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ARTICLE

Effects of Different Arbuscular Mycorrhizal Fungi on Physiology of *Viola prionantha* under Salt Stress

Yajie Liu, Linlin Fang, Wenna Zhao and Chunxue Yang*

College of Landscape Architecture, Northeast Forestry University, Harbin, 150040, China

*Corresponding Author: Chunxue Yang. Email: senxiu99@163.com

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi distribute widely in natural habits and play a variety of ecological functions. In order to test the physiological response to salt stress mediated by different AM fungi, *Viola prionantha* was selected as the host, the dominant AM fungus in the rhizosphere of *V. philippica* growing in Songnen saline-alkali grassland, *Rhizophagus irregularis*, and their mixtures were used as inoculants, and NaCl stress was applied after the roots were colonized. The results showed that *V. philippica* could be colonized by AM fungi in the field and the colonization rate ranged from 73.33% to 96.67%, and *Claroideoglomus etunicatum* was identified as the dominant AM fungi species in the rhizosphere of *V. philippica* by morphology combined with sequencing for AM fungal AML1/AML2 target. Inoculation with both the species resulted in the formation of mycorrhizal symbiosis (the colonization rate was more than 70%) and AM fungi significantly enhanced plants' tolerance to salt stress of varying magnitude. Higher activity of antioxidant enzymes and augmented levels of proline and other osmoregulators were observed in AM plants. The content of MDA in CK was higher than that in the inoculations with the stress of 100, 200, and 250 mM. All indices except soluble protein content and MDA content were significantly correlated with AM fungal colonization indices. The analysis for different AM fungal effects showed that the mixtures and *R. irregularis* worked even better than *C. etunicatum*. These results will provide theoretical support for the exploration and screening of salt-tolerant AM fungi species and also for the application of AM-ornamental plants in saline-alkali urban greening.

KEYWORDS

Dominant AM fungi species; *Viola philippica*; salt stress; *Viola prionantha*; physiological response

1 Introduction

Soils around the world were severely salinized and the area of agricultural land decreased sharply in recent years [1]. The global saline-alkali area is approximately 956 million hectares at present [2]. China's salinized land covers an area of 520 million acres [3], and the salinized region is classified into five areas according to the differences in bio-climate and other environmental factors. Among which, Songnen saline-alkali grassland is situated in the northeast region [4], which is one of the three soda salinized soil distribution areas in the world and usually occurs salinization simultaneously with sodication [5]. Several measures have been taken to alleviate the pressure on ecological and economic development brought by the increasingly serious soil salinization in this area. The method of growing

plants can greatly reduce the cost and improve the soil in a depth [4]. Therefore, it is necessary to promote the plants' development growing in saline-alkali land.

As an excellent ground cover, *V. philippica* is important for afforestation and enjoying in early spring in Northern China. Relevant studies have proved that *V. philippica* had strong saline-alkali tolerance [6,7] and naturally distributed in some areas of Songnen saline-alkali grassland. Arbuscular mycorrhizal (AM) fungi can form a symbiosis with 90% of vascular plants and play important roles in the ecosystem [8], such as uptaking and transporting nutrients, regulating soil properties, and improving the relationships between plants and other biogroup [9]. Nowadays, many scholars have tried to investigate the effects of AM fungi on plant salt tolerance [10,11], which to the benefit of exploring more effective measures to restore salty land. When the hosts are salt-stressed, AM fungi can improve root viability and reduce leaf water loss [12], effectively maintaining the ionic balance by promoting the accumulation of K^+ and Ca^{2+} and reducing the Na^+ content [13], in addition, it can also regulate the salt tolerance of plants in molecular level [14,15]. Previous investigations proved that Songnen saline-alkali grassland was rich in AM fungi resources [16]. However, no paper reported the AM fungal species in the rhizosphere of *V. philippica* and it was not clear whether AM fungi were involved in the growth regulation. It is worth noting that pH directly affected the AM fungal diversity and the distribution of some species [17,18]. Therefore, the exploration of *V. philippica*-AM in different pH conditions would provide a basis for realizing the relationship between AM fungi and the salt tolerance of *V. philippica* growing in saline-alkali grassland and also for the application of *V. philippica*-AM symbiosis in salty cities. Molecular biological analysis and morphological identification were the major methods to identify AM fungi. The species obtained by morphology possess more comprehensive information, but the researchers should equip with an overall understanding for the morphological characteristics of the discovered and newly recorded species [19]. AM fungal AML1/AML2 target showed interspecific differences and the alignment result could accurately annotate the species [20]. The combination of the above-mentioned methods contributes to the correct analysis of AM fungal species.

V. prionantha, which is the related species of *V. philippica*, has an earlier flowering period and different morphology characteristics, although they belong to the same genus and both of them possess salt tolerance [21]. It has been verified that *Rhizophagus irregularis* performed well in improving the physiology and secondary metabolites of *Viola tricolor* in the *Viola* genus [22]. To widen the extent of AM fungal application and compare the effects among different AM fungi species, *V. prionantha* was selected as the host and after which inoculate *R. irregularis* and the dominant species isolated in the experiment. After the salt stress for a while, enzyme activities, membrane lipid peroxidation, and the content of osmoregulators in hosts were measured, so that the physiological response to salt stress mediated by different AM fungi was clarified. The results will provide theoretical support for the application of plant-AM symbionts in salty habitats.

