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Research article

Comparative physiological and metabolomic analyses reveal mechanisms of *Aspergillus aculeatus*-mediated abiotic stress tolerance in tall fescue

Yan Xie[a], Xiaoyan Sun[b], Qijia Feng[a], Hongji Luo[c], Misganaw Wassie[a], Maurice Amee[a], Erick Amombo[a], Liang Chen[a,*]

[a] CAS Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan City, China
[b] The Key Laboratory of Horticultural Plant Genetic and Improvement of Jiangxi, Institute of Biology and Resources, Jiangxi Academy of Sciences, Nanchang City, China
[c] Sichuan Changhong Green Environmental Science &Technology Co., Ltd, Chengdu City, China

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ABSTRACT

Aspergillus aculeatus has been shown to stimulate plant growth, but its role in plants abiotic stress tolerance and the underlying mechanisms are not fully documented. In this study, we investigated the mechanisms of *A.aculeatus*-mediated drought, heat and salt stress tolerance in tall fescue. The results showed that *A.aculeatus* inoculation improved drought and heat stress tolerance in tall fescue as observed from its effect on turf quality (TQ) and leaf relative water content (LWC). In the same stress conditions, *A.aculeatus* alleviated reactive oxygen species (ROS) induced burst and cell damage, as indicated by lower H_2O_2, electrolyte leakage (EL) and malondialdehyde (MDA) levels. Additionally, the *A.aculeatus* inoculated plants exhibited higher photosynthetic efficiency than uninoculated plants under drought, heat and salt stress conditions. The fungus reduced the uptake of Na^+, and inoculated plants showed lower Na^+/K^+, Na^+/Ca^{2+} and Na^+/Mg^{2+} ratios compared to uninoculated ones under salt stress. Furthermore, comparative metabolomic analysis showed that *A.aculeatus* exclusively increased amino acid (such as proline and glycine) and sugar (such as glucose, fructose, sorbose, and talose) accumulation under drought and heat stress. However, there were no differences between inoculated and uninoculated plants except for changes in H_2O_2 level, Na^+ in the root and photosynthetic efficiency under salt stress. Taken together, this study provides the first evidence of the protective roles of *A.aculeatus* in the tall fescue response to abiotic stresses, partially via protection of photosynthesis and modulation of metabolic homeostasis.

1. Introduction

In the natural environment, plants are often exposed to harmful abiotic stresses such as drought, extreme temperature, and high salt concentration, which induce various physiological and developmental alterations that adversely affect plant growth and development. As a primary cause of crop loss, abiotic stress accounted for more than 50% of the average yield loss of major crops (Valliyodan and Nguyen, 2006). When plants experience adverse environmental stresses, various metabolic, physiological, biochemical and defense mechanisms are activated to ensure survival and sustain growth.

It has been reported that drought, extreme temperature, and salinity stresses are often interconnected, and may induce similar cellular damages (Wang et al., 2003). The biochemical and physiological alterations that are associated with these stress conditions include changes in membrane fluidity and composition, solute concentration, and turgor loss (Chaves et al., 2003). These diverse environmental stresses are manifested primarily as osmotic stress, which then leads to turgor loss and the disruption of homeostasis and ion distribution (Zhu, 2001). In addition, abiotic stresses also cause oxidative stress via over generation of reactive oxygen species (ROS), such as hydroxyl radicals, singlet oxygen and hydrogen peroxide (Shi et al., 2015). The oxidative stress as a result of the production of ROS, thereby leads to metabolic dysfunction, photosynthesis inhibition, and cellular structures damage

Abbreviations: ROS, reactive oxygen species; PSII, photosystem II; CAT, catalase; SOD, superoxide dismutase; POD, peroxidase; H_2O_2, hydrogen peroxide; ACC, 1-aminocyclopropane- 1-carboxylate; TQ, turf quality; SGR, shoot growth rate; LWC, leaf relative water content; Chla, chlorophyll *a*; Chlb, chlorophyll *b*; Chlt, chlorophyll; EL, electrical conductivity; MDA, malondialdehyde; OJIP curve, chlorophyll fluorescence transients curve; PCA, principal component analysis; HCA, hierarchical clustering analysis; GC-MS, gas chromatography-mass spectrometry

* Corresponding author.
E-mail address: chenliang1034@126.com (L. Chen).

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(Krasensky and Jonak, 2012).

In higher plants, photosystem II (PSII) which consists of a multi-subunit complex, is the core portion of the process of photosynthesis. The PSII reaction centers provide the basis for photosynthetic recovery processes and acclimation and are susceptible to any type of stress (Allakhverdiev et al., 2008). On the other hand, plants have evolved several tremendous defense mechanisms to protect themselves from abiotic stress. Plant traits that are associated with defense mechanisms are multigenic, and different plant species are highly variable in response to harsh abiotic stresses (Munns and Tester, 2008). For example, plants have developed numerous antioxidant strategies including enzymatic and non-enzymatic antioxidant systems (Wang et al., 2003) to scavenge the excess ROS, and hence increase tolerance to different stress conditions. The enzymatic antioxidant system consists of enzymes such as catalase (CAT), superoxide dismutase (SOD) and Peroxidase (POD), and can scavenge hydrogen peroxide (H_2O_2), free radicals and oxy-intermediates (Lee et al., 2007). Moreover, plants fine-tune their metabolism to cope with different harmful environmental stresses, and the adjustments depend on the magnitude and type of stress and the plant species. Recently, our knowledge of the importance of metabolic adjustments in response to abiotic stress has increased considerably. For instance, the metabolic profiles of different plant species including *Arabidopsis thaliana*, rice, bermudagrass, *Thellungiella halophile* and ryegrass have been analyzed under temperature, drought, and salinity stresses (Caldana et al., 2011; Fan et al., 2015; Gong et al., 2010; Hu et al., 2011; Kaoru et al., 2010; Zuther et al., 2007). Studies have shown that some metabolic profile changes are common to drought, extreme temperatures, and salt stress conditions, whereas others are specific to the particular stress. For example, proline accumulation increased in response to different stress conditions. In opposite, tricarboxylic acid cycle intermediates and organic acids decreased in glycophytes after salt stress but increased after temperature and drought stress (Kaoru et al., 2010; Sanchez et al., 2010). Thus, analyzing the metabolic profiles of plants under unfavorable conditions can help to improve our understanding of the role of metabolites in regulating plant responses to different abiotic stresses.

