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Research Article

Arbuscular mycorrhizae enhanced the growth and freezing tolerance of Mongolian crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.)

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Abstract

Agropyron cristatum (L.) Gaertn. (Crested wheatgrass) is a dominant endemic grass species of the Mongolian steppe. Our earlier study showed that the arbuscular mycorrhizal fungus (AMF) *Acaulospora scrobiculata* Trappeis capable of forming a symbiotic association with Mongolian crested wheatgrass. In this study, the effects of the arbuscular mycorrhizae (AM) on growth and freezing tolerance of crested wheatgrass were determined. Plant height, biomass and concentrations of chlorophyll, minerals and proline were significantly higher in mycorrhizal (M+) than non-mycorrhizal (M-) crested wheatgrass when grown at 20± 3°C in greenhouse. To test its cold tolerance, cold-acclimated crested wheatgrass seedlings were subjected to a freezing temperature of -8, -11, -14, -15, -16 or-17°C for 2 h and then cultivated at 12 ± 2°C for 10 d. The leaf LT_{50} (lethal temperature for causing 50% mortality) of the M- and M+ crested wheatgrass were -8 and -12.5°C, respectively. Consistently, the seedling LT_{50} of M- and M+ crested wheatgrass were -11 and -15.5°C, respectively. These results demonstrated that *A. scrobiculata* could effectively form arbuscular mycorrhizae with crested wheatgrass, which significantly improves its nutrition concentrations, growth, and freezing tolerance.

Keywords: *Agropyron cristatum*, *Acaulospora scrobiculata*, Arbuscular mycorrhizae, Chlorophyll, Freezing Tolerance, Proline.

Introduction

Agropyron cristatum (L.) Gaertn. (Crested wheatgrass) is a dominant endemic species in the Mongolian steppe. Available forages in this area consist primarily of *Stipa krylovii*, crested wheatgrass and *Allium polyrrhizum* (representing 80% of available phytomass), all of which are regarded as desirable forage plants (Retzer 2007).Crested wheatgrass is widely used in the restoration of the Mongolian grassland.

Arbuscular mycorrhizae (AM) is one of the most common symbioses worldwide and about 80% of the known plant species form AM (Smith and Read 2008).The symbiosis contributes to improved water use and nutrient uptake, especially for elements with low soil mobility, such as P and Zn, and it increases plant tolerance to environmental stresses, such as nutrient deficiency, diseases, drought and salinity (Smith and Read 2008; Gupta and Kumar 2000).Previous research has examined the distribution of AMF in sandy area (Blaszkowski et al.2002), in agricultural soils (Oehl et al.2009), and in certain natural ecosystems (Guadarrama and Alvarez-Sanchez 1999), but few of them have looked into grasslands

(Smith and Read 2008), especially in arid and semiarid areas (Lugo and Cabello 2002), or areas with freezing temperatures.

Low temperature is one of the most important stress factors that reduce plant growth by affecting various physiological and metabolic processes (Charest and Phan 1990; Guy 1990). Low temperature decreases the capacity and efficiency of photosynthesis through changed pigment composition, declined electron transport and impaired chloroplast development (Farooq et al. 2009). Mycorrhizae enhance protection against low temperature stress, via better access to nutrients and maintenance of active physiology, i.e. intercellular CO_2 concentration, electron transport and photosynthetic enzyme activity (Zhu et al. 2010; Wu and Zou 2010).Charest et al.(1993) reported that mycorrhizae counteract chilling injury in maize *(Zea mays* L.). Volkmar and Woodbury (1989) also demonstrated that AMF are beneficial to the growth of barley *(Hordeum vulgare* L.) under different soil temperatures.