The view that *Glomus* was a broad-spectrum symbiotic system has been proved in previous research, and *Glomus* was the dominant AM fungal genera in various environments [23,24]. Many studies also pointed out that the colonization was better than single inoculation when two species were inoculated simultaneously [25,26]. Besides, mixtures have worked best in several studies [25,27], and local fungus might be more effective than commercial fungi due to the local adaptation [28]. Therefore, the following hypotheses will be tested in this experiment: (a) The dominant species in the rhizosphere of *V. philippica* belongs to *Glomus.* (b) The colonization of mixtures is better than that of single inoculation. (c) All the inoculations significantly enhance plants' tolerance to salt stress of varying magnitude and the mixtures work the best, followed by the dominant species.

2 Materials and Methods

2.1 Samples Collection and Determination of Soil pH

The sampling sites were located in the saline-alkali land around Zhaodong, Heilongjiang Province in the middle of Songnen Plain (125°54'2"–125°54'15.13" E, 46°2'52"–46°2'58.16" N). The climate of this

region was characterized by semi-humid-semi-arid and the soil was soda salinized. On April 27, 2019, a total of nine sites of *V. philippica* growth were selected randomly within 90 hectares. According to the "multipoint parallel sampling method" and the "five sampling method" [29], plants in each plot were randomly harvested from four directions to ensure that the samples collected in this study were widely representative. After being dug up, the plants were vigorously shaken to remove the excess soil, and those still stuck to the roots were considered the rhizosphere soil [30]. Subsequently, the materials including roots and rhizosphere soils (approximately 0.5 Kg) from the depth of 10–20 cm were collected at each site. The roots were immersed in FAA fixing solution (5 ml formalin, 5 ml glacial acetic acid, and 90 ml of 70% ethyl alcohol) after they were cut into the 1 cm-long segments. 300 g of rhizosphere soil was reserved, numbered, and marked, then stored at 4°C.

Soil pH was measured by a pH meter (METTLER TOLEDO FE20, China). 10 g rhizosphere soil was weighed from each sample and it was mixed into suspension (water:soil = 2.5:1). After the solution precipitated for 0.5 h, its pH value was measured using the pH meter. The average of three measurements, determined after the pH meter reading stabilized, was taken as the pH of each sampling site.

2.2 AM Fungal Colonization in the Roots of V. philippica

The root segments soaked in FAA solution were taken out, and they were incubated in 10% KOH to soften and transparentize after being washed with distilled water. Subsequently, the roots were bleached with 10% hydrogen peroxide. After that, the root segments were neutralized with 2% hydrochloric acid for 30 min, and then they were stained with the 0.05% trypan-blue reagent in accordance with Philips et al. [31]. The decolorization was performed with glycerin lactate solution. Finally, the morphology of arbuscular, vesicles, and hyphae were observed under the microscope (OLYMPUS-DSX500, Japan). The AM fungal colonization in *V. philippica* was evaluated as the description in Trouvelot et al. [32].

2.3 Isolation and Morphological Identification of AM Fungal Spores

AM fungal spores in rhizosphere soil (50 g) were separated using wet screening and sucrose density-gradient centrifugation method [33]. The spores were enumerated under a dissecting microscope (Leica MDG33, Germany) after they were cleaned in an ultrasonic washer (AS, Tianjin Autoscience Instrument Co., Ltd., China). Subsequently, a single spore was sucked onto a glass slide and so that the sporal characteristics which involved walls, content, color, size, hyphae were observed under a digital optical microscope (OLYMPUS-DSX500, Japan). Then the spores were stained using Melzer's reagent and the resulting changes were recorded. Finally, the species identification was performed based on the characteristics obtained in the above-mentioned operation and was also referred to as "Chinese AM fungal resources and germplasm resources" [34], INVAM International website (http://invam.wvu.edu/the-fungi/species-descriptions), Janusz Blaszkowski of Poland agricultural university (http://www.zor.zut.edu.pl/Glomeromycota) and morphological descriptions, pictures, and literature newly published.

2.4 Determination of Dominant AM Fungal Species by Morphology

The separation frequency, relative abundance, and importance value of the spores identified by morphology were calculated referring to the method proposed by Yang et al. [35]. The dominant species was determined according to the importance value.

Separation frequency (F) = occurrence frequency of individual species/total sample number × 100%

Relative abundance (RA) = spore number of individual species/total quantity of AM fungal spores × 100%

Importance value (IV) = (F + RA)/2 × 100%.

2.5 Molecular Identification of Dominant AM Fungal Spores

The dominant AM fungal spores cleaned by an ultrasonic washer were transferred into a sterilized petri dish filled with ddH_2O. A single spore (1 μl) that was used as the amplification template was aspirated repeatedly until it was clean and then it was placed in sterilized PCR tube. After which the spore was thoroughly crushed with a sterile pipette tip under a stereoscope. Subsequently, a two-step nested PCR was performed to amplify the AML1/AML2 target of AM fungal 18 s rDNA and the amplified fragment was about 800 bp in length. The AM fungal universal primers GeoA2 (5′-CCAGTAGTCATATGCTTGTCTC-3′) and Geo11 (5′-ACCTTGTTACGACTTTTACTTCC-3′) were used for the first PCR. And the second PCR employed specific primers AML1 (5′-ATCAACTTTCGATGGTAGGATAGA-3′) and AML2 (5′-GAACCCAAACACTTTGGTTTCC-3′) [36]. The reaction system and process were shown in Appendix A.