Tall fescue (*Festuca arundinacea* Schreb) is a self-incompatible allohexaploid out-crossing cool-season forage and turf grass species that is distributed in the temperate regions (Tao et al., 2015). Currently, tall fescue is widely cultivated as turf grass and forage due to its yield, adaptability, persistence, and ecosystem services. However, tall fescue is sensitive to high temperature, which is a major growth limiting factor (Zhang et al., 2005). Moreover, marginal land such as saline soils is necessary for planting forage species because of the competition between the decreasing farmland and increasing population. Therefore, improving the abiotic stress tolerance of forage species is quite necessary.

Recently, the utilization of globally abundant microbes to evoke plant abiotic-stress tolerance has received great attention (Hosseini et al., 2016; Waller et al., 2005; Xie et al., 2014b). Microorganisms can improve plant stress tolerance in many different ways which include: stimulating growth, increasing mineral nutrient uptake, improving plant disease resistance, among others (Xie et al., 2014b). Previous studies have indicated that *Epichloë coenophiala* (tall fescue endophytic fungus) significantly increased plant available water and soil available water, which could result in water rise for productivity in drought periods (Hosseini et al., 2015a, 2015b, 2016). In addition, *Piriformospora indica* was shown to provide growth promoting activity as a plant root-colonizing fungus during its symbiosis with barley (Waller et al., 2005). *Aspergillus aculeatus* (*A.aculeatus*) which was isolated from the metal polluted soil (Xie et al., 2014b), could colonize plant roots and improve salt tolerance in bermudagrass and ryegrass (Li et al., 2017; Xie et al., 2017). In our previous study, *A.aculeatus* has been confirmed to produce indole-3-acetic acid, siderophores, and enzyme 1-aminocyclopropane- 1-carboxylate (ACC) deaminase, and solubilize the natural forms of phosphorus (Xie et al., 2017). However, little is known about the role of *A.aculeatus* in tall fescue tolerance to drought, extreme temperatures, and high salt stress. Therefore, the aim of the present study was to investigate the effect of *A.aculeatus* in protecting tall fescue from drought, high temperatures and high salt stresses. In order to elucidate the physiological responses of *A. aculeatus*–colonized tall fescue to drought, high temperatures, and salinity stresses, important indicators such as plant growth and productivity, lipid peroxidation, photosynthetic rate, mineral nutrient, and metabolic homeostasis were measured.

2. Materials and methods

2.1. Preparation of growth substrate

The growth substrate was prepared with the following components; sawdust/sand (3:1 v:v), organic matter content, 22.32 g kg^{-1}, N 638 mg kg^{-1}, P 572 mg kg^{-1}, K 1.58%, total soil porosity 54.9%, and pH 6.5. The growth substrate were heated at 180 °C in an oven for 8 h and divided into two groups. Meanwhile, the spores of *A.aculeatus* were cultivated in liquid Martin medium (10.0 g glucose, 5.0 g peptone, 1.0 g KH_2PO_4 and 0.5 g $MgSO_4{\cdot}7H_2O$ per liter, and plus 1% rose bengal solution 3.3 mL, pH 6.0). When the germination rate of spores reached 80%, the spores were collected by centrifugation at 2500 × *g* for 10 min, and then washed five times with 0.85% normal saline and resuspended. For half of the substrate, a 10 mL of spore suspension (ca. 10^6 cfu mL) was inoculated into the per kg substrate. For the other half of the substrate, 10 mL of 0.85% normal saline was inoculated into per kg substrate.

2.2. Plant materials

The seeds of Tall Fescue (cv. Houndog 5, a commercial type tall fescue) were obtained from Beijing Clover Seed & Turf CO. Ltd., China. It has wide adaptability to different climates and soils, and is highly resistant to abiotic stress, especially with strong drought resistance and heat resistance. Seeds were surface sterilized with H_2O_2 (8%), washed with deionized water five times, and then germinated on filter paper at 22 °C. After germination, uniformly germinated plantlets were grown in plastic pots (15 cm in diameter and 20 cm deep) filled with 1.5 kg pre-prepared growth substrate. All the plastic pots were sterilized using UV for 2 h before planting. All the pots were placed into a growth chamber with standard conditions of 22/18 °C (day/night), average photosynthetically active radiation of 400 μmol m^{-2} s^{-1}, 60% relative humidity, and 14 h light/10 h dark. The grasses were fertilized weekly with half strength-Hoagland's solution (Hoagland and Arnon, 1950).

2.3. Stress treatments

After one month of pre-culture, the seedlings grown in the above-mentioned substrate were divided into four experimental regimes, namely, CK (control), S (300 mM NaCl stress), H (heat stress: 39/35 °C for day/night) and D (drought stress: without water for 7 days). Each treatment regime contained two groups (uninoculated and inoculated). All the treatments were defined as follows: (i) CK-uninoculated, optimum growth condition without the spores of the *A.aculeatus*; (ii) CK-inoculated, optimum growth condition with the spores of the *A.aculeatus*; (iii) S-uninoculated, 300 mM NaCl stress without the spores of the *A.aculeatus*; (iv) S-inoculated, 300 mM NaCl stress with the spores of the *A.aculeatus*; (v) D-uninoculated, drought stress without the spores of the *A.aculeatus*; (vi) D-inoculated, drought stress with the spores of the *A.aculeatus*; (vii) H-uninoculated, heat stress without the spores of the *A.aculeatus*; (viii) H-inoculated, heat stress with the spores of the *A.aculeatus*. The experiments were arranged in a randomized complete block design with four replicates and the pots were rotated randomly every two days. After 7 d of treatment, the visual turf quality, canopy height, chlorophyll content, and leaf relative water content were determined. The fully expanded 3rd leaves were collected and stored at −80 °C for subsequent analyses.

2.4. Measurements

2.4.1. Measurements of phenotypic and physiological parameters

The turf quality (TQ) was assessed visually using a scale of 0–9 score, where 0 score indicates withered, yellow, and dead grass; 9 score indicates dense green, and uniform; and 6 score indicates minimum acceptable level according to color, density, and uniformity (Turgeon, 1991).

The vertical canopy height was determined by the difference before and after treatment according to Xie et al. (2017). In brief, four positions in each pot were measured using a rule and averaged to determine the canopy height. Shoot growth rate (SGR) was the difference in the canopy height before and after treatment.