Frost injury to plants during the reproductive stage is a common problem in temperate region. Injury to leaves caused by late spring and early autumn frosts significantly reduces the growing season, and exerts a strong influence over plant production and its distribution (Woodward 1987). Late spring frosts are particularly damaging because they occur at a time when most plants have broken dormancy, and introduce significant costs for leaf replacement. Freezing injury is caused primarily by the physical disruption of cellular structures by ice crystal and desiccation resulting from the higher water potential of cellular contents than extracellular ice (Pearce 2001). However, the protective effect of mycorrhizae on plants subjected to cold temperatures has not yet been extensively studied.

Mongolia has a continental climate with extreme fluctuations in temperature, both daily and seasonally. Freezing events are critical to the ecology of the Mongolian steppe during spring time. Grasses in Mongolia usually begin to grow at the end of April or early May. Late spring frosts generally occur during May. Freezing injury can cause serious mortality of crested wheatgrass, the important endemic species of Mongolian steppe. Therefore, cold tolerance and survival play an important role in growth and survival of crested wheatgrass. Some measures must be taken to maintain or restore Mongolian grasslands. Thus, the aims of this study were to assess the effects of the native AMF on growth and freezing tolerance of Mongolian crested wheat grasses seedlings through mycorrhizal inoculation and freezing test. It is hoped that the findings from this study may contribute to the application of mycorrhizal technique in restoration of Mongolian grasslands.

Materials and Methods

Isolation, identification and propagation of AMF

Spores of AMF in rhizosphere soils of crested wheatgrass were extracted by wet sieving and decanting method (Gerdermann and Nicolson 1963; Tommerup 1992) and sucrose density gradient centrifugation (Daniels and Skipper, 1982), and then identified with reference to the key provided by the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM, http://www.invam.caf.wvu.edu). After identification, spores were subsequently propagated with corn seeds germinated in sterilized sand pot culture for AM fungal inoculum preparation. Spore sand was quantified for a further inoculation test.

Plant material and growth conditions

Seeds of crested wheatgrass were collected from natural grassland in the vicinity of Ulaanbaatar city (107°08′31″ E, 47°45′767″N, at an elevation of 1597m) of Mongolia. Seeds were first sterilized with a 10% sodium hypochlorite solution for 15 min, rinsed three times with sterile distilled water, and then germinated on sterilized mixture of peat moss, vermiculite and per litre (1:1:1, v/v). As the seedlings attained4cm in height, one plant was transferred to each pots (12 cm diameter and 10 cm height) filled with sterilized sand for AMF inoculation.

AMF inoculation

The grass seedlings were inoculated with 10 g sand spore (*Acaulospora scrobiculata* containing 15 ± 5 spores/g sand). Non-inoculated seedlings treated with the sand-spore filtrate served as the control. All seedlings were cultured in a greenhouse set at 20°C and 1000 ± 200 μmole photons m^{-2} sec^{-1} photosynthetic photon flux density (PPFD), and

watered with deionized water as needed without supplemental fertilization.

Examination of mycorrhizae

After three months of culture, the roots of seedling were sampled and cleaned with water in a supersonic oscillator (Upson et al., 2007). Roots were cut into 1 cm segments, cleared in 10% KOH, treated with 3% H_2O_2 and 1% HCl, and then stained with 0.05% Trypan blue. The morphology of mycorrhizae was observed with a stereomicroscope (Abbott 1982; Brundrett et al.,1996). Mycorrhizal root colonization was assessed by grid line intersection method (Giovannetti and Mosse 1980).

Cold acclimation

Plants were subjected to four treatments (40 pots/treatment) as follows:

- **Non-stressed treatment**: plants, inoculated with AMF (*A. scrobiculata*) or non-inoculated (control), were grown in a greenhouse at 20±3°Cfor 9 months.
- **Cold acclimation treatment**: plants, inoculated with AMF (*A. scrobiculata*) or non-inoculated (control), were hardened for two weeks at 12°C under a 10 h photoperiod, and then cold acclimated at 2°C under an 8 h photo period for two weeks, 0°C for 24 h, and 2°C for 24 h (Pociecha et al. 2009). The cold acclimated plants were used for freezing test subsequently**.**