The OMEGA Gel Extraction Kit (D2500-01) was used to purify the amplified products that the length had been examined by 1.0% agarose gel electrophoresis. Sequencing was completed by Qingke Biotechnology Co., Ltd. and the GeneBank accession number was MW349795. A phylogenetic tree (Maximum-likelihood) was constructed using MEGA 7.0.

2.6 Experimental Conditions

The seeds of *V. prionantha* were purchased from Small Ant Shop in Ningxia, China. The dominant species identified in this experiment and the fungi which performed well in *Viola* (*R. irregularis*, RI) were selected as the experimental inoculants and they were purchased from China AMF Germplasm Bank (BGC). The spore density was about 18/g.

The *V. prionantha* was seeded into the sterilized substrate (peat soil: vermiculite = 3:1) after the seeds were disinfected with 0.3% $KMnO_4$. The seedlings were transplanted when two euphylla appeared and four inoculations were added at the same time: 12 g CE, RI, and CE + RI were placed around the roots of *V. prionantha* in three inoculation groups, respectively, and the control group which without inoculation (CK) was applied equal amount of sterilized substrate. Maintaining light 14 h, light intensity 5000lx, the temperature at 20°C–25°C, in addition, irrigating 100 ml distilled water every two days. 80 days after the inoculation, NaCl stress (0, 50, 100, 200 and 250 mM) was applied. The plants were irrigated with 50 ml NaCl solution every day and the experiment lasted for 10 days. All the groups were replicated three times. At the end of the 10-day salt tolerance test, the roots were harvested and immersed in FAA fixing solution at 4°C, the leaves were sub-packaged with foil and then they were frozen in liquid nitrogen and preserved at −80°C till further use.

2.7 Physiological Response of V. prionantha to Salt Stress Mediated by AM Fungi

The AM fungal colonization in the roots was observed referring to the staining method of Philips et al. [31]. The osmoregulators concentration, enzyme activities, and membrane lipid peroxidation were measured by referring to “Principles and Techniques of Plant Physiological Biochemical Experiment (Version 2)” [37]. The specific test methods were as follows: the content of malondialdehyde (MDA) was determined by thiobarbituric acid method, proline by acid ninhydrin, soluble sugar by anthrone colorimetry, and soluble protein by Coomassie Brilliant Blue G-250, the activities of peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) were determined using guaiacol, NBT and ammonium molybdate, respectively.

2.8 Statistical Analysis

MYCOCALC was used for the analysis of AM fungal colonization, SPSS 25.0 for the one-way ANOVA test, and also for the Pearson correlation analysis, the post hoc applied LSD Duncan. And chart drawing was completed with Sigmaplot12.5 and Photoshop 2019.

3 Results

3.1 The Soil pH and AM Fungal Colonization in V. philippica

The pH of sampling sites ranged from 6.74 to 7.95. *V. philippica* growing in all the sites were colonized with the colonization rate of 73.33%–96.67%. The differences analysis of pH and various AM fungal colonization indices were performed at the 95% confidence interval (Table 1). The results showed that there were significant differences in soil pH, colonization intensity, arbuscular abundance, and vesicle abundance among different plots, while colonization rate showed insignificant difference in 9 plots. The analysis proved that the soil pH varied among sampling sites, and it also indicated that the investigation results of AM fungal species in 9 sampling sites were convincing for understanding the resources in the rhizosphere of *V. philippica* growing in Songnen saline-alkali grassland.

Table 1: The soil pH and AM fungal colonization in *V. philippica*

Samples ID	pH value	Colonization rate (%)	Colonization intensity (%)	Arbuscular abundance (%)	Vesicular abundance (%)	AM type
1	7.52 ± 0.01d	73.33 ± 11.55b	32.63 ± 1.36b	0.57 ± 0.51def	23.05 ± 8.69b	I
2	7.65 ± 0.01b	73.33 ± 5.77ab	30.93 ± 2.32b	1.53 ± 0.45bc	13.46 ± 1.79cd	I
3	6.74 ± 0.06e	76.67 ± 5.77b	21.10 ± 1.85d	1.29 ± 0.49bcd	12.98 ± 1.34cd	I
4	7.67 ± 0.06b	86.67 ± 5.77ab	19.80 ± 1.14d	1.97 ± 0.22b	7.57 ± 2.2de	I
5	7.52 ± 0.01d	96.67 ± 5.77a	10.73 ± 0.87f	0.72 ± 0.25def	2.60 ± 0.65e	I
6	7.65 ± 0.01b	86.67 ± 5.77ab	30.27 ± 3.63b	2.70 ± 0.73a	17.45 ± 3.81bc	I
7	7.61 ± 0.01bc	76.67 ± 15.28b	15.50 ± 2.8e	0.30 ± 0.05ef	7.99 ± 2.95de	I
8	7.95 ± 0.02a	86.67 ± 5.77ab	42.40 ± 2.61a	0.01 ± 0.01f	36.38 ± 2.28a	I
9	7.54 ± 0.32cd	80.00 ± 10.00b	24.90 ± 1.44c	0.85 ± 0.25cde	14.28 ± 0.77cd	I

Note: Different letters within the same column indicated significant differences among the sites, and we marked the largest value with a, followed by b, c, etc. Standard deviation (SD) was used for data statistics.

