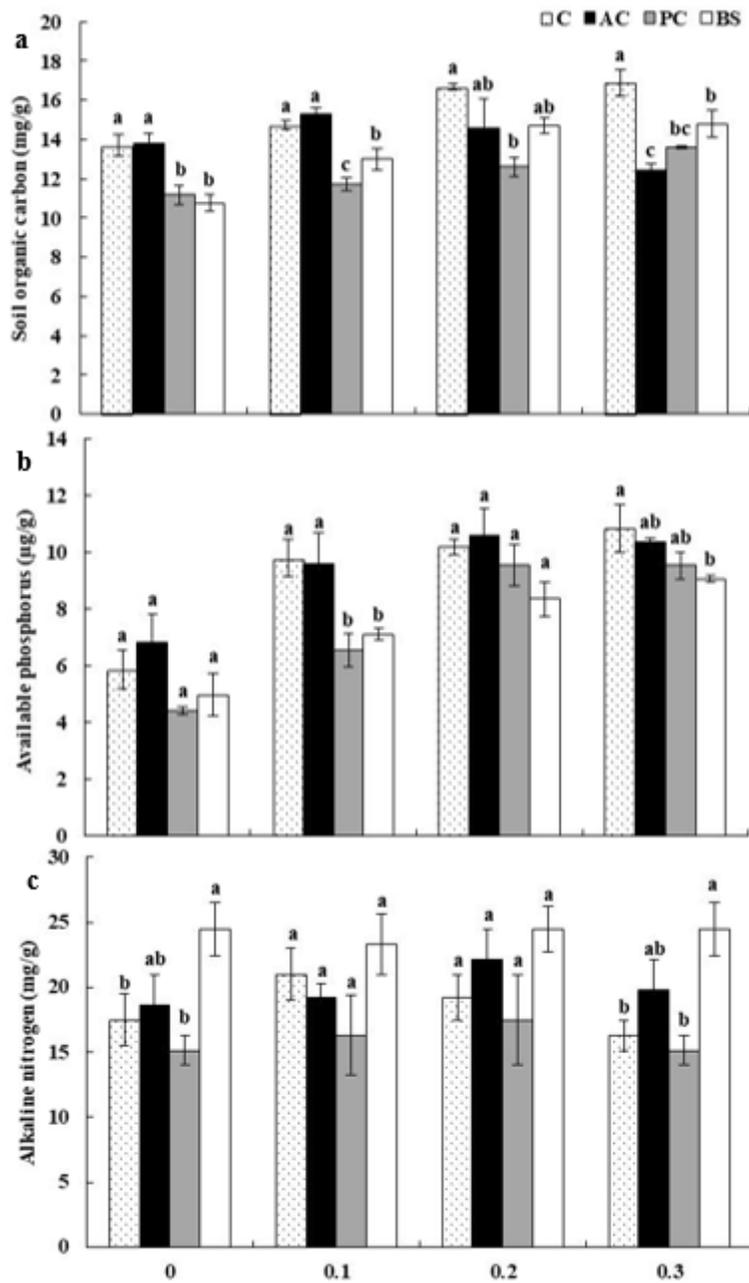


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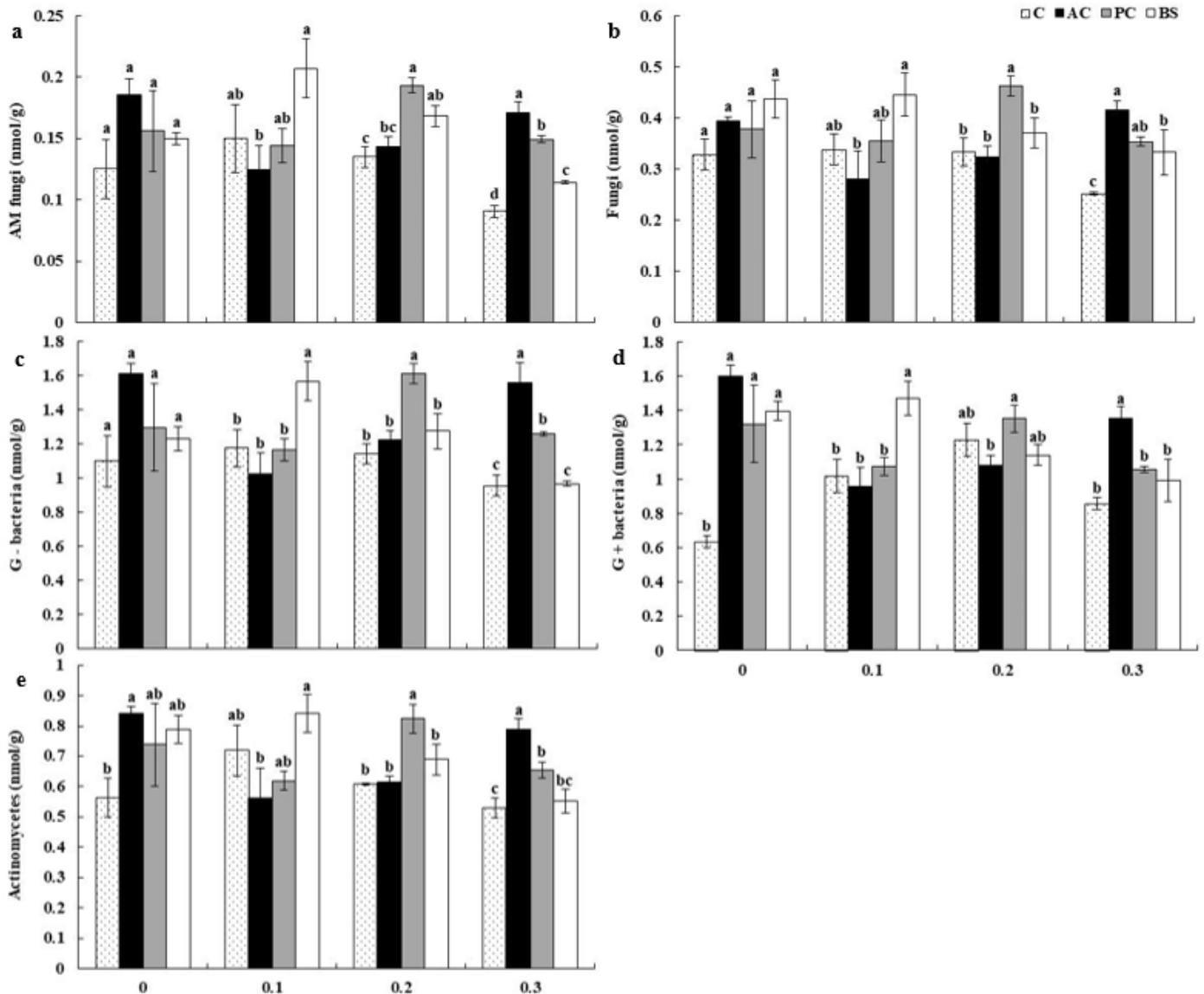


Figure 8

Effects of dark septate endophytes (DSE) inoculation and NaCl stress on the soil microbial community composition in the rhizosphere of *Artemisia ordosica*. The error bars represent the standard errors (SE) of the means. The different letters above the error bars indicate significant differences at $P < 0.05$ according to Duncan's multiple-range test. 'C' indicates noninoculated plants. 'AC' indicates plants inoculated with *Alternaria chlamyosporigena*. 'PC' indicates plants inoculated with *Paraphoma chrysanthemicola*. 'BS' indicates plants inoculated with *Bipolaris sorokiniana*.

