Amelioration of Salt Stress on Bermudagrass by the Fungus *Aspergillus aculeatus*

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There is considerable evidence that plant abiotic-stress tolerance can be evoked by the exploitation of a globally abundant microbe. *A. aculeatus*, which was initially isolated from the rhizosphere of bermudagrass, has been shown to increase heavy metal tolerance in turfgrasses. Here, we report on the potential of *A. aculeatus* to induce tolerance to salt stress in bermudagrass. Physiological markers for salt stress, such as plant growth rate, lipid peroxidation, photosynthesis, and ionic homeostasis were assessed. Results indicated that strain *A. aculeatus* produced indole-3-acetic acid (IAA) and siderophores and exhibited a greater capacity for Na⁺ absorption under salt stress. The plant inoculation by *A. aculeatus* increased plant growth and attenuated the NaCl-induced lipid peroxidation in roots and leaves of bermudagrass. The fungus significantly elevated the amount of IAA and glutathione and slightly enhanced photosynthetic efficiency of salt-treated bermudagrass. Tissues of inoculated plants had significantly increased concentrations of K⁺ but lower Na⁺ concentrations than those of uninoculated regimes. It appears that the role of *A. aculeatus* in alleviating bermudagrass salt stress is partly to produce IAA, to increase the activity of anti-oxidases, to absorb Na⁺ by fungal hyphae, and to prevent the plant from ionic homeostasis disruption.

Soil salinization is a serious factor restricting the expansion of agriculture, animal husbandry, and forestry around the globe. Salinity problems are prominent in arid and semiarid regions, including fertile alluvial plains, valleys, densely populated areas, and the coastal regions (Pessarakli and Szabolcs 2011). Salinity is becoming particularly widespread in many regions. Globally, more than 20% of agricultural land and approximately 50% of irrigated land is affected by salinity, which poses a serious threat to more than 50% of all arable lands by the year 2050 (Flowers and Yeo 1995; Wang et al. 2003).

Highly saline soil from irrigation water leads to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity (Wang et al. 2000). Under saline conditions, plants are exposed to three types of stresses, including ion toxicity, desiccation, and disruptions in the mineral nutrition of the plant (Blumwald 2000). Excessive ions (Na⁺ and Cl[−]) in the rhizosphere causes injury

to plant roots, followed by their gradual accumulation in the aerial parts, with heavy damage to plant metabolism, which leads to stunted growth and subsequently reduced yield (Shannon 1997). Furthermore, the excessive uptake of Na⁺ and Cl[−] can limit the uptake of other mineral nutrition (e.g., K⁺, Ca²⁺, Mg²⁺), and the lack of nutritional elements causes adverse effects on ionic homeostasis, which restrains cell elongation and division, causing premature leaf aging and reducing leaf and root growth (Munns 2002; Zhu 2001). Dionisio-Sese and Tobita (2000) reported that the net photosynthetic rate declined with increasing levels of salinity stress of four rice varieties (Dionisio-Sese and Tobita 2000). This might be due to the direct effect of salt on stomatal resistance via a reduction in guard cell turgor (Maxwell and Johnson 2000). Therefore, salinity is considered as one of the most decisive environmental factors limiting plant growth and productivity.

To counter negative effects of soil salinity, selection of salt tolerant plants, desalination of soil by leaching excessive salts, and application of biological processes such as plant-microbe interactions, alone or in combination, can be employed (Bandou et al. 2006; Feng et al. 2002; Munns 2005). Among the remediation tools, the desalination of soils relies on a high chemical input, hence, it is not economically viable for sustainable agriculture and is achieved at the expense of the environment (Bandou et al. 2006; Waller et al. 2005). There is considerable evidence that abiotic-stress tolerance can be evoked in plants by the exploitation of globally abundant microbes, which live in reciprocally beneficial relationships with plants (Waller et al. 2005). For example, a fungal endophyte of tall fescue (*Epichloë coenophiala*) significantly increases plant-available water and aggregate stability of rhizosphere soil and, thereby, might raise water productivity in drought periods (Hosseini et al. 2015a and b, 2016). *Piriformospora indica*, a plant root-colonizing basidiomycete fungus, was shown to provide strong growth-promoting activity during its symbiosis with barley (Baltruschat et al. 2008; Waller et al. 2005). Previous studies have demonstrated that the *Epichloë* spp. usually infect cool-season grasses such as tall fescue, meadow fescue, and perennial ryegrass and alter soil biological and biochemical properties through the production of alkaloids, phenolic compounds, and other secondary metabolites, such as carbohydrates and proline (Hosseini et al. 2015a, 2016; Malinowski et al. 2000). Microorganisms have been reported to enhance the growth of plants by stimulating plant growth, increasing mineral nutrition, and minimizing excessive ion uptake (Na⁺ and Cl[−]) under salt stress (Li et al. 2012; Smith and Read 2010; Waller et al. 2005). Furthermore, mutualistic symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants grown in saline soils (Baltruschat et al. 2008). Arbuscular

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mycorrhizal fungi enhance the growth of host plants by improving nutritional mineral uptake in soils (Al-Karaki 2000; Bandou et al. 2006; Tian et al. 2004). Mycorrhiza can help moderately salt-tolerant plants to prevent excessive translocation of ions to the shoot and leaf tissues, eventually improving salt tolerance (Giri and Mukerji 2004). However, mycorrhizal plants often show enhanced susceptibility to biotrophic leaf pathogens (Shaul et al. 1999).