3.2 Dominant Species in the Rhizosphere of V. philippica Identified by Morphology

Thirty species of 11 AM fungal genera were identified from the rhizosphere soil of *V. philippica* by morphology. Among these, 9 species of *Glomus*, 7 species of *Acaulospora*, 2 species each of *Ambispora*, *Rhizophgus*, *Septoglomus*, *Scutellospora*, and *Claroideoglomus*, and 1 species each of *Sclerocystis*, *Funneliformis*, *Entrophospora*, and *Gigaspora*. The No. 119 distributed in 7 sampling sites and it was identified as the dominant species with the isolation frequency, relative abundance, and importance values of 88.89%, 25.5%, and 57.2%, respectively, while the relative abundance and importance values of other species only were 0.5%–12.5% and 5.81%–52.5%, respectively. Images of the thirty species could be found in the previous investigation of our team [38].

The morphological characteristics of No. 119 were shown in Fig. 1: The sporal color was orange to red-brown, the shape was globose or subglobose and the diameter was 60–160 μm with an average of 129 μm. The spore wall consisted of two layers (L1 and L2) that differentiated consecutively as spores develop. As an outer layer, L1 had some plasticity with an uneven outer surface and it became pink to reddish-purple after staining in Melzer's reagent. L1 degraded and sloughed as spores age so that it might be present in patches or appeared as a granular layer. In addition, the hypha connected to the spore was shaped as cylindrical to slightly flared. The characteristics of No. 119 were highly similar to the *Claroideoglomus etunicatum*.

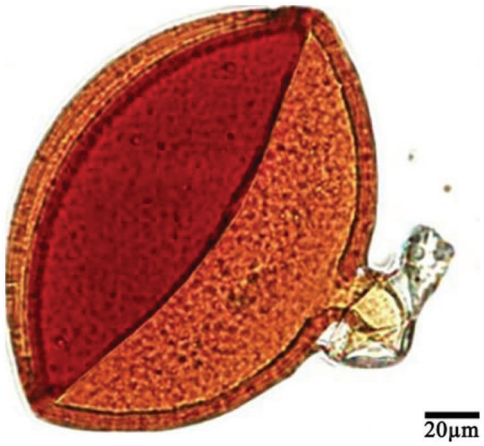

Figure 1: Dominant AM fungi (No. 119) in the rhizosphere of *V. philippica*

3.3 Results of Molecular Identification

The length of the AML1/AML2 target of No. 119 was 764 bp (Fig. 2a). The maximum-likelihood phylogenetic tree (Fig. 2b) was constructed using MEGA7.0 according to the results of sequence alignment conducted with the NCBI database. The 12 sequences with high homology of more than 98% to No. 119 were selected. The results showed that No. 119 clustered with the MN726592.1 *C. etunicatum*. Therefore, No. 119 was identified as *C. etunicatum* by molecular sequencing combined with morphological identification.

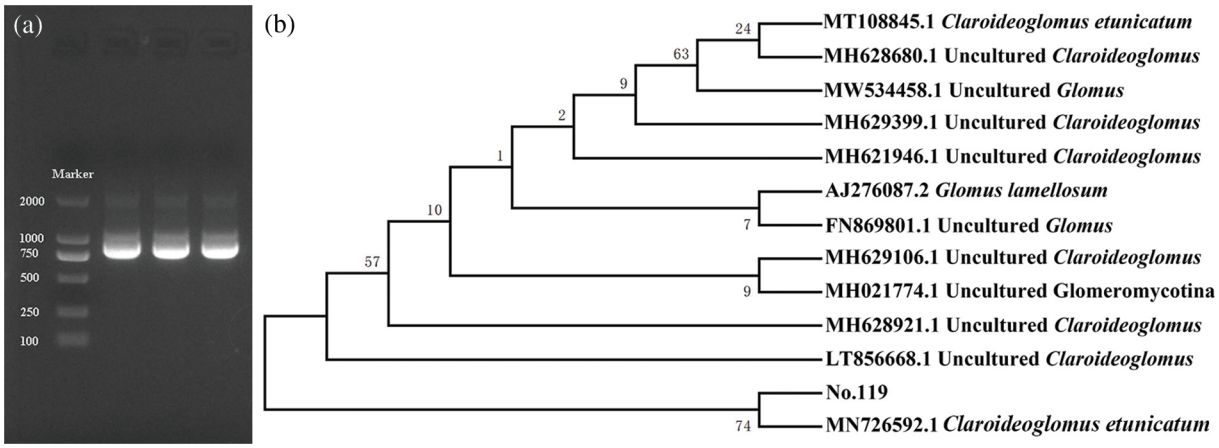

Figure 2: (a) Electrophoresis map of nested-PCR amplified products; (b) Phylogenetic tree of No. 119

3.4 AM Fungal Colonization in the Roots of V. prionantha under Salt Stress

Inoculation with both species resulted in the formation of mycorrhizal symbiosis (Fig. 3). AM fungi formed hyphae outside the roots of *V. prionantha* (Fig. 3a), besides, large numbers of hyphae would be present in the cortical cells after colonizing the roots and some of them expanded to be vesicles (Figs. 3b, 3c). In addition, some hyphae branched densely to form arbuscules (Figs. 3d–3h). The roots inoculated with CE formed *Paris*-type (P) mycorrhizal structure in which hyphae circles could be observed (Figs. 3c, 3g, 3i). A typical *Arum*-type (A) mycorrhizal structure was observed in the roots of inoculating RI.