Assessment of freezing tolerance

After hardening and cold acclimation, all dead leaves were removed and six plants in each treatment were subjected to freezing test at -8, -11, -14, -15, -16,or-17°C for 2 h (Rapacz et al. 2004). Then, the plants were transferred to a greenhouse at 12± 2°C, 1000 ± 200µmole photons m^{-2} sec^{-1} PPFD, and watered with deionized water. The extent of leaf injury was assessed in all plants exposed to freezing treatments. The total numbers of dead (>67% leaf area with necrotic symptom) and green leaves were counted in the following 3-7 days, and used to calculate leaf LT_{50}. LT_{50} was determined by controlled freezing treatment followed by visual rating of plants after 10 days of cultivation at 12± 2°C (Palonen and Buszard 1998).

Estimation of photosynthetic pigment concentration

The photosynthetic pigments (chlorophyll a, b and a+b) were extracted and determined in the fresh leaves of crested wheatgrass, according to the spectrophotometric method of Tseng et al. (1991).Fresh tissue (0.05g) was sampled from the youngest fully expanded leaf, and chlorophyll was extracted with 10 ml 80% acetone and read at 663 and 645 nm using a UV/visible spectrophotometer.

Proline analysis

Determination of free proline concentration was performed according to Bates et al. (1973). Leaf samples (0.5 g) from each plant were homogenized in 3% (w/v) sulphosalycylic acid and homogenate filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, the resulting mixture was heated at 100°C for 1 h in a water bath. Reaction was then stopped by cooling in an ice bath. The mixture was extracted with toluene, and the absorbance of fraction with toluene aspired from liquid phase was read at 520 nm. Proline concentration was determined using a calibration curve and expressed as mg/g fresh weight.

Growth and yield measurements

After cold acclimation, four plants per treatment of non-inoculated and inoculated seedlings were harvested. Growth parameters including plant height, root length, number of leaves, areas of leaves, fresh and dry weights of leaves, stems and roots were determined. Dry weights were measured after drying the samples in an oven at 70 ± 2°C for 48 hr. Plant water contents were calculated using the following formula.

Plant water content = (fresh weight of plant – dry weight of plant / fresh weight of plant) * 100%

Leaf area was measured using a Li-3100 leaf area meter (Li-COR, Inc., Lincoln, Nebraska, USA) and specific leaf area (SLA) was calculated according to the following equation.

(SLA (cm^2/g) = leaf area (cm^2) / leaf dry weight (g)).

Leaf anatomy

Leaves were sampled and cut into 1cm long pieces, fixed in F.A.A. (Formalin: Acetic acid: Alcohol, 5: 5: 50, v/v) overnight, then rinsed with distilled water 3 times and dehydrated in 70%ethanol and a series of TBA concentration of 20, 35, 55, 75 and 100%. The specimens were embedded in paraffin wax (m.p. 56°C), and transverse sections of 10-12 µm thickness were cut with a rotary microtome. Paraffin was removed with xylol and sections were stained with Safranin and Fast green (Ruzin 1999).

Mineral concentration analysis

For mineral concentration analysis, root, shoot, and leaf samples were oven-dried at 70±2°C and digested with concentrated H_2SO_4 and H_2O_2. Nitrogen concentrations of root, shoot, and leaf were estimated by microkjeldahl method (MacDonald 1977). Phosphorus, potassium, calcium, sodium, and magnesium concentrations were estimated by inductively coupled plasma atomic emission spectrometry.

Quantification of mycorrhizal dependency

Mycorrhizal dependency was defined as the ratio of the dry weight of seedlings with and without inoculation with AMF (Graham and Syvertsen 1985).

Statistical analysis

Statistical analysis was performed using the software Statistical Package for the Social Science (SPSS 12.0, IL. USA) for window program. All data represent means of 4 separate experiments ± standard error (n = 4). Differences in growth and physiological characteristic rates among treatments were analyzed by Tukey's multiple range tests at $p \leqq 0.05$ significant level.