Aspergillus aculeatus is a fungus, belonging to the genus *Aspergillus*, that was isolated from the rhizosphere of bermudagrass in Hunan province, Central China (Xie et al. 2014). Our previous study showed that root colonization and association of fungal hyphae with roots resulted in promotion of plant growth, higher yields, and reduction of Cd translocation to plant shoots under unfavorable conditions (Xie et al. 2014). In addition, *A. aculeatus* can be easily cultivated on synthetic media, allowing for large-scale propagation in contrast to arbuscular mycorrhizal fungi. Therefore, *A. aculeatus* might be possibly used in plant production in saline environments. The mechanism of *A. aculeatus*-induced salt tolerance has not yet been investigated. Bermudagrass (*Cynodon dactylon*) is a perennial grass distributed across all continents and on most oceanic islands between latitudes of approximately 45 degrees north and south (Xie et al. 2016). Bermudagrass is a widely used turf and forage that is well-adapted to salt sprays and coastal regions (Hu et al. 2015). In addition, this grass is naturally associated with *A. aculeatus* fungi, but the role of *A. aculeatus* fungi in bermudagrass growth, photosynthesis, and nutrition under salt stress remains unknown. The aim of the present study was to investigate the *A. aculeatus*-mediated protective plant responses to moderate (200 mM NaCl) and high (400 mM NaCl) salt stress in

bermudagrass. In order to elucidate physiological responses of *A. aculeatus*-colonized bermudagrass to salinization, we measured important indicators of salt stress, such as plant growth and productivity, lipid peroxidation, photosynthetic rates, and mineral nutrition.

RESULTS

Salt resistance and uptake of the strains.

The *A. aculeatus* strain was found to exhibit strong salt resistance characteristics, as shown by the markedly increased biomass by moderate salt stress when compared with unsalinized control (Fig. 1A). The inhibition effect of NaCl on growth was observed when *A. aculeatus* strains were subjected to 400 mM NaCl. The Na⁺ uptake and the percentage of adsorption by the *A. aculeatus* strains were determined over a concentration range of 100 to 800 mM NaCl for 24 h of adsorption is represented in Figure 1B. The accumulation of Na⁺ in *A. aculeatus* strains significantly increased with increasing salinity level. The removal efficiency was found to be higher at lower initial concentrations of NaCl. The adsorptive rate of Na⁺ reached maximum (62%) at 200 mM NaCl concentrations and, then, decreased as NaCl concentrations increased.

Characteristics of the strains.

The *A. aculeatus* strains had the capacity to produce indole-3-acetic acid (IAA) and siderophore in culture when the medium was supplemented with L-tryptophan (Table 1). When salinity increased from 200 to 800 mM, the concentration of IAA decreased, whereas the concentration of siderophore significantly increased when compared with the control.

A. aculeatus accelerates the growth of bermudagrass under salt stress.

Bermudagrass treated with high concentrations of salt (400 mM NaCl) for 2 weeks showed stunted growth and underwent early senescence. The shoot growth rate slightly decreased under moderate (200 mM NaCl) saline conditions (Fig. 2A). Moderate salinity caused a slight but not significant increase in root growth rate of bermudagrass plants (Fig. 2B). When inoculated bermudagrass plants were exposed to high salt concentrations, they showed reduced shoot and root growth rate compared with the control. However, the detrimental effect of high salt stress was partially ameliorated by *A. aculeatus*, as shown by the fact that inoculated plants showed higher shoot and root growth rate than their control plant counterparts (Fig. 2A and B). More interestingly, inoculated bermudagrass plants showed obviously greater root growth rate compared with uninoculated plants regardless of NaCl level (Fig. 2B). Moderate saline stress increased the root/shoot growth rate ratio for uninoculated plants (Fig. 2C). *A. aculeatus* inoculation caused a significant increase in the root/shoot growth rate ratio, when compared with uninoculated plants, regardless of NaCl level.

Turf quality of both treated plants declined with the increase in NaCl concentration and, to a larger extent, in uninoculated bermudagrass than inoculated plants. Inoculations with

Fig. 1. The influence of the salt treatment on the **A**, dry biomass and **B**, total Na⁺ uptake (in milligrams per gram of dry weight [DW]) of *Aspergillus aculeatus*. Error bars are $\pm$ standard deviation ($n = 4$). Columns marked with the same lower case letter were not significantly different among treatments ($P < 0.05$).

Table 1. Characteristics of the *Aspergillus aculeatus* strains^z

	NaCl concentration (mM)				
	0	100	200	400	800
Indole-3-acetic acid (pmol per liter)	11.3 a	9.7 b	10.3 ab	8.1 c	8.2 c
Siderophore production (%)	25.0 b	16.8 c	22.2 b	25.0 b	34.0 a

^z Values are means of five samples. Columns marked with the same lower case letter were not significantly different among treatments ($P < 0.05$).