And both the two types were found in the segments colonized with CE + RI and the mycorrhizal structure was determined as *Intermediate*-type (I).

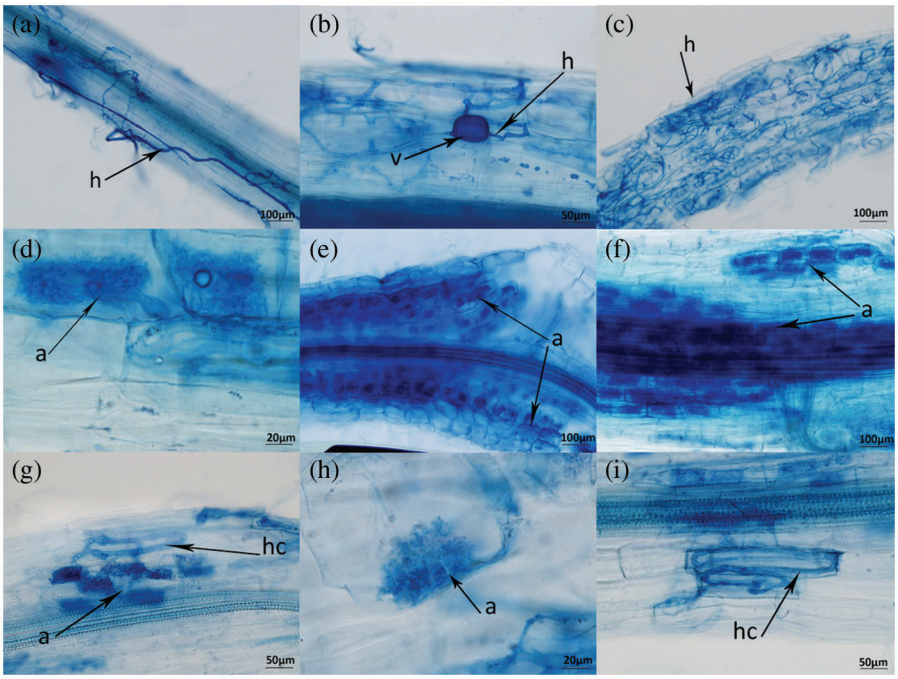

Figure 3: Symbiont of AM-*V. prionantha*. h-hypha, v-vesicule, a-arbuscule, hc-hyphal coil

All the root segments inoculated RI were colonized. The colonization rate, colonization intensity, and vesicle abundance were by the tendency of RI > CE + RI > CE. The results of the one-way ANOVA test for different inoculations were as follows (Table 2). There was an inconspicuous difference in colonization indices of CE while arbuscular abundance in RI decreased distinctly with the increasing NaCl concentrations ($P < 0.05$). Arbuscular abundance and colonization intensity in CE + RI were significantly different ($P < 0.05$) and all of them decreased with the continuously aggravated NaCl stress. It was noteworthy that the colonization of mixtures was not better than that of single inoculation.

Table 2: AM fungal colonization in the roots of *V. prionantha*

Treatment	Colonization rate (%)	Colonization intensity (%)	Arbuscular abundance (%)	Vesicle abundance (%)	Type of mycorrhiza
CE0	86.67 ± 5.77a	26.6 ± 3.16b	22.03 ± 4.35b	0.32 ± 0.13a	P
CE50	86.67 ± 5.77a	40.77 ± 4.44a	29.91 ± 1.62a	0.10 ± 0.10b	P
CE100	83.33 ± 11.55a	21.30 ± 3.55b	13.01 ± 2.68c	0.01 ± 0.01b	P
CE200	80.00 ± 10.00a	28.87 ± 5.34b	21.64 ± 2.24b	0.00 ± 0.00b	P
CE250	76.67 ± 5.77a	26.97 ± 5.11b	23.50 ± 4.93b	0.00 ± 0.00b	P
RI0	100.00 ± 0.00a	36.07 ± 4.58c	21.44 ± 4.88a	4.72 ± 1.03c	A
RI50	100.00 ± 0.00a	43.70 ± 5.20ab	25.47 ± 4.42a	7.53 ± 1.11b	A
RI100	100.00 ± 0.00a	48.60 ± 6.63a	11.85 ± 1.61b	12.98 ± 2.68a	A

(Continued)

Table 2 (continued)

Treatment	Colonization rate (%)	Colonization intensity (%)	Arbuscular abundance (%)	Vesicle abundance (%)	Type of mycorrhiza
RI200	100.00 ± 0.00a	42.83 ± 1.08ab	5.07 ± 0.33c	8.38 ± 0.53b	A
RI250	100.00 ± 0.00a	36.23 ± 4.01c	8.60 ± 2.25bc	8.90 ± 1.03b	A
CE + RI0	100.00 ± 0.00a	44.80 ± 5.81a	25.99 ± 2.88a	2.52 ± 0.43b	I
CE + RI50	100.00 ± 0.00a	41.43 ± 3.45ab	16.55 ± 5.80b	1.17 ± 0.30b	I
CE + RI100	96.67 ± 5.77a	32.23 ± 2.15c	11.44 ± 2.92b	4.02 ± 1.43a	I
CE + RI200	93.33 ± 11.55a	35.67 ± 4.21bc	16.08 ± 2.50b	4.99 ± 0.86a	I
CE + RI250	90.00 ± 10.00a	16.42 ± 1.96d	1.82 ± 0.31c	1.29 ± 0.30b	I

Note: CE, RI, and CE + RI were different inoculations. 0, 50, 100, 200, and 250 were the concentration of NaCl stress. Different letters in the same inoculation indicated significant differences among stresses, and we marked the largest value with a, followed by b, c. Standard deviation (SD) was used for data statistics.