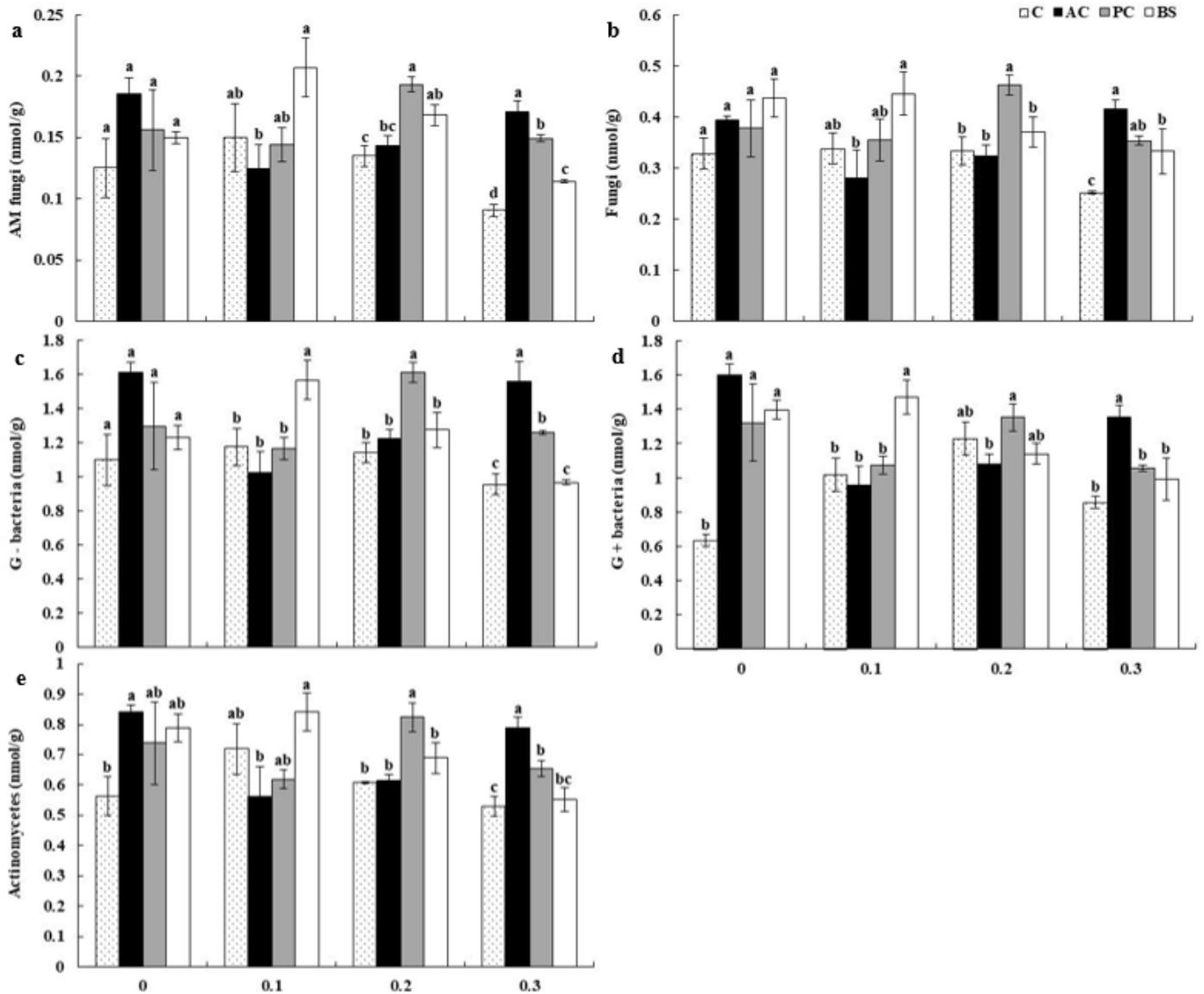


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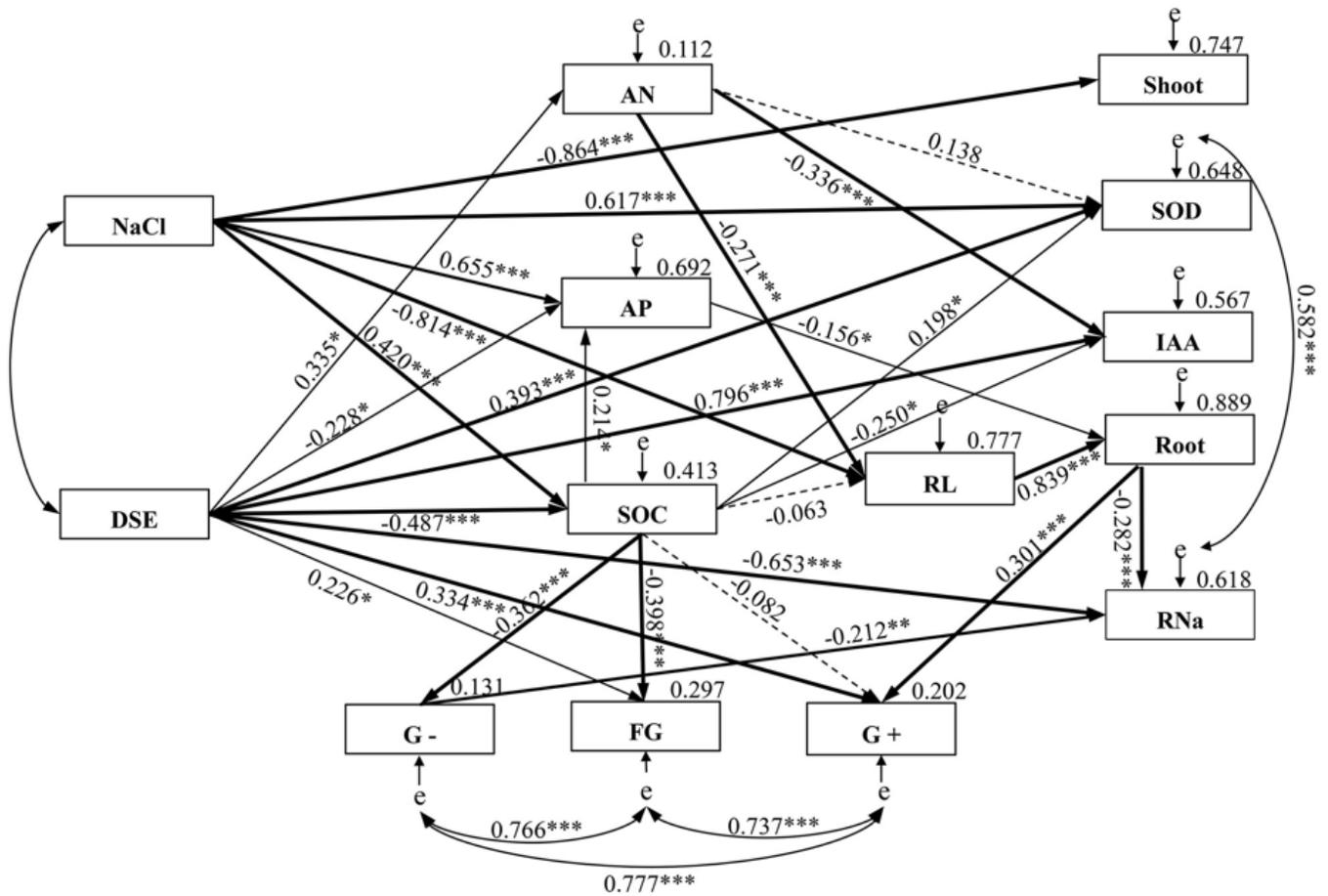


Figure 9

Structural equation model (SEM) showing the causal relationships among DSE species, NaCl stress, soil factors, the soil microbial community, and the growth parameters of *Artemisia ordosica*. The final model fit the data well: maximum likelihood, $\chi^2 = 78.527$, $df = 60$, $P = 0.055$, Tacker-Lewis index (TLI) = 0.953, Comparative fit index (CFI) = 0.969, and Root mean square error of approximation (RMSEA) = 0.081. The solid lines and dashed lines indicate significant and nonsignificant pathways, respectively. The width of the solid lines indicates the strength of the causal effect, and the numbers near the arrows indicate the standardized path coefficients. Significant values are indicated by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$). The numbers in the upper-right corner of the box indicate the R² values and represent the proportion of variance explained for each variable. SOC = soil organic carbon. AP = soil available phosphorus. AN = soil alkaline nitrogen. G - = gram-negative bacteria. FG = fungi. G + = gram-positive bacteria. Shoot = shoot biomass. Root = root biomass. SOD = superoxide dismutase activity. IAA = indoleacetic acid content. RL = root length. RNa = Na⁺ content in the root.