The leaf relative water content (LWC) was determined according to the method of Sun et al. (2013). About 0.5 g fresh leaves were immediately weighed for the fresh weight (FW), soaked in water for 12 h and weighed for the turgid weight (TW), dried in an oven at 80 °C for 72 h then weighed for the dry weight (DW). The LWC was calculated using the following formula:

$$\mathrm{LWC} = \frac{(FW - DW)}{(TW - DW)} \times 100\%$$

The chlorophyll content was measured following the method described by Xie et al. (2014c). The electrical conductivity (EL) was measured according to the method of Bi et al. (2016). The fully expanded leaves (0.15 g) were washed 3 times and excised into 0.5 cm long segments and transferred into a tube filled with 15 mL deionized water. The tubes were then shaken at 180 rpm for 24 h at 25 °C. The initial conductivity (Ci) was measured using a conductance meter (JENCO3173, Jenco Instruments, Inc., YSIModel32, USA). To release the electrolytes completely, the tubes were autoclaved for 15 min at 121 °C and the final conductivity (Cmax) was measured after cooling to room temperature. The EL was calculated using the formula:

$$\mathrm{EL}\ (\%) = \frac{Ci}{Cmax} \times 100\%$$

The malondialdehyde (MDA) content was measured by thiobarbituric acid (TBA) method according to Xu et al. (2012). The H_2O_2 content was determined according to the previous study (Velikova et al., 2000).

The fully expanded 3rd leaves were used for chlorophyll *a* fluorescence measurements. All measurements were determined by a pulse-amplitude modulation portable chlorophyll fluorimeter (PAM 2500, Heinz Walz GmbH) with high time resolution (10 μs) at the end of the experiment according to our previously described methods (Chen et al., 2013; Xie et al., 2017). Detailed information about the JIP-test parameters is described in Supplementary Table 1. For each treatment, measurements were repeated 8 times.

2.4.2. Metabolites extraction and derivatization

The metabolite extraction and sample derivatization were conducted according to our previous study (Xie et al., 2014a). Briefly, fourth fully expanded leaves (0.1 g) were grounded to a fine powder with liquid nitrogen, and then the powder was transferred in to a 2-mL tube containing 1.4 mL of 80% (v/v) aqueous methanol. Subsequently, the tubes were shaken for 2 h under 200 rpm at ambient temperature. Ten μL of ribitol solution (2 mg mL^{-1}) was added as an internal standard. The mixture was incubated in a water bath at 70 °C for 15 min and centrifuged for 15 min at 3000 × *g*. The supernatant was transferred to a new 10 mL tube with 1.5 mL of water and 0.75 mL of chloroform. The solution was vortexed thoroughly for 15 s and centrifuged at 10,000 g for 10 min. The supernatant (0.5 mL) was transferred into 2 mL HPLC vials and dried at 900 g for 4 h in a Centrivap benchtop centrifugal concentrator (Labogene, Denmark). The dried polar phase was derivatized with 80 μL methoxyamine hydrochloride (20 mg mL^{-1}, in pyridine) at 30 °C for 2 h followed by trimethylsilyl with 50 μL MSTFA at 37 °C for 2 h. The standards and reagents used in this section were purchased from Sigma-Aldrich Co.Ltd. (Poole, UK). The metabolites were determined using gas chromatography-mass spectrometry, (GC-MS, Agilent 7890A/5975C, Agilent Technologies, Palo Alto, CA, USA) as described by Xie et al. (2014a).

2.4.3. Determination of ion concentration

For Na^+, K^+ and Ca^{2+} determined, the oven-dried roots and leaves were finely ground and measured as the method described by Li et al. (2017). Briefly, about 0.1 g samples were digested in a mixture of concentrated 5 mL HNO_3 and 1 mL H_2O_2 using an ETHOS ONE microwave sample preparation system with digestion procedure: 130 °C for 12 min, 160 °C for 8 min, and finally 160 °C for 30 min. The Na^+, K^+ and Ca^{2+} concentration was defined with inductively coupled plasma optical emission spectroscopy (Perkin Elmer, USA).

2.5. Statistical analysis

For metabolism analysis, principal component analysis (PCA) and hierarchical clustering analysis (HCA) were conducted using the MetaboAnalyst webpage (https://www.metaboanalyst.ca/faces/home.xhtml) (Chong et al., 2018). All mean data were subjected to analysis of variance. Significance of differences in metabolite ratios and other parameters between means were assessed using one-way ANOVA combined with Tukey test at a significance level of 0.05.

3. Results

3.1. Effects of *A. aculeatus* on growth of tall fescue under abiotic stress

Compared with the control, salt stress significantly decreased TQ and LWC (Fig. 1A, C) of uninoculated tall fescue. There were no significant differences between inoculated and uninoculated plants for TQ, SGR, LWC, Chla, Chlb and Chlt content under saline conditions.

Drought stress significantly decreased the TQ and LWC of both uninoculated and inoculated plants (Fig. 1A, C). However, *A. aculeatus* inoculated plants exhibited significantly higher TQ and LWC than uninoculated plants under drought stress. Surprisingly, inoculated plants showed significantly lower Chla, Chlb and Chlt content compared to uninoculated plants under drought stress (Fig. 1D and E, F). Heat stress declined the TQ and LWC of both inoculated and uninoculated tall fescue, with a more pronounced decrease in uninoculated plants (Fig. 1 A, C). The SGR of both inoculated and uninoculated plants decreased under heat stress, and there was no significant difference between them (Fig. 1 B). Furthermore, inoculated plants showed lower Chla, Chlb and Chlt content compared to uninoculated plants under heat stress (Fig. 1D and E, F).

3.2. *A. aculeatus* reduced the EL, MDA and H_2O_2 levels under abiotic stress

As shown in Fig. 2, salt, drought and heat stress markedly increased the EL, MDA and H_2O_2 levels of uninoculated plants compared with the control. However, *A. aculeatus* inoculation significantly reduced the EL and MDA levels compared to uninoculated plants under heat- or drought-treated (Fig. 2A and B), but no significant difference was observed under salt stress. Furthermore, inoculated plants showed obviously lower H_2O_2 levels than uninoculated plants under control, salt, and drought stress conditions (Fig. 2C).