Results and Discussion

Identification and morphology of arbuscular mycorrhizae

After extraction, spores of AMF were collected. The AMF species was identified as *Acaulospora scrobiculata* using the synoptic keys and species morphology of the INVAM website (http://invam.caf.wvu.edu). *A. scrobiculata* were formed singly in soil, orange yellow to orange brown and globose to subglobose (Fig. 1a). Three layers (L1, L2 and L3) of spore wall were observed in the *A. scrobiculata* (Fig. 1a). Also, germinal wall (gw), hypha remnant and pits were found (Fig. 1a).

Significant AM colonization was observed in crested wheatgrass inoculated with *A. scrobiculata.* The roots of inoculated seedlings produced network of external hyphae (Fig. 1b). Staining of root samples revealed that AM developed well in the roots of inoculated crested wheatgrass seedlings. Arbuscules and vesicles were also present abundantly in the roots of inoculated crested wheatgrass seedlings (Fig. 1c and d).Other AMF, such as *Glomus macrocarpum*, *G. macrocarpum* var. *macrocarpum*, were also found to form AM with *Agropyron smithii* in Colorado, USA (Singh, 2004). Caldwell et al. (1985) reported that the Asian *Agropyron desertorum* (Fisch. Ex Link) Schultes had a greater frequency of arbuscule formation than the North American species *Agropyron spicatum* (Pursh) Scribner & Smith in response to phosphorus fertilization. In our study, three months after inoculation, 65% root colonization and external hyphae, spores, vesicles, and arbuscules, were observed in roots of crested wheatgrass seedlings (Fig. 1b, c and d).

Plant growth

The cold temperature treatments significantly decreased all growth parameters of crested wheat grasses, such as plant height, root length, dry weight of AMF inoculated and non-inoculated plants (Tables 1and 2).However, the height and root length of AMF inoculated crested wheat grass were significantly higher than those of non-inoculated ones under both normal growth and cold acclimation conditions (Table 1). At normal growth condition, the enhancements in these two parameters were 103% and 90%, respectively, whereas at cold acclimation, the enhancements in plant height and root length were 134% and 186%, respectively. In addition, the dry weights of roots, stems and leaves of AMF inoculated crested wheat grass were also significantly higher than those of the controls under both conditions (Table 2).In our study, the beneficial effect of AMF symbiosis on plant growth and dry weight under different temperature conditions was in agreement with previous studies using other plant species (Anderson et al. 1987; Volkmar and Woodbury 1989; Charest et al. 1993; Gavito et al. 2003; Zhu et al.