A. aculeatus subsequently improved the turf quality compared with NaCl-only regimes when bermudagrass was subjected to high level salt treatment (400 mM NaCl). Chl decreased with the increasing NaCl concentration for uninoculated bermudagrass plants (Supplementary Fig. 1). No significant reduction in Chl was observed for inoculated plants with the increase in NaCl concentration. Among plants grown in a highly saline environment, Chl of inoculated bermudagrass plants was significantly higher when compared with the uninoculated regime.

A. aculeatus reduces lipid peroxidation and H₂O₂ level in salt-treated bermudagrass.

The degree of oxidative damage in the root and leaf was examined by determining the level of lipid peroxidation, which was estimated from the malondialdehyde concentration (MDA) concentration. As shown in Figure 3A and B, salt stress markedly increased the MDA concentrations in the root and leaf of NaCl- and NaCl + *A. aculeatus*-treated plants when compared with the control. No significant difference was observed in root and leaf MDA concentrations between uninoculated and inoculated plants subjected to 200 mM NaCl. Under high saline conditions (400 mM NaCl), the MDA concentration from the root and leaf of salt-treated bermudagrass plants increased by 109 and 267%, respectively, when compared with the unsalinized control. However, high salt exposure accelerated the MDA concentration by only 62% and 140% in root and leaf of *A. aculeatus*-inoculated plants.

There was no significant effect of moderate (200 mM) salt treatment on the H₂O₂ level in the root of both treatments, when compared with the unsalinized control (Fig. 3C). When inoculated plants were exposed to both moderate and high salt concentrations, the H₂O₂ accumulation in roots was significantly lower than that in uninoculated plants (Fig. 3C). Furthermore, inoculated plants showed obviously lower H₂O₂ levels in leaves compared with uninoculated plants under high saline conditions (400 mM NaCl) (Fig. 3D).

A. aculeatus enhances photosynthetic efficiency of salt-treated bermudagrass.

As shown in Figure 4, with the increase in salinity level, there was no obvious effect to OJIP fluorescence transient of bermudagrass leaves after dark adaption. In addition, no clear K-step was observed in all curves at 0.3 ms. However, we found that it slightly enhanced fluorescence intensity for inoculated plants when compared with uninoculated plants, regardless of NaCl level.

The JIP test was used to analyze basic fluorescence in order to determine the structural and functional parameters quantifying the photosynthetic behavior of the bermudagrass plants

(Supplementary Table 1). Inoculated plants treated with a high concentration (400 mM NaCl) salt stress showed significantly higher ϕP_0 , ϕE_0 , PI_{ABS} , and PI_{total} than uninoculated plants. In detail, PI_{ABS} and PI_{total} were 33 and 40% higher than that of uninoculated, respectively. There were no differences among other parameters between uninoculated and inoculated plants under three NaCl levels.

A. aculeatus induces IAA and glutathione (GSH) content of salt-treated bermudagrass.

As shown in Table 2, the IAA concentration in leaves and roots of both treatments significantly decreased as the salinity level increased. No significant difference was observed in IAA concentration in leaves between uninoculated and inoculated plants, regardless of NaCl level. Strikingly, IAA concentration was also enhanced in roots for inoculated plants relative to uninoculated ones, regardless of NaCl level.

GSH content of both treatment plants increased with the increasing in NaCl concentration, to a larger extent in inoculated bermudagrass plant than uninoculated plants (Table 2). Inoculations with *A. aculeatus* subsequently improved the GSH in both leaves and roots compared with NaCl only regimes when bermudagrass was subjected to moderate (200 mM) and high concentration (400 mM) salt stress.

Changes in ionic uptake and ionic homeostasis.

The accumulation of Na⁺ in the leaves and roots of both uninoculated and inoculated plants significantly increased as the salinity level increased but to a great extent in uninoculated plant (Fig. 5A and B). Compared with NaCl-only treated, inoculation of *A. aculeatus* significantly decreased the Na⁺ concentration in roots of bermudagrass under high (400 mM NaCl) saline conditions. The K⁺ content decreased as salinity levels increased in the leaf and root of both treatment plants (Fig. 5C and D). There was no difference for K⁺ concentration in leaves between uninoculated and inoculated plants, regardless of NaCl level. However, among plants grown in a high-saline environment, K⁺ concentration in roots of inoculated plants was significantly higher when compared with the uncolonized plants. Salt stress caused a decline of Ca²⁺ concentration in leaves of bermudagrass (Fig. 5E). At the same time, no significant differences in Ca²⁺ concentrations in roots were observed with an increasing salinity level (Fig. 5F). *A. aculeatus* inoculation caused a significant increase in Ca²⁺ concentration of roots when compared with uninoculated plants under highly saline conditions (400 mM NaCl).