3.3. Effects of *A.aculeatus* on photosynthetic efficiency under abiotic stress

Salt, drought and heat stress adversely affected the chlorophyll fluorescence transient curve (OJIP curve) of uninoculated tall fescue

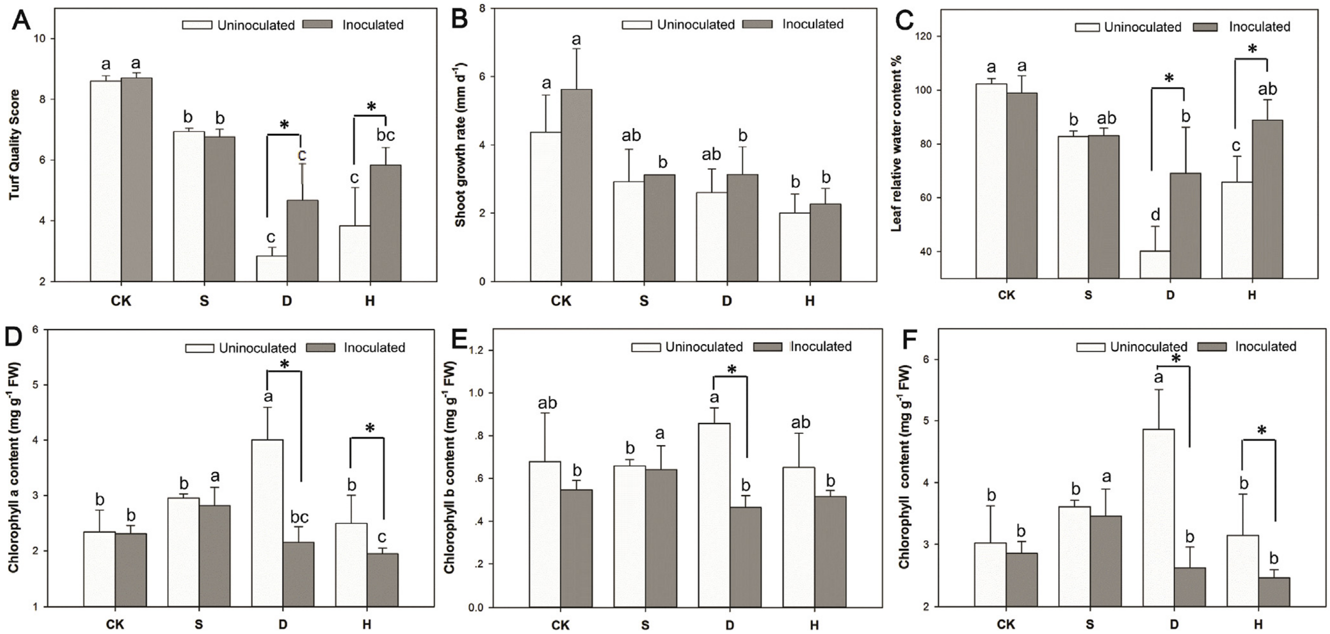

Fig. 1. Impacts of *A.aculeatus* on turf quality (A), shoot growth rate (B), leaf relative water content (C), chlorophyll *a* content (D), chlorophyll *b* content (E) and chlorophyll content (F) of tall fescue under abiotic stress. Error bars are ± standard deviation (n = 4). Columns marked with identical lower-case letter indicate that there is no significant differences under only inoculated or un-inoculated conditions for comparisons among different stresses base on Turkey test ($P < 0.05$) while the asterisks indicate significant difference between un-inoculated and inoculated conditions within the same stresses based on Turkey test ($P < 0.05$). CK, S, D and H are control, salt stress, drought stress and heat stress, respectively.

leaves (Fig. 3). As shown in Fig. 3C, heat stress dramatically caused the OJIP curves of both inoculated and uninoculated plants to decline. Regardless of the stress conditions, *A.aculeatus*-inoculated plants exhibited slightly higher OJIP curves compared to uninoculated plants (Fig. 3).

Basic photosynthetic parameters including F_0, F_K, F_J, F_I, F_M, and M_0 were extracted from the OJIP curve (Supplementary Table 2). Compared with the control, there were no obvious changes in these parameters in uninoculated or inoculated plants under salt stress. However, drought and heat stress significantly decreased the F_I and F_M and increased the M_0 of both inoculated and uninoculated tall fescue compared with the control. But, *A.aculeatus*-inoculated plants showed significantly higher F_K under salt stress, F_I under drought and heat stresses, and lower F_0 and F_M under drought stress than uninoculated plants (Supplementary Table 2).

Performance indexes (PI_{ABS} and PI_{total}) are important indicators to describe the overall activity of PSII. Our results showed that inoculated plants treated with drought and heat stress had significantly higher PI_{ABS} than uninoculated plants (Supplementary Fig. 1). However, there was no difference for PI_{total} between uninoculated and inoculated plants regardless of the stress conditions. On the other hand, quantum yield and efficiency parameters including ϕP_0, ϕE_0, ϕR_0, $\gamma RC2$, and RC/ABS showed an obvious difference in tall fescue under different treatment (Supplementary Fig. 2 A-E). The ϕP_0, ϕE_0, $\gamma RC2$ and RC/ABS of uninoculated and inoculated plants declined under drought and heat stress, with a more pronounced decrease observed in uninoculated plants. However, only ϕP_0 showed a significant improvement in inoculated plants compared to uninoculated plants. Specific energy flux parameters including ET_0/RC, RE_0/RC, and ABS/RC were altered remarkably in all stress conditions (Supplementary Fig. 2 F-H). In both drought and heat stress, these three parameters were increased compared with the control, but inoculated plants showed lower RE_0/RC compared to uninoculated plants. However, there were no significant differences in ϕP_0, ϕE_0, ϕR_0, $\gamma RC2$, RC/ABS, ET_0/RC, RE_0/RC and ABS/RC between uninoculated and inoculated salt-treated plants.

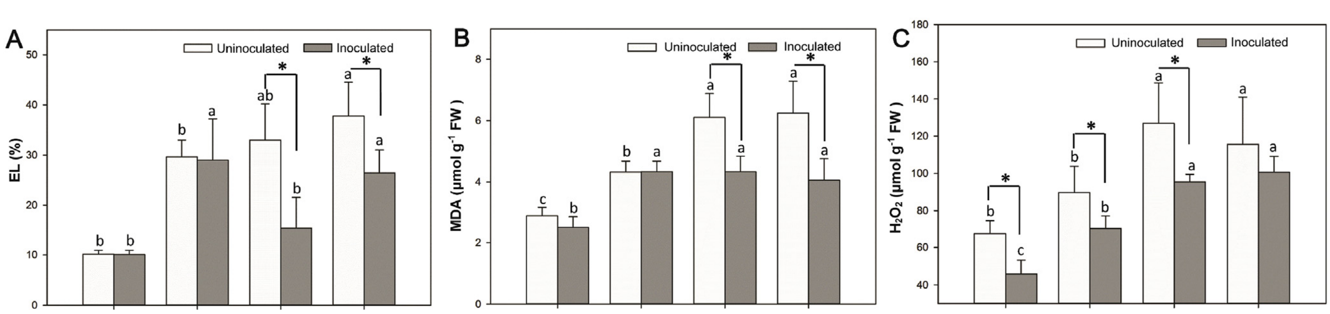

Fig. 2. Electrolyte leakage (EL) level (A), malondialdehyde (MDA) concentration (B) and H_2O_2 accumulation (C) of tall fescue grown with or without *A.aculeatus* under abiotic stress. Error bars are ± standard deviation (n = 4). Columns marked with identical lower-case letter indicate that there is no significant differences under only inoculated or un-inoculated conditions for comparisons among different stresses base on Turkey test ($P < 0.05$) while the asterisks indicate significant difference between un-inoculated and inoculated conditions within the same stresses based on Turkey test ($P < 0.05$). CK, S, D and H are control, salt stress, drought stress and heat stress, respectively.