3.5 Physiological Response of V. prionantha to Salt Stress Mediated by AM Fungi

3.5.1 The Response of Membrane System to Salt Stress Mediated by AM Fungi

In keeping with common sense, MDA showed an upward trend in CK with the NaCl stress aggravated (Fig. 4). The content of MDA in CK was higher than that in the inoculations with the stress of 100, 200, and 250 mM. Comparing with CK, MDA concentrations in CE, RI and CE + RI decreased by 41.15%, 53.86% and 3.35% at 100 mM NaCl stress, respectively, 65.52%, 55.58% and 29% at 200 mM, 54.73%, 31.69% and 53.04% at 250 mM, respectively. The CE reduced the content significantly compared with other inoculations at concentrations of 0, 50, and 200 mM ($P < 0.05$). The effect of CE + RI on alleviating membrane lipid peroxidation was relatively poor and that of RI was the worst under extremely heavy salt stress (250 mM). In conclusion, CE worked the best in reducing membrane damage of *V. prionantha* under salt stress.

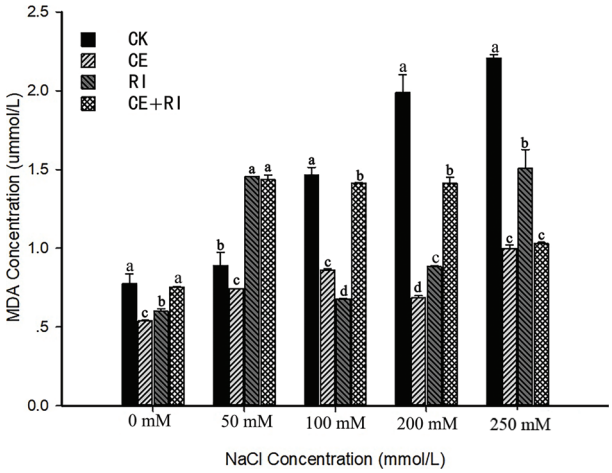

Figure 4: MDA content of *V. prionantha* under salt stress mediated by AM fungi

Note: Different letters in a certain stress indicated significant differences among inoculations, and we marked the largest value with a, followed by b, c, d.

3.5.2 The Response of Antioxidant Enzymes to Salt Stress Mediated by AM Fungi

SOD activity (Fig. 5a) in CK decreased with the augment of NaCl stress. The activity in CE only increased (175.31%) at 250 mM compared with CK. The performance of CE + RI was the similarity to that of CE, and the activity of CE + RI was 33.12% higher than CK at a NaCl concentration of 250 mM. The results also showed that the SOD activity of *V. prionantha* increased significantly in three inoculations at the extreme stress of 250 mM ($P < 0.05$). RI positively regulated the SOD when the NaCl concentration reached 100 mM. In conclusion, RI worked perfectly in SOD regulation.

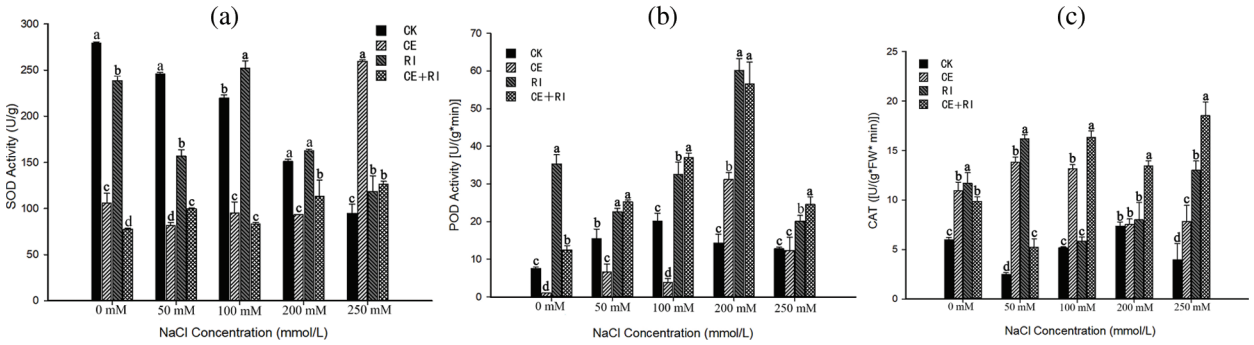

Figure 5: The activities of antioxidant enzymes under salt stress mediated by AM fungi (a) SOD activity; (b) POD activity; (c) CAT activity. Different letters in a certain stress indicated significant differences among inoculations, and we marked the largest value with a, followed by b, c, d

POD activity increased first and then decreased with the stress augmented (Fig. 5b). The value of CE, RI, and CE + RI reached the maximum at 200 mM, which were increased by 117.83%, 319.29%, and 294.58%, respectively. RI and CE + RI increased the activity significantly at every NaCl concentration ($P < 0.05$) while CE inhibited the enzyme activity at a lower concentration. In brief, RI and the mixtures (CE + RI) worked even better.