The Na⁺/K⁺ and Na⁺/Ca²⁺ ratios were calculated from the Na⁺, K⁺, and Ca²⁺ concentrations. Salt treatment significantly

Fig. 2. Impacts of *Aspergillus aculeatus* on **A**, shoot growth rate, **B**, root growth rate, and **C**, root/shoot growth rate ratio of bermudagrass at 0, 200, and 400 mM NaCl. Error bars are $\pm$ standard deviation ($n = 5$). Bars marked with the same lower case letter for a given treatment (i.e., NaCl or NaCl + *A. aculeatus*) were not significantly different for the comparison of NaCl concentrations, based on Student's t test ($P < 0.05$). Asterisks indicate significant difference for comparisons between NaCl and NaCl + *A. aculeatus* treatments at a given NaCl concentration, based on Student's t test ($P < 0.05$).

increased the Na^+/K^+ and $\text{Na}^+/\text{Ca}^{2+}$ ratios in shoots and roots of both treatments, when compared with uninoculated plants (Fig. 6). It is intriguing to note that inoculated plants showed lower Na^+/K^+ ratios in leaves and roots when compared with uninoculated plants under moderate (200 mM) and high (400 mM) salt stress (Fig. 6A and B). Fundamentally, under moderate and high saline conditions, the Na^+/K^+ ratio in roots of inoculated plants

decreased by 43 and 49%, respectively, when compared with uninoculated plants. However, there was no significant difference in the $\text{Na}^+/\text{Ca}^{2+}$ ratio in leaves between uninoculated and inoculated plants regardless of NaCl level (Fig. 6C). When inoculated plants were exposed to high salt concentrations (400 mM), the $\text{Na}^+/\text{Ca}^{2+}$ ratio in roots was decreased by 56% compared with uninoculated plants (Fig. 6D).

Fig. 3. A and B, Malondialdehyde (MDA) concentration and C and D, H_2O_2 accumulation in the root and leaf of bermudagrass grown with or without *Aspergillus aculeatus* under 0, 200, and 400 mM NaCl stress. Error bars are $\pm$ standard deviation ($n = 5$). Bars marked with the same lower case letter for a given treatment (i.e., NaCl or NaCl + *A. aculeatus*) were not significantly different for the comparison of NaCl concentrations, based on Student's *t* test ($P < 0.05$). Asterisks indicate significant differences for comparison between NaCl and NaCl + *A. aculeatus* treatments at a given NaCl concentration, based on Student's *t* test ($P < 0.05$).

Fig. 4. Comparison of chlorophyll a fluorescence (OJIP) transient between NaCl and NaCl + *Aspergillus aculeatus* treatment under A, 0 mM, B, 200 mM, and C, 400 mM NaCl stress in bermudagrass leaves. OJIP transients were induced by 10-s light pulse of $3,000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. Data for the JIP test are sampled at 10- μs intervals for the first 320 ms.

EC and pH value in the soil affected by *A. aculeatus*.

The soil pH and EC (effective concentration) value were obtained, to determine the influence of *A. aculeatus* on soil properties (Supplementary Fig. 2). Salt stress decreased soil pH, as compared with the unsalinized control. Furthermore, in the inoculated treatment, the pH value was markedly decreased compared with the noninoculated regime, under moderate (200 mM) and high (400 mM) salt stress. Concomitantly, we found, when soil was exposed to high salt concentrations (400 mM), the EC value was increased by 23% by *A. aculeatus* compared with the uninoculated.

DISCUSSION

In this study, 100 and 200 mM NaCl enhanced the biomass of the tested *A. aculeatus* strain (Fig. 1). The inhibition effect of NaCl on growth was observed when *A. aculeatus* strain was subjected to 400 mM NaCl. This result is accordant with other studies, which indicated that moderate salinity had a positive effect on the metabolism of cytoplasm, whereas high salinity restricted the growth of mycelium and decreased the content of protein (Kernaghan et al. 2002; Nagarajan and Natarajan 1999). Chen et al. (2001) found that a variety of different *Pisolithus* species showed growth reduction in the presence of 200 mM NaCl. Blomberg and Adler (1993) indicated that 50 to 200 mM NaCl significantly decreased the growth of both mycelium and plants. *A. aculeatus* was more salt tolerant than the above-mentioned fungus species, since the strain remained at a biomass similar to that in controls at 300 mM NaCl.

In order to get a better understanding of the impact of *A. aculeatus* on the establishment of salt tolerance, we assessed important indicators of salt stress, such as plant growth and productivity, lipid peroxidation, photosynthetic behavior, and mineral nutrition. Previous studies have demonstrated that growth parameters, such as plant growth rate, turf quality, and chlorophyll concentrations in leaves has been reported to be excellent criteria to determine salinity tolerance in turfgrasses (Hu et al. 2012; Marcum 1999). In our study, higher growth rates, turf quality, and chlorophyll concentrations were observed for inoculated plants under salt-stress conditions, when compared with uninoculated plants. This showed that inoculation by *A. aculeatus* can alleviate the deleterious effects of salt stress. These findings are consistent with previous reports, which indicated that some microorganisms, particularly fungi and bacteria, can improve plant performance under salt stress (Baltruschat et al. 2008; Brown 1974; Waller et al. 2005). It is possible that salinity resulted in a progressive decline in the level of IAA in the root system of plants (Sakhabutdinova et al. 2003). It is also suggested that root-colonizing microorganisms that produce phytohormones may act as mechanisms for plant growth stimulation and can prevent the deleterious effects of stresses from the environment (Frankenberger and Arshad 1995). In our

study, the *A. aculeatus* strains had the capacity to produce IAA. Strikingly, IAA concentration was also enhanced in roots for inoculated plants. Therefore, the IAA-producing fungus strain *A. aculeatus* also alleviated, effectively, the reductive effect of salt stress on plant growth rates, probably through its ability to produce IAA. It is essential to point out that the root growth rate of *A. aculeatus* on the bermudagrass plants occurred not only during salt stress but also in unsalinized control conditions, which implies that the fungal effect improving plant growth was not a specific process induced by salinity stress.