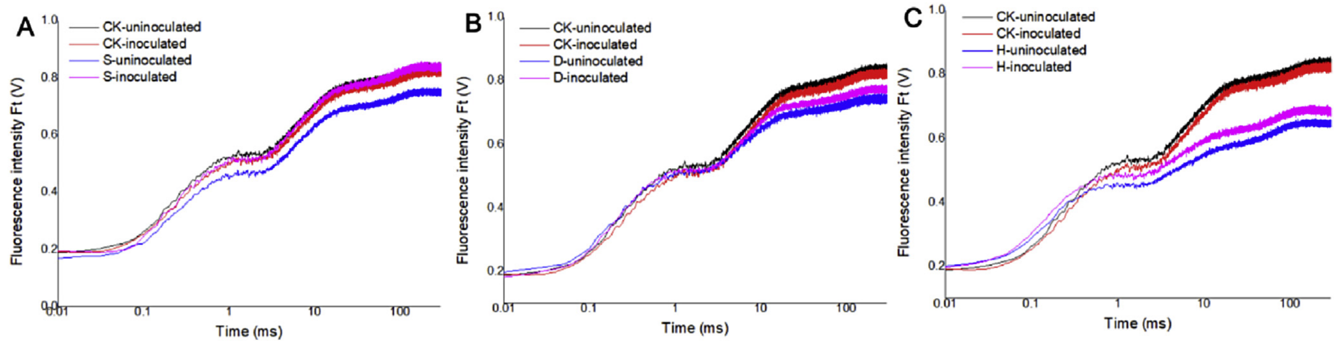

Fig. 3. Comparison of chlorophyll fluorescence transient curve (OJIP curve) between un-inoculated and inoculated plants under salt (A), drought (B) and heat (C) stress in tall fescue leaves. OJIP curve were induced by 10 s light pulse of 3000 μmol photons $m^{-2}s^{-1}$. Data for the JIP-test were sampled at 10 μs intervals for the first 320 ms.CK, S, D and H are control, salt stress, drought stress and heat stress, respectively.

3.4. Effects of *A.aculeatus* on metabolites of tall fescue under abiotic stresses

A total of 42 metabolites comprising 8 amino acids, 10 organic acids, 14 sugars, 3 fatty acids, 2 sugar alcohol and 5 others were identified across all samples (Supplementary Table 3). The Venn diagram analysis demonstrated that different metabolites were significantly changed in response to different stress treatments between inoculated and uninoculated tall fescue (Fig. 4). All stress conditions up-regulated a number of metabolites in tall fescue, but the largest number of metabolites (18) were detected in *A.aculeatus* inoculated plants under heat stress.

In addition, more uniquely up-regulated metabolites were detected in inoculated plants than uninoculated ones under drought and heat stress, while the same amount of uniquely up-regulated metabolites was detected in inoculated and uninoculated plants under salt stress. Few uniquely down-regulated metabolites were noted in inoculated plants under drought and salt regimes. However, more unique down-regulated metabolites in inoculated plants were detected under heat stress. Under drought stress, the uniquely up-regulated metabolites of inoculated plants were mainly amino acids (alanine, glycine, threonine, valine, and proline), and sugar (fructose, talose, galactopyranose). In addition, the uniquely up-regulated metabolites in inoculated plants under heat stress were largely sugar molecules (glucose, xylulose, arabinopyranose, sorbose, and fructose) and amino acids (glycine and proline). Furthermore, five metabolites includingγ-Aminobutyric acid (GABA), galactopyranose, galactinol, arabinopyranose, and xylulose were uniquely up-regulated in inoculated plants under salt stress. Six metabolites (glycine, ribonic acid, proline, fructose, talose and galactopyranoside) were commonly up-regulated in inoculated plants under drought and heat stress. However, only two fatty acids including hexadecanoic acid and octadecanoic acid were uniquely down-regulated in inoculated plants under heat stress.

3.5. Principle component analysis (PCA) and hierarchical cluster analysis (HCA) of steady-state metabolite concentrations

PCA analysis was applied to separate the metabolites of tall fescue leaves under salt, drought and heat stress conditions. The first principal component section showed clear separations between salt, drought or heat stress treatments, which were represented as 73.9%, 61.1% and 73.8% of the total variation, respectively (Fig. 5). The second principal component revealed separations between inoculated or uninoculated plants by 13.1%, 22.1% and 17.6% of the variation for drought, salt and heat stress, respectively. In addition, the HCA results showed that there were two major clusters corresponding to the control and abiotic stress treatment groups (Supplementary Fig. 3). However, the subgroups were disordered.

3.6. Changes in ionic uptake and homeostasis under salt stress

The accumulation of Na^+ in the leaves and roots of both uninoculated and inoculated plants were significantly increased under salt stress (Table 1). But, inoculated tall fescue plants showed obviously lower Na^+ concentration in the root compared to uninoculated ones. The Na^+/K^+, Na^+/Ca^{2+} and Na^+/Mg^{2+} ratios were calculated from the Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations. The results showed that salt treatment significantly increased the Na^+/K^+, $Na^+/Ca^{2+}$and Na^+/Mg^{2+} ratios in the leaves and roots of both uninoculated and inoculated plants, with a pronounced effect in uninoculated plants (Table 1). In both inoculation and uninoculated plants, there was no significant difference between the control and salt treatment for K^+, Ca^{2+}, and Mg^{2+}concentrations in the leaves and roots of tall fescue. *A.aculeatus* inoculated plants showed lower Na^+/K^+, Na^+/Ca^{2+} and Na^+/Mg^{2+} ratios in the root than uninoculated plants. However, when leaves samples were used, there were no significant differences for Na^+/K^+, $Na^+/Ca^{2+}$and Na^+/Mg^{2+}ratios between uninoculated and inoculated plants.