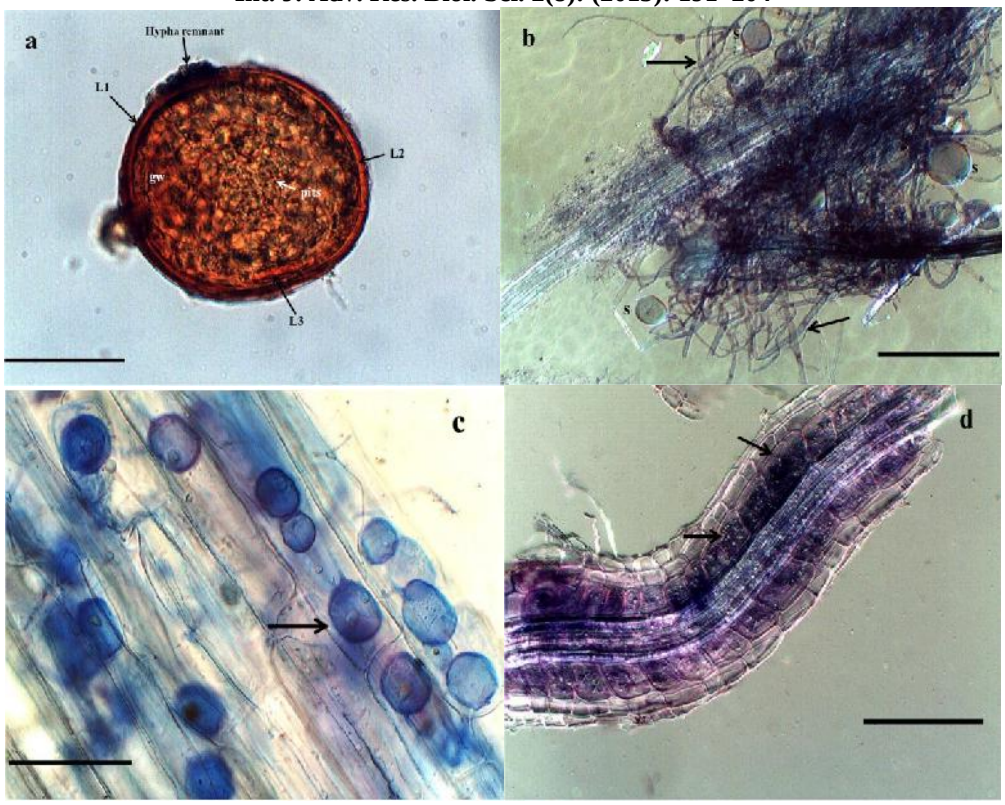

Fig. 1 Morphology of root of crested wheat grass inoculated with *A. scrobiculata*. ***a*** Morphology of *A. scrobiculata*. **b** External hyphae (arrowhead) and chlamydospores (s) produced by *A. scrobiculata*. **c** Structure of root with vesicles (arrowhead). **d** Structure of root with arbusculus (arrowhead).*Bars*=100 µm.

Table 1 Growth of inoculated and control (non-inoculated) seedlings under normal growth and cold acclimation conditions

Treatment	Plant height (cm)	Root(cm)
As	55.8 ± 4.35^{a}	27.5 ± 2.08^{a}
AsC	33.5 ± 8.02^{b}	19.3 ± 1.50^{b}
C	27.5 ± 2.08^{b}	14.5 ± 2.65^{c}
CC	14.3 ± 4.19^{c}	6.75 ± 0.96^{d}

All values were means± standard error of four replicates.
Values in the same column with different superscript letters are significantly different at 5% significant level. **As** *A. scrobiculata* + normal condition. **AsC** *A. scrobiculata* +cold acclimation. **C** control + normal condition. **CC** control + cold acclimation

Table 2 Dry biomass of inoculated and control seedlings under normal growth and cold acclimation conditions

Treatment	Dry biomass (g)		
	Root	Stem	Leaf
As	0.99 ± 0.31^{a}	1.09 ± 0.34^{a}	1.32 ± 0.27^{a}
AsC	0.54 ± 0.07^{b}	0.82 ± 0.31^{ab}	0.82 ± 0.60^{b}
C	0.15 ± 0.03^{c}	0.34 ± 0.01^{bc}	0.49 ± 0.11^{bc}
CC	0.07 ± 0.04^{c}	0.12 ± 0.07^{c}	0.18 ± 0.06^{c}

All values were means± standard error of four replicates.
Values in the same column with different superscript letters are significantly different at 5% significant level.
The treatments were the same as those specified in Table 1.

2010; Wu and Zou 2010). Di and Allen (1991) reported that root dry biomasses of AM inoculated 2 diploid cultivars (*Agropyron cristatum* cv. 'Fairway' and *A. cristatum* ssp. *puberulum*) were significantly lower than non-ionculated plants, but AMF inoculated other cultivars (*A. desertorum* cv. 'Nordan', *A.cristatumxdesertorum* cv. 'Hycrest', *A. cristatum* (Iran) and *A. cristatum* (USSR))showed no significant differences in dry biomass. Another study showed that *A. smithii* had increased biomass with AM inoculation in low P soils, but had no significant change in biomass with inoculation in high P soils (Duce 1987; Miller et al. 1987). Our study revealed that *Acaulospora scrobiculata* inoculation largely promoted the growth and biomass of crested wheatgrass seedlings under normal and cold acclimation (Tables 1 and 2). The mycorrhizal influence was more pronounced in aerial biomass than in root biomass (Table 2) which may be due to a proportionally greater allocation of carbohydrates to the shoot than to the root tissues after AMF colonization (Schwab et al. 1982).