To better unravel the impact of *A. aculeatus* on the establishment of salt tolerance, we assessed biochemical markers for salt stress, such as lipid peroxidation, H_2O_2 , and photosynthetic efficiency. Salt-induced lipid peroxidation was significantly attenuated in *A. aculeatus*-treated plants. Previous studies have demonstrated that cellular damage as a result of salt stress is associated with an accumulation of reactive oxygen species (ROS) (Hernández et al. 2000). ROS can be toxic to living cells and act as signaling molecules for stress responses. According to a recent report, endophytic fungi characterized by their broad host ranges can confer effective tolerance to ROS under salinity conditions (Rodriguez et al. 2008). Our results indicated that inoculated plants showed obviously lower H_2O_2 levels in leaves and roots compared with uninoculated plants under high salt conditions. Consistent with the H_2O_2 accumulation, the amount of lipid peroxidation (i.e., the MDA concentration) was lower in inoculation with *A. aculeatus*. Therefore, the *A. aculeatus* seemed to overcompensate the salt-induced accumulation of H_2O_2 and lipid peroxidation. GSH plays a critical role in oxidative stress by converting the oxidized GSSG to GSH (Fadzilla et al. 1997). It is also a major antioxidant that is known to protect cells from oxidative stress (Noctor et al. 1998). Moreover, GSH seems to be a signal molecule acting as a direct link between environmental stress and key adaptive responses and it plays a protective role by increasing stress tolerance (Kok and Oosterhuis 1983; May and Inzé 1998). *A. aculeatus* improved GSH concentration in both leaves and roots compared with NaCl-only regimes. The increased GSH content in the inoculated plants may reflect, at least partially, a higher antioxidant defense existed in the detoxification of membrane lipid peroxidation. This suggests that enhanced tolerance to salt stress can be associated with alleviation of lipid peroxidation in *A. aculeatus*-inoculated plants.

Chlorophyll a fluorescence was expected to be a useful tool for investigation of plant photosynthetic efficiency under abiotic stress conditions (Chen et al. 2013). Previous studies demonstrated that salt stress caused an inhibition to PSII activity (Allakhverdiev and Murata 2008). However, other research has shown that salinity did not affect the PSII of most barley leaves (Morales et al. 1992). In this study, there is no obvious effect to OJIP fluorescence transient of bermudagrass leaves with the increase in salinity level, even when growth was seriously restricted (Fig. 4). Our results suggest that, although salinity

Table 2. Effects of *Aspergillus aculeatus* on indole-3-acetic acid (IAA) and glutathione (GSH) concentrations in the leaf and root of bermudagrass under 0, 200 and 400 mM NaCl stress^z

	NaCl (mM)	IAA (pmol per liter)			GSH (μmol per liter of fresh weight)		
		NaCl	NaCl+A. <i>aculeatus</i>	% of NaCl	NaCl	NaCl+A. <i>aculeatus</i>	% of NaCl
Leaf	0	124.3	129.5	104	68.7	67.4	94
	200	98.6	96.4	97.7	77.9	85.6 *	109
	400	68.3	70.5	103	95.6	109.2 *	114
Root	0	65.4	87.9 *	134	25.2	29.6	117
	200	59.6	79.1 *	133	36.7	45.2 *	123
	400	47.2	67.5 *	143	53.1	68.5 *	129

^z Values are means of five samples. Asterisks indicate significant differences for comparison between NaCl and NaCl + *A. aculeatus* treatments at a given NaCl concentration, based on Student's *t* test ($P < 0.05$).

impairs the growth of bermudagrass plants, plants can efficiently dissipate excessive light not needed for photosynthesis, as shown by the maintenance of an efficient photochemistry (Morales et al. 1992). This phenomenon may be due to salt-induced physiological changes leading to leaf death and not to salt itself (Morales et al. 1992). More interestingly, our results showed that fluorescence intensity was slightly enhanced for inoculated plants, as compared with uninoculated (Fig. 4). Meanwhile,

φP_0 (Fv/Fm), φE_0 , PI_{ABS} , and PI_{total} was slightly enhanced for inoculated plants under high salt concentration. The alterations of φP_0 , φE_0 , and performance indices (PI_{ABS} and PI_{total}) substantiate the increase in the efficiency of photosynthetic electron transport for inoculated plants. The performance indices used as an indicator of plant vitality increased significantly under the high-salinity condition for inoculated plants, as evidenced by increased chlorophyll content and high turf quality.

Fig. 5. A and B, Effects of *Aspergillus aculeatus* on Na⁺, C and D, K⁺, and E and F, Ca²⁺ concentrations in the leaf and root of bermudagrass grown with or without *A. aculeatus* under 0, 200, and 400 mM NaCl stress. Error bars are $\pm$ standard deviation ($n = 5$). Bars marked with the same lower case letter for a given treatment (i.e., NaCl or NaCl + *A. aculeatus*) were not significantly different for the comparison of NaCl concentrations, based on Student's t test ($P < 0.05$). An asterisk indicates a significant difference for comparison between NaCl and NaCl + *A. aculeatus* treatments at a given NaCl concentration, based on Student's t test ($P < 0.05$).