4. Discussion

Plants abiotic-stress tolerance can be evoked by the exploitation of globally abundant microbes. *A. aculeatus* has been shown to increase Cd and salt tolerance in bermudagrass and ryegrass (Li et al., 2017; Xie et al., 2014b, 2017). However, prior to this study, there was no direct evidence on the role of *A.aculeatus* in tall fescue tolerance under unfavorable environmental conditions. In this study, the protective role of *A.aculeatus* to abiotic stress (drought, salt and heat stress) and the underlying mechanisms in tall fescue were revealed.

Under control conditions, *A.aculeatus* had no significant effect on tall fescue growth or physiological responses (Fig. 1). Under drought and heat stress conditions, however, *A. aculeatus* inoculation significantly increased the TQ and LWC levels compared to uninoculated plants. These results indicate that exogenous *A. aculeatus* application improved drought and heat tolerance in tall fescue. Similar results were reported under heavy metal stress (Xie et al., 2014b), and salt stress (Li et al., 2017; Xie et al., 2017). Previous studies have indicated that root-colonizing fungi which can produce phytohormones such as indole-3-acetic acid (IAA) could mitigate the deleterious effects of various stresses and stimulate plant growth (Egamberdieva, 2009). Our previous research also indicated that *A. aculeatus* had the capacity to produce IAA (Xie et al., 2017), which could be evidence for the higher TQ and LWC that were observed in tall fescue under drought and heat stress conditions in this study. Surprisingly, no obvious changes were observed in plant growth or physiological responses between inoculated and uninoculated plants under salt stress (Fig. 1), which is inconsistent with previous studies in bermudagrass and ryegrass (Li et al., 2017; Xie et al., 2017). Under in vivo conditions, the effect of

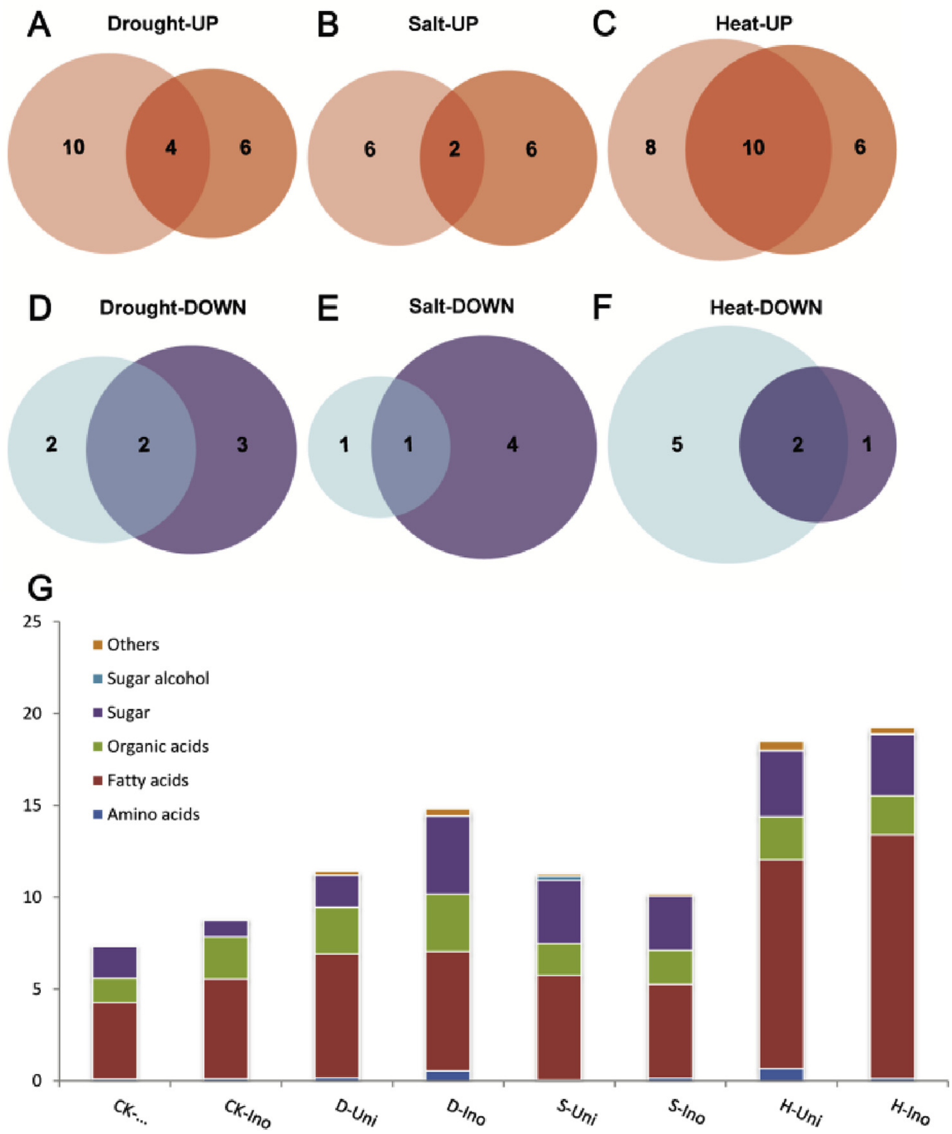

Fig. 4. Global view of the distinct and common metabolites in tall fescue plants in response to salt, drought and heat stress. The Venn diagram of metabolites found in the GC-MS analyses between uninoculated (the circle on the right) and inoculated (the circle on the left) leaves of tall fescue. The total number in each unique or overlapping set of metabolic analysis is shown with graphical representation of functional categories for each set. (A–C) present the number of up-regulated metabolites relative to control under drought, salt and heat stress, respectively (D–F) present the number of down-regulated metabolites relative to control under drought, salt and heat stress, respectively. (G) present the metabolite distributions in tall fescue.