Furthermore, under normal and cold acclimation conditions, the inoculated crested wheatgrasses had significantly higher leaf blade number, leaf area and specific leaf area compared with non-inoculated plants (Table 3).Under normal condition, the enhancements in leaf blade number, leaf area and specific leaf area were 91, 182 and 25%, respectively, whereas after cold acclimation, the enhancements in these parameters were 88, 311 and 130%, respectively. Taken together, these results showed that *A. scrobiculata* could effectively stimulate leaf growth of crested wheatgrass. Consistently, Berta et al. (1995) found that inoculated arbuscular mycorrhizal colonization with either of the arbuscular mycorrhizal (AM) fungi *Glomus mosseae* or *Glomus intraradices* increased root, stem and leaf weights, leaf area, root length and specific leaf area of *Prunus cerasifera*. Busquets et al. (2010) also reported that plants of *Anthyllis cytisoides* inoculated with *Glomus intraradices* produced more leaves than the control. Di and Allen (1991) found AM inoculated a hexaploid cultivar (*A. cristatum* from U.S.S.R) produced significantly higher tillers, while AM inoculated the tetraploid *A. desertorum* cv. 'Nordan' had fewer tillers and wider leaves.

Table 3 Leaf area, leaf blade and specific leaf area of seedlings after cold acclimation

Treatment	Leaf area (cm^2)	Leaf blade (*n*)	Specific leaf area (cm^2g^{-1})
As	110 ± 9.2^{a}	42 ± 3.3^{a}	121 ± 3.5^{a}
AsC	78 ± 3.7^{b}	30 ± 4.8^{b}	106 ± 6.5^{ab}
C	39 ± 2.8^{c}	22 ± 3.9^{bc}	97 ± 10.2^{b}
CC	19 ± 1.4^{d}	16 ± 1.7^{c}	46 ± 2.9^{c}

All values were means± standard error of four replicates.
Values in the same column with different superscript letters are significantly different at 5% significant level.
The treatments were the same as those specified in Table 1.

Under cold acclimation, the water content of stem and leaf of non-inoculated crested wheat grasses were significantly higher than normal treatments, while there was no differences between root water content of cold acclimated and normal control plants (Table 4). However, the water content of root and stem of *A. scrobiculata* inoculated grasses were significantly decreased by cold acclimation, while the water content of leaf was significantly higher under cold acclimation (Table 4). Little is known about the influence of AM inoculums on water status of plants under low temperature. Zhu et al. (2010) reported that relative water content and water saturation deficit in mycorrhizal and non-mycorrhizal plant leaves were similar at all temperature treatments, but water conservation in the leaves and water use efficiency were higher in mycorrhizal than non-mycorrhizal plants at all temperature treatments. EI-Tohamy et al. (1999) pointed out that mycorrhizal bean plants had higher leaf water potential during chilling stress. However, our study showed that leaf water content of mycorrhizal and non-mycorrhizal plants had no significant differences under normal condition, while water contents of root, stem and leaf of mycorrhizal plants were significantly lower than non-mycorrhizal plants under cold acclimation.

The mycorrhizal dependency of crested wheat grass on arbuscular mycorrhiza with *A. scrobiculata* was estimated to be 291%based on biomass accumulation under normal growth condition and 489%under cold condition. These results showed that mycorrhizal

dependency is more significant at low temperatures. The mycorrhizal dependency of crested wheatgrass with *A. scrobiculata* indicated a high degree of responsiveness of crested wheatgrass growth to mycorrhizal colonization. A similar effectiveness of AMF for tree species in arid land was also