Previous research indicated that the primary effects of salt stress are through ion toxicity (Levitt 1980). The excessive accumulation of Na^+ in plant cells decreases photosynthesis in leaves. Salinity stress results in rapid accumulation of Na^+ and decreases in K^+ , because Na^+ can enter plant cells through a high-affinity K^+ transporter (Haro et al. 2005). This disturbs the ionic balance, causing injury. Therefore, a crucial question addressed in this study was whether *A. aculeatus* strains would also protect the bermudagrass plants from the uptake of excess NaCl. It is noteworthy that *A. aculeatus*-inoculated plants exhibited reduced Na^+ uptake in root and shoot tissues as compared with uninoculated controls in our study. Under high saline conditions, the Na^+ concentration in leaves and roots of inoculated plants was 8 and 23% lower than in uninoculated plants. The diminished buildup of salt in leaf tissue is likely the result of salt uptake and transport restriction in roots (Chen et al. 2002). Our data show that *A. aculeatus* fungal hyphae exhibited a greater capacity for Na^+ absorption under saline conditions. The adsorptive rate of Na^+ reached maximum (62%) at 200 mM NaCl concentrations. Therefore, an obvious assumption was that the fungus might provide a physical barrier against salt uptake into the root. It appears that the role of *A. aculeatus* in alleviating salt stress is partly to prevent Na^+ absorption to root and translocation to shoot tissues.

In addition to an influence on Na^+ concentrations, *A. aculeatus* fungus improved the general nutrient status of bermudagrass, causing significant increases in Ca^{2+} and K^+ concentrations in root under high (400 mM) saline conditions (Fig. 5). Excess Na^+ causes injury in plants because Na^+ competes with K^+ , leading to a depletion of K^+ (Tester and Davenport 2003). Our data show that *A. aculeatus*-inoculated plants accumulated significantly more K^+ in their leaves and roots than uninoculated plants. Therefore, *A. aculeatus*-inoculated plants were able to maintain a low Na^+/K^+ ratio, which is a hallmark of salinity tolerance (Munns and Tester 2008). This result indicated that the disruption of ionic homeostasis was prevented in inoculated plants. Such beneficial effects on K^+ accumulation have also been reported for arbuscular mycorrhiza (Giri and Mukerji 2004; Langenfeld-Heyser et al. 2007). We suggest that the maintenance of a high level of K^+ may also contribute the alleviation of the negative effects of sodium. However, it is currently not clear how *A. aculeatus* fungus affect plant transport and uptake systems for K^+ .

In conclusion, our results demonstrated that a high-saline environment is well tolerated by *A. aculeatus*-inoculated bermudagrass plants. *A. aculeatus* facilitated bermudagrass growth, alleviated the NaCl toxic effects, and inhibited Na^+ uptake and transport in bermudagrass. Based on our results, we

Fig. 6. Effects of *Aspergillus aculeatus* on **A** and **B**, the Na^+/K^+ ratio and **C** and **D**, the $\text{Na}^+/\text{Ca}^{2+}$ ratio in the leaf and root of bermudagrass grown with or without *A. aculeatus* under 0, 200, and 400 mM NaCl stress. Error bars are $\pm$ standard deviation ($n = 5$). Bars marked with the same lower case letter for a given treatment (i.e., NaCl or NaCl + *A. aculeatus*) were not significantly different for the comparison of NaCl concentrations, based on Student's t test ($P < 0.05$). Asterisks indicate significant differences for comparison between NaCl and NaCl + *A. aculeatus* treatments at a given NaCl concentration, based on Student's t test ($P < 0.05$).

deduced that differences in salt tolerance and accumulation in bermudagrass inoculated with *A. aculeatus* might be explained by the following mechanisms. *A. aculeatus* i) stimulated plant growth through its ability to produce IAA; ii) increased activity of antioxidants, which could decrease ROS concentrations; iii) provided a physical barrier against salt uptake into the root; and iv) prevented, in the inoculated plant, disruption of ionic homeostasis under salt stress. To the best of our knowledge, this is the first research report elucidating the characteristics and salt tolerance of the *A. aculeatus* strains and elucidating the role of *A. aculeatus* in salt tolerance and the oxidative system by bermudagrass with concurrent promotion of plant growth in a pot experiment. *A. aculeatus* might be a suitable candidate for phytoremediation processes and revegetation of salinity soil.

MATERIALS AND METHODS

Effect of NaCl on growth and ion relations in *A. aculeatus*.

Fungal cultures. The fungus used in this study was *A. aculeatus*, which was obtained from isolates from the rhizosphere of bermudagrass (Xie et al. 2014). *A. aculeatus* isolates were cultured in Martin agar medium and liquid Martin medium. The Martin agar medium contained following components in grams per liter: glucose, 10.0 g; peptone, 5.0 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; and agar, 18.0 g; diluted with water to 1.0 liters, plus 1% rose bengal solution, 3.3 ml, pH 6.0. The *A. aculeatus* isolates, stored at -80°C , were activated with Martin agar medium on petri dishes (diameter 90 mm). The petri dishes were kept in permanent darkness at 30°C and relative humidity was 60%.