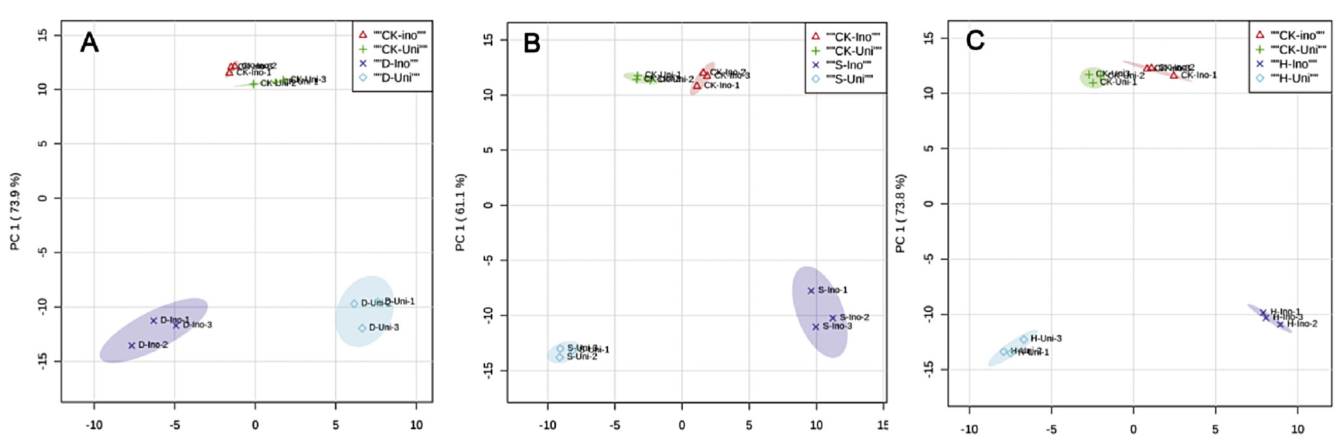

Fig. 5. Principal component analysis of the metabolic profiles of tall fescue leaves under abiotic stress. Drought (A), Salt (B) and Heat (C) stress. CK-Uni, CK-Ino, S-Uni, S-Ion,D-Uni, D-Ion, H-Uni, and H-Ionare represent CK-un-inoculated (optimum growth condition and un-inoculated the spores of *A.aculeatus*), CK- inoculated (optimum growth condition and inoculated the spores of *A.aculeatus*), S-un-inoculated (300 mM NaCl stress and un-inoculated the spores of *A.aculeatus*), S-inoculated (300 mM NaCl stress and inoculated the spores of *A.aculeatus*), D-un-inoculated (drought stress and un-inoculated the spores of *A.aculeatus*), D-inoculated, (drought stress and inoculated the spores of *A.aculeatus*), H-un-inoculated (heat stress and un-inoculated the spores of *A.aculeatus*) and H-inoculated (heat stress and inoculated the spores of *A.aculeatus*), respectively.

Table 1
Effects of *A.aculeatus* on Na^+, K^+, Ca^{2+}, Mg^{2+}, Na^+/K^+, Na^+/Ca^{2+} and Na^+/Mg^{2+} in the leaves and roots of tall fescue grown with or without *A.aculeatus* under salt stress.

	Leaf				Root			
	CK-Uni	CK-Ino	S-Uni	S-Ion	CK-Uni	CK-Ino	S-Uni	S-Ion
K^+	22.27 a	24.26 a	22.21 a	21.92 a	16.30 a	15.30 a	17.53 a	17.30 a
Na^+	1.03 b	1.28 b	15.62 a	14.73 a	2.84 c	3.48 c	13.38 a	10.59 b
Ca^{2+}	6.18 a	6.32 a	6.20 a	6.61 a	6.00 a	6.54 a	6.52 a	6.59 a
Mg^{2+}	4.27 a	5.02 a	3.44 a	3.95 a	5.58 a	6.27 a	5.53 a	5.41 a
Na^+/K^+	0.05 b	0.05 b	0.70 a	0.67 a	0.18 c	0.23 c	0.78 a	0.61 b
Na^+/Ca^{2+}	0.17 b	0.21 b	2.70 a	2.23 a	0.47 c	0.53 c	2.06 a	1.60 b
Na^+/Mg^{2+}	0.25 b	0.25 b	5.13 a	4.12 a	0.51 c	0.56 c	2.55 a	1.95 b

Data in the table are mean (n = 4). Means in a row for leaf or root followed by the same lower-case letter were not significantly different among treatments based on Tukey test ($P < 0.05$). CK-Uni, CK-Ino, S-Uni and S-Ion are CK-un-inoculated (optimum growth condition and un-inoculated the spores of *A.aculeatus*), CK- inoculated (optimum growth condition and inoculated the spores of *A.aculeatus*), S-un-inoculated (300 mM NaCl stress and un-inoculated the spores of *A.aculeatus*) and S-inoculated (300 mM NaCl stress and inoculated the spores of *A.aculeatus*), respectively.

salinity depends on the salt concentration, exposure time and the plant species. In support, Munns and Tester (2008) reported that plants tolerance to salinity varies greatly as reflected by different growth responses.

In this study, important indicators such as MDA, EL, and H_2O_2 were assessed in order to investigate the role of *A.aculeatus* for abiotic stress tolerance in tall fescue (Fig. 2). When plants are subjected to abiotic stresses, ion leakage and lipid peroxidation increased as a result of free radical-induced membrane deterioration or damage (Allen et al., 2010). Our results indicated that inoculated plants showed remarkably lower EL and MDA levels compared with uninoculated plants under drought and heat stress conditions. Nevertheless, there was no difference in the EL and MDA levels between inoculated and uninoculated plants under salt stress. This suggests that drought- and heat-induced ion leakage and lipid peroxidation were significantly attenuated in *A. aculeatus* treated plants. It is reported that the cellular damage as a result of abiotic stress is associated with the accumulation of reactive oxygen species (ROS) (Fadzilla et al., 1997). Excess ROS can act as a signaling molecule, but also toxic to living cells under stress conditions. Consistent with the EL and MDA accumulation, the level of H_2O_2 was lower in inoculated plants, indicating that *A. aculeatus* could protect the plant from stress-induced oxidative damage. Taken together, the results suggest that enhanced tolerance to drought and heat stress in inoculated tall fescue could be associated with the alleviation of ion leakage, lipid peroxidation and H_2O_2 accumulation by *A. aculeatus*.

Chlorophyll *a* fluorescence is a useful tool for studying the photosystem of plants under abiotic stress conditions (Chen et al., 2013). Compiled studies have shown that, abiotic stress causes an inhibition of PSII activity (Bi et al., 2016; Chen et al., 2013; Li et al., 2017). In this study, the OJIP curve was analyzed in *A.aculeatus* inoculated and uninoculated tall fescue leaves by JIP-test (chlorophyll fluorescence analysis method) to explore the role of *A. aculeatus* in regulating PSII activity under abiotic stress. *A.aculeatus*-inoculated plants showed less damage in the OJIP curve than uninoculated plants under salt, drought and heat stress (Fig. 3). Subsequently, a decrease in F_K and F_I were observed in inoculated plants than uninoculated ones, indicating that *A.aculeatus* could alleviate the effect of abiotic stresses on PSII in tall fescue.