All inoculations improved the CAT activity of *V. prionantha* under salt stress of varying magnitude (Fig. 5c). A remarkable effect was presented in CE + RI under the serious stress of 100, 200, and 250 mM compared with others ($P < 0.05$). CE, RI, and CE + RI increased the activity by 98.6%, 230.1%, and 370.0% contrasted with CK at 250 mM, respectively. The results indicated that CE + RI improved CAT activity the best at NaCl ≥ 100 mM while the RI performed well at slight stress.

3.5.3 The Response of Osmoregulators to Salt Stress Mediated by AM Fungi

The response of soluble sugar content (Fig. 6a) to various NaCl concentrations mediated by AM fungi was different. The content in the CE-plants outnumbered the CK at stress concentration ≤ 100 mM and it increased by 47.19% as the NaCl at 100 mM. However, the contents in RI and CE + RI were higher than that of CK at every NaCl concentration almost, and the effects were significant at multiple concentrations ($P < 0.05$). The highest increment in RI was 148.27% as the concentration at 50 mM and the maximum (43.67%) in CE + RI presented at the stress of 250 mM.

The inoculations of CE, RI, and CE + RI increased the content of soluble protein under adversity (Fig. 6b), and the content in each inoculation was significantly augmented at 250 mM ($P < 0.05$), which increased by 52.08%, 54.11%, and 185.44%, respectively. In CE plants, the content first increased and then decreased with the strengthening of NaCl stress, the result indicated that CE performed well at all the concentrations ($P < 0.05$). The optimum effect of RI presented at 200 mM (increased by 152.28%) and CE + RI only performed well at 250 mM (increased by 185.44%). In conclusion, the effect of CE was the most stable, followed by RI.

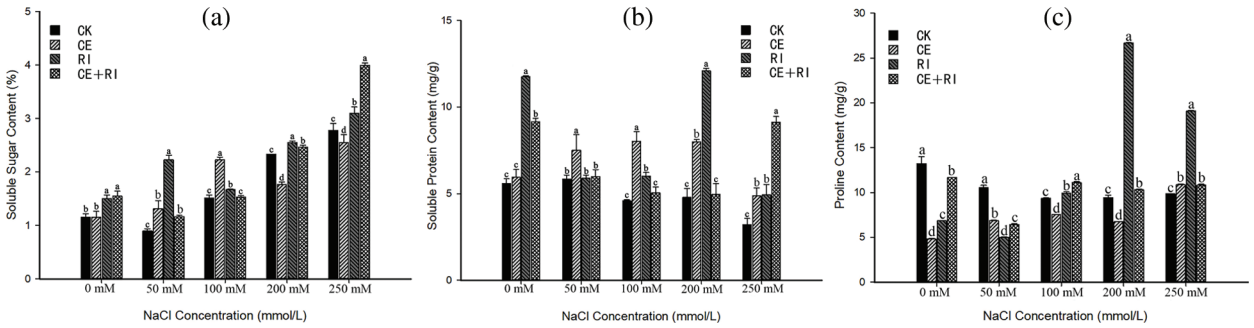

Figure 6: The osmoregulators under salt stress mediated by AM fungi (a) Soluble sugar content; (b) Soluble protein content; (c) Proline content. Different letters in a certain stress indicated significant differences among inoculations, and we marked the largest value with a, followed by b, c, d

Proline content (Fig. 6c) in *V. prionantha* under NaCl stress was improved to varying degrees in inoculations so that the osmoregulators in AM-plants were balanced. The content in CE only increased when the NaCl reached 250 mM (increased by 8.13%). The inoculation effects of RI and CE + RI were consistent and their values were higher than that of CK at salinity ≥ 100 mM. Furthermore, the preponderance of RI expressed significantly at 200 and 250 mM ($P < 0.01$) with the increment of 184.32% and 93.83%, respectively. Therefore, the effects of the three inoculations were as follows: RI > CE + RI > CE.

3.6 Correlation between AM Fungal Colonization and Physiology of Host under NaCl Stress

The correlation between AM fungal colonization and the physiology of host was analyzed to understand the relationship between them (Table 3). There was a significant negative correlation between soluble sugar content, CAT activity, and colonization intensity, and a negative association also existed between arbuscular abundance and the proline, soluble sugar, and POD. It was worth noting that a significant positive correlation existed between the vesicle abundance and some physiological indices (proline content, POD, and SOD). In addition, the colonization rate was only related to POD activity.

Table 3: Correlation analysis between AM fungal colonization and physiology of *V. prionantha*

	MDA content	Proline content	Soluble sugar content	Soluble protein content	POD activity	SOD activity	CAT activity
Colonization rate	0.276	0.269	−0.074	0.145	0.372*	0.033	0.003
Colonization intensity	0.080	0.177	−0.409**	−0.021	0.293	0.146	−0.389**
Arbuscular abundance	−0.263	−0.564**	−0.600**	−0.111	−0.426**	−0.042	−0.165
Vesicle abundance	0.209	0.462**	0.152	−0.009	0.508**	0.434**	−0.093

Note: **indicate that the correlation is significant at 0.01 level; * indicate that the correlation is significant at 0.05 level.