Salt treatment. For salt treatment, 100, 200, 300, 400, 600, and 800 mM NaCl was added to the liquid Martin medium, respectively. Controls were grown in the absence of NaCl. Discs of mycelium were cut from the actively growing edge of 10-day-old colonies using a 6-mm diameter cork borer. Eventually, the discs were immersed in a 250-ml Erlenmeyer flask filled with 100 ml of liquid Martin medium (3 discs per flask). The flasks were placed on the rotary shaker at 180 rpm and 30°C . The mycelium pellet and liquid Martin medium were harvested after 48 h of salt treatment.

The biomass of the mycelium pellet. After 48 h of salt treatment, the liquid medium was filtrated with qualitative filter paper. The obtained mycelium pellet was washed with deionized water to remove culture medium and, then, was air-dried at room temperature (72°C) and dry weight was obtained.

Cations. For Na^+ determination, the over-dried mycelium pellets (0.05 g) were digested in a mixture of concentrated HNO_3 , HF, and HCl (5:2:2, vol/vol/vol). The mixture was digested in an ETHOS ONE microwave sample preparation system (Milestone), using the following procedure: 12 min at 130°C , 25 min at 160°C , and finally, 25 min at 180°C . The mineral extract was adjusted to 50 ml and was then filtrated with a $0.45\text{-}\mu\text{m}$ filter membrane. The Na^+ concentration was determined using inductively coupled plasma optical emission spectroscopy. Each experiment was repeated at least five times.

IAA production. The IAA concentration in culture was detected with L-tryptophan, following the procedures described by Sheng et al. (2008). In brief, the *A. aculeatus* strain was cultured for 48 h in an Erlenmeyer flask containing 100 ml of liquid Martin medium supplemented with tryptophan. After incubation, a 1-ml cell suspension was mixed with 2 ml of Salkowski's reagent at room temperature for 15 min, after which a pink color developed in the suspensions. The IAA concentration in culture was calculated by examining the absorbance of pink color developed after 25 min of incubation at 530 nm. Absorbance values were calibrated using a calibration curve of pure IAA.

Effect of *A. aculeatus* on salt tolerance and ionic homeostasis in bermudagrass.

Plant materials and growth conditions. Uniform stolon (4 cm long) of bermudagrass 'WBD 242' were planted in solid growth substances (1:1 peat soil/sand, vol/vol) in plastic pots (15 cm diameter and 20 cm tall) on May 20, 2015. The pots were placed in a greenhouse for two months to allow for the establishment of roots and shoots, with the day and night temperatures set at $35 \pm 3^\circ\text{C}$ and $20 \pm 3^\circ\text{C}$, respectively, and 14 h light and 10 h dark. During the growth period, plants were irrigated three times per week and were fertilized weekly with 60 ml of half strength Hoagland's solution (Hoagland and Arnon 1950). The grasses were hand clipped to 10 cm in height every week.

Fungal growth and plant-fungus cocultivation. The substrate used was a mixture of coarse silica sand and sawdust (3:1; vol/vol, pH = 6.5). The substrate was sieved with stainless steel mesh (1 mm) and was sterilized at 127°C for 120 min. Subsequently, the matrices were divided into two groups, namely NaCl treatment only (NaCl) and NaCl plus *A. aculeatus* treatment (NaCl + *A. aculeatus*).

The fungus used in this study was the *A. aculeatus*. The isolate, stored at -80°C , was activated with Martin agar medium (the medium contains the following components (measured in grams per liter): glucose, 10.0 g; peptone, 5.0 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; agar, 18.0 g; diluted with water to 1.0 liters; plus 1% rose bengal solution, 3.3 ml (pH 6.0) on July 18, 2015, and then, was inoculated in a 250-ml Erlenmeyer flask filled with 150 ml of liquid Martin medium. The flask was placed on the rotary shaker at 180 rpm and 30°C for 48 h. For the NaCl + *A. aculeatus* group, the inocula of fungi isolates in liquid Martin medium were filtered with gauze and were then transferred into the substrate and mixed before the plants were transferred.

For plant-fungus cocultivation, an equal amount of grass was rinsed thoroughly, using distilled water, and then, the grass was transplanted into plastic pots (15 cm in diameter and 20 cm deep) filled with inoculated substrate on July 20, 2015. All the pots were set into the growth chamber with standard conditions (32 and 25°C day and night temperatures, 14-h light and 10-h dark cycle, 60% relative humidity and photosynthetically active radiation levels at $720 \text{ mmol m}^{-2} \text{ s}^{-1}$) for cocultivation for two weeks.