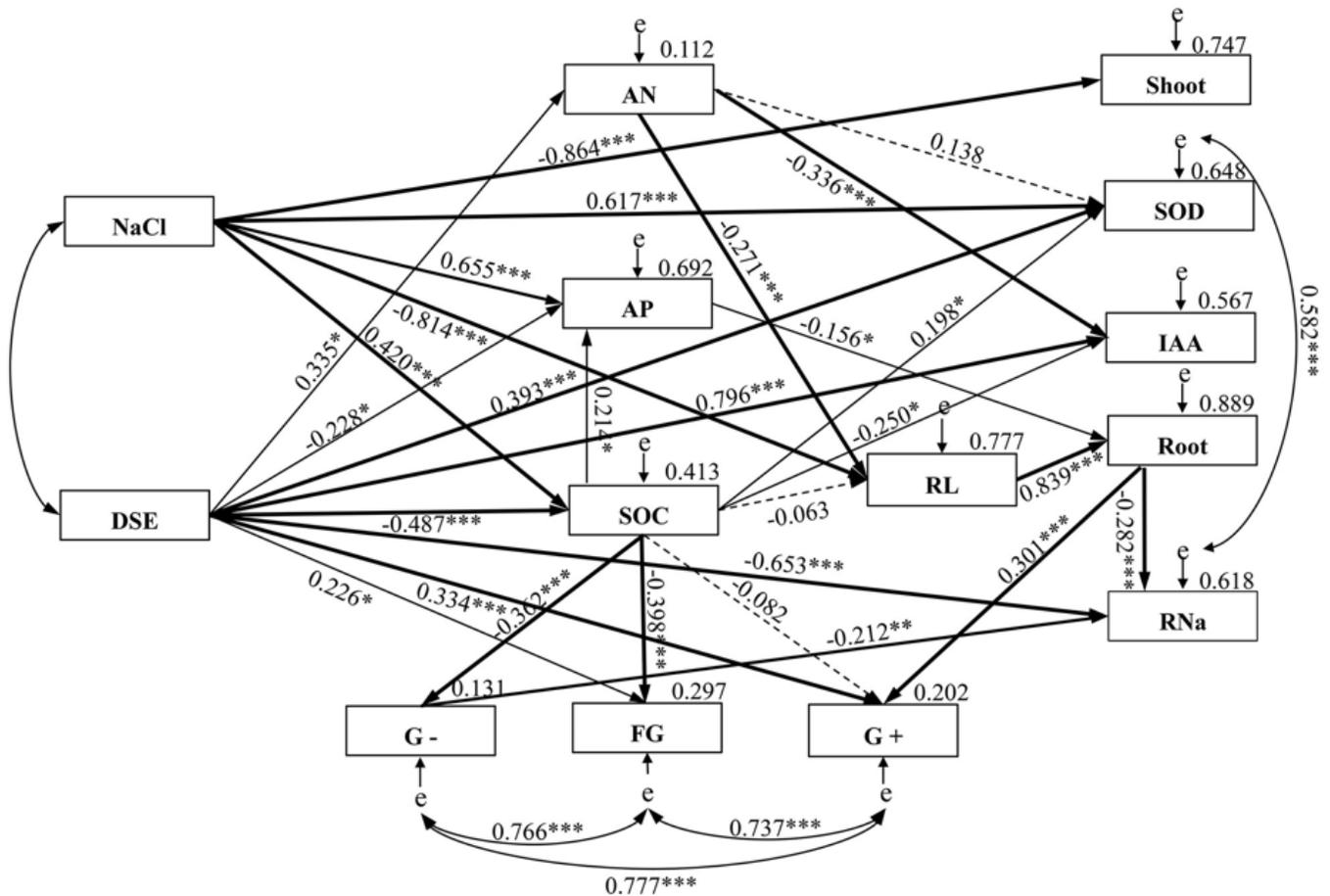


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