The performance index (PI) is a sensitive parameter for assessing plant photochemical activities under abiotic stress conditions (Chen et al., 2013; Fan et al., 2015). Performance index (PI_{ABS}) incorporates three primary functional steps including excitation energy trapping, light energy absorptions and conversion of excitation energy to electron transport (Fan et al., 2015). In this study, PI_{ABS} was higher in inoculated plants than uninoculated plants under drought and heat stress, indicating the positive role of *A.aculeatus* in protecting the photosynthetic activity of tall fescue under drought and heat stress. Similarly, *A. aculeatus* inoculation significantly improved the ϕP_0 (maximum quantum yield for primary photochemistry) under drought and heat stress, suggesting that *A. aculeatus* enhanced the quantum yield on the donor and acceptor sides of PSII. Taken together, the results revealed that *A. aculeatus* play a role in maintaining plants photosynthesis under drought and heat stress. It has been reported that the negative effect of stresses on photosynthesis could be further magnified by the ROS production (Freeman et al., 2010). In our study, protecting the plant from oxidative damage by *A. aculeatus* might be a critical mechanism for a positive regulatory role in the photosynthesis of tall fescue under drought and heat stress.

Furthermore, the comparative metabolomic analysis showed the action of *A.aculeatus* in regulating the metabolic profiles of tall fescue under abiotic stress conditions. Perhaps unsurprisingly, the accumulation of metabolites first showed a greater difference between stress treatments than between inoculated and uninoculated plants, as reflected in the PCA (Fig. 4). Apart from this, inoculated plants exhibited higher concentrations of some metabolites compared to uninoculated plants. Among these metabolites, proline, glycine, and some carbohydrates are important compatible solutes in response to abiotic stress and are helpful for osmotic adaptation (Krasensky and Jonak, 2012; Shi et al., 2015). For many years, proline is considered to act as a ROS scavenger, osmolyte and a molecular chaperone in stabilizing the structure of proteins (Szabados and Savourcb, 2010), and can protect cells from stress-induced damage. It is pointed out that proline accumulates in many plant species in response to abiotic stress (Krasensky and Jonak, 2012). However, previous studies have shown that heat stress did not lead to proline accumulation (Dobra et al., 2010; Lv et al., 2011; Rizhsky et al., 2004). Similarly, in this study, proline accumulation was not observed under abiotic stress conditions in uninoculated tall fescue plants. However, proline and glycine were exclusively up-regulated in *A.aculeatus*-inoculated tall fescue under drought and heat stress. Furthermore, we demonstrated that inoculated tall fescue exhibited higher cell membrane stability, lower lipid peroxidation and H_2O_2 levels, which might be attributed to the greater accumulation of proline and glycine in inoculated plants under drought and heat stress.

In addition, *A.aculeatus* altered carbohydrate metabolites in tall fescue under abiotic stress. Soluble sugar is the major form of signaling molecule and carbohydrate metabolite, which is crucial for plant cell structure and metabolism (Hu et al., 2013). When plants are exposed to abiotic stress, soluble sugar such as fructose, glucose, sucrose and sorbose accumulate as storage substances that can be mobilized during the period of limited energy supply (Krasensky and Jonak, 2012). In this study, uniquely up-regulated sugars such as fructose, talose, and galactopyranose under drought stress, glucose, xylulose, arabinopyranose, sorbose, and fructose under heat stress, and galactopyranose, arabinopyranose and xylulose under salt stress were detected in inoculated plants but were unchanged in uninoculated plants. The up-regulated soluble sugars in inoculated tall fescue could contribute to the superior abiotic stress tolerance, as soluble sugar accumulation acts as a triggering molecule of plant defense (Kano et al., 2013). In addition, higher levels of other metabolites in inoculated tall fescue indicate the beneficial role of *A.aculeatus* in physiological processes during abiotic stress treatment. Comparison of metabolomics under different stress conditions further revealed the diversity and complexity of the role of metabolites in abiotic stress tolerance.

The previous study indicated that salt stress commonly caused higher Na^+ accumulation and lower K^+ concentrations (Haro et al., 2005). Thus, re-establishing ionic homeostasis under stressful condition is one of the strategies to achieve higher tolerance to salt stress (Zhu, 2001). In the current study, inoculated tall fescue plants reduced the uptake of Na^+ in the root compared to uninoculated plant, indicating that *A. aculeatus* is crucial to reduce the uptake of excess ion under salt stress. However, there was no observable significant difference in Na^+ in the leaves of inoculated and uninoculated plants both under control and salt treatment. Our previous study indicated that the *A. aculeatus* hyphae exhibited a greater capacity for Na^+ absorption under salt stress condition (Xie et al., 2017). Therefore, we infer that the *A. aculeatus* hyphae might provide a physical barrier against salt uptake into the plant root. In addition, our results showed that higher Na^+ content was observed in the leaf samples of in comparison with root samples. Previous studies have shown that a negative correlation between salinity tolerance and Na^+ accumulation in leaves is often seen when comparing different genotypes within a species (Munns, 2002; Tester and Davenport, 2003), but this is not the case when comparing different species, such as wheat and barley (Munns and Tester, 2008). Therefore, we speculate that in addition to Na^+ exclusion, other mechanisms may be important for tall fescue. Further research is needed on the salt tolerance mechanism of tall fescue.

5. Conclusion

In conclusion, our results demonstrated that *A. aculeatus* application enhanced drought and heat stress tolerance in tall fescue, as evidenced by higher TQ and LWC. Consequently, from our previous and current research, we deduce the following four mechanisms for *A. aculeatus* induced drought and heat tolerance in tall fescue: (i) improved drought and heat tolerance; (ii) alleviated lipid peroxidation and H_2O_2 accumulation; (iii) enhanced plant photosynthetic efficiency; (iv) regulated the concentration of metabolites such as proline, glycine and soluble sugar. *A. aculeatus* had an effect only in H_2O_2 level, Na^+ in the root and photosynthetic efficiency in the leaf of tall fescue under salt stress. Overall, this study demonstrated the first evidence for the role of *A.aculeatus* in protecting tall fescue from abiotic stresses.

Author contributions

C. L. and Y. X. conceived and designed the experiments; Y. X. and X. S. analyzed the data and wrote the manuscript; H. L cultivated the experimental materials; Y. X and Q. F performed the experiments; C. L., M. W, M. A. and E. A revised the manuscript; all authors have contributed to, seen and approved the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.07.022.

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