Treatments and experimental design. After two weeks of cocultivation, the transpiration rate was measured by the difference in the plant-pot system weight on a 24-h interval. Plant-pot systems with similar transpiration rates were grouped as a same replication, in order to reduce the initial differences. Subsequently, grasses were subjected to three salinity levels: 0 mM (control), 200 mM (moderate), and 400 mM (high) NaCl. Salinity was increased by daily increments of 50 mM NaCl, until a final salinity level of 200 and 400 mM was reached. After the final salinity, the shoot was clipped. The containers with plants were kept in the above-mentioned conditions for two weeks. During the period of salt exposure, the plants were irrigated daily, to maintain sufficient water and nutrition supply, with half-strength Hoagland's solution. At the end of the experiment (on August 18, 2015), the visual turf quality, canopy height, root length, and chlorophyll content were determined. Plants were separated into roots and leaves and were then immediately frozen in liquid nitrogen and stored at -80°C for further subsequent analysis. The salt concentrations and treatments were arranged in a randomized complete block design with five replicates.

Measurements. Turf quality was visually assessed based on plant density, grass color (extent of chlorosis and leaf senescence), and degree of leaf wilting, using a nine-point system, in which 0 is withered yellow and dead grass, 9 is fully turgid,

green, dense, and uniform grass, and 6 is minimum acceptable quality.

Chl was recorded using the chlorophyll meter (SPAD-502, Minolta Japan) at the end of the experiment. The content of each individual was recorded from nine fully expanded leaves with three measurements on each leaf.

Canopy height was determined by the difference in turf canopy height before and after treatment, following the procedures described by Huang and Liu (2009). In brief, canopy height was estimated by measuring four positions in each pot, using a ruler, and measurements were averaged. Shoot growth rate was the difference in canopy height before and after treatment at 2-week intervals. Root length was determined only for roots elongating before and after treatment. Subsequently, the root/shoot length ratio (average root length/canopy height) of the grass was determined.

Chlorophyll fluorescence measurements were assessed with a pulse-amplitude modulation portable chlorophyll fluorimeter (PAM 2500; Heinz Walz GmbH) with high time resolution (10 μ s), from the fourth fully expanded leaves at the end of the experiment. To ensure sufficient closure of PSII reaction centers and estimate the maximum fluorescence yield, the leaves were given a dark-adaptation for 30 min. The chlorophyll fluorescence transients (OJIP curve) were determined by the measuring a red light of 3,000 μ mol photons $\text{m}^{-2} \text{s}^{-1}$, which was sufficient excitation intensity to obtain a true fluorescence intensity of F_M . The chlorophyll a fluorescence emission that was induced by the strong light pulses was measured and digitized between 10 μ s and 320 ms. The OJIP curve was analyzed using the JIP test (chlorophyll fluorescence analysis method) (Chen et al. 2013).

MDA was determined according to the method described by Heath and Packer (1968), with slight modifications, as we described before. Briefly, fresh leaves and roots (0.3 g) were homogenized in trichloroacetic acid (0.1% wt/vol) solution. The homogenate was then centrifuged at 10,000 $\times g$ for 15 min at 4°C and 1 ml of supernatant was added to 1 ml 0.5% thiobarbituric acid in 20% trichloroacetic acid. The mixture was heated in a water bath at 90°C for 30 min and was then cooled on ice and centrifuged at 10,000 $\times g$ for 10 min. The MDA concentration was calculated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm and was calibrated by using the extinction coefficient of 155 $\text{mM}^{-1} \text{cm}^{-1}$ (Heath and Packer 1968).

To measure the H_2O_2 content, frozen leaves and roots (0.1 g) were homogenized in sodium phosphate buffer (100 mM, pH 7.8) at 4°C. The H_2O_2 content was measured with a hydrogen peroxide assay kit (S0038; Beyotime), according to the manufacturer protocols. The H_2O_2 content was calculated by examining the absorbance of the reaction buffer at 560 nm. Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2 .

Quantification of endogenous IAA was performed with the enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer protocols, as previously described by Lin et al. (2005). Briefly, frozen tissues (0.3 g) were ground into a fine powder with liquid nitrogen. The obtained powder was suspended in 80% methanol and centrifuged at 8,000 $\times g$ for 10 min at 4°C, to extract IAA. Then, the collected supernatant was immediately passed through a pre-equilibrated C18 Sep-Pak cartridge (Millipore). The efflux was collected, was dried in N_2 gas, was dissolved in 100% methanol for methylation with freshly synthesized ethereal diazomethane, was dried under N_2 gas, again, and was redissolved in 300 μ l of phosphate buffered saline for IAA ELISA, using a monoclonal antibody of high specificity for IAA methyl ester, a standard procedure. Plant endogenous IAA was assayed and determined three times on the same extract, and samples were determined in three biological replicates.

The GSH content was measured using a commercial colorimetric assay kit, GSH-400, from OXIS International, according to the manufacturer's instructions. The GSH activity was calculated by measuring the absorbance at 420 nm.

For Na^+ , K^+ , and Ca^{2+} determination, the oven-dried tissues were finely ground and determined as described above. Each experiment was repeated at least five times.

At the end of the experiment, the pH values and electrical conductivity of the substrate were determined using an Accumet AR10 meter (Fisher Scientific Inc.) and W.E.T. sensor kit (Delta-T Device Ltd.), respectively.

Statistical analysis.

All mean data were subjected to analysis of variance. Significant differences between means were performed using Student's t test and multivariate analysis of variance. Differences were considered to be significant at $P < 0.05$. All the statistical analyses were carried out using SPSS 20.0.

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