4 Discussion

C. etunicatum has been reported in previous investigations operated in Songnen saline-alkali grassland [16,33,39–41]. However, *C. etunicatum* was identified as the dominant species for the first time and it only

occurred as a common species in the rhizosphere of other plants. These might be related to the characteristics and genotype of hosts, which could play an impact on AM fungal community composition [42]. In addition, the AM fungal composition affected the reestablishment of degraded grassland [28], *C. etunicatum*, which appeared in songnen saline grassland frequently, might accelerate the vegetation restoration in saline-alkali land. The dominant species obtained in this research deserved further study.

Environmental conditions exerted an influence on AM fungal colonization [43] and that was proved in this experiment. The average colonization rate of CE and CE + RI in *V. prionantha* gradually declined with the aggravating NaCl stress, which was consistent with the relevant studies of AM fungi under salt stress [44]. A similar result as previous studies was obtained, in which the colonization of mixtures was not better than that of single AM fungi [45]. It could be speculated that the spores of multiple AM fungal species were inoculated and germinated simultaneously in the laboratory and the drastic competition for plant nutrients occurred in high-concentration NaCl stress, resulting in the decrease in colonization rate.

Inoculations could improve the physio-biochemical characteristics of hosts under stress and AM fungi played a significant role in improving the salt tolerance of *V. prionantha* in this experiment. AM fungal effects in multiple indices similar to the colonization, in which the mixtures was not better than single inoculation. The reason was likely that the malignant competition for living space and nutrients after colonizing simultaneously weakened the effects [46]. All the physiological indices of *Trifolium repens* inoculated with *C. etunicatum* were positively improved under salt stress of each concentration [45]. However, inoculations sometimes exerted the negative effects of inhibiting enzyme activities, increasing MDA content, and reducing osmolytes content in this study. These controversial indices differed among studies [47,48]. These might be related to the mechanism of salt tolerance mediated by AM fungi. Little damage will be brought to *V. prionantha* with salt tolerance by slight NaCl stress and the AM-plant symbiosis increased the nutrients output so that the negative effects appeared. Nevertheless, AM fungi transferred water and nutrients from the soil to hosts and regulated the physiology of plants to ensure the stability of symbiosis under high-concentration stress [49]. AM fungal regulation effects on various substances were different, improvement in certain indices was often accompanied by the interference to other indices [50], which was consistent with the experiment. The results showed that CE only worked well in the regulation of MDA and soluble protein, while *R. irregularis* and mixtures played good effects on four indicators. Studies have shown that some species in *Rhizophagus* could induce the specific expression of transporters and improve plant growth under adversity [51], besides, AM fungi played roles by community in the field, some species would appear together to stabilize the community composition but performed minor biological functions [52], moreover, there was almost no vesicle in the roots colonized with CE, suggesting that CE was underdeveloped in *V. prionantha* [53], which would affect the functions. These were likely the reasons for the better effects of RI and CE + RI. Though *R. irregularis* was not isolated from the rhizosphere of *V. philippica*, the role of *R. irregularis* in a community should be further verified to provide theoretical support for its application in saline-alkali land.

The colonization intensity represented the amounts of symbionts in roots and the arbuscular provided sites for exchanging nutrients and transferring carbohydrates, the augment of them would accelerate the nutrient consumption of hosts [54]. These were the reasons that colonization intensity and arbuscular abundance were negatively associated with multiple indices. Besides, the results showed that multiple indicators positively correlated to vesicle abundance. It was might relate to the function of the vesicle that reserved nutrients [8]. A large vesicle abundance indicated that sufficient nutrient was supplied for fungi growth and host development, and it ensured that AM fungi could play a stable ecological role and reduce the nutrient uptake from hosts. But that's just a guess based on the existing information. The relationship between AM fungal colonization and physiological responses of hosts under stress was necessary to further explore.

5 Conclusion

The results showed that our hypothesis was incorrect. The dominant species (*C. etunicatum*) in the rhizosphere of *V. philippica* didn't belong to *Glomus*. The colonization of mixtures was not better than that of single inoculation. Inoculations improved the physio-biochemical characteristics of *V. prionantha* under salt stress, a higher activity of antioxidant enzymes and augmented levels of osmoregulators were observed in AM plants. RI and the mixtures (CE + RI) worked even better because they exerted a good effect on multiple indices. Besides, all indices except soluble protein and MDA were significantly correlated with AM fungal colonization indices.

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Appendix A: The reaction system and process of nested PCR

Reaction system			Reaction process		
	First PCR	Second PCR		First PCR	Second PCR
Premix TaqTM	10 μl	25 μl	Inital denaturation 94°C	4 min	4 min
DNA	1 μl	1 μl	Denaturation 94°C	30 s	30 s
10 mmol/l F Pri	1 μl	2 μl	Annealing 55.2°C	30 s	30 s
10 mmol/l R Pri	1 μl	2 μl	Extension 72°C	1 min	1 min
ddH_2O	7 μl	20 μl	Final extension72°C	7 min	7 min
Total	20 μl	50 μl	Save at 4°C		

Note: F-Forward, R-Reverse, Pri-Primer, the denaturation, annealing and extension cycled 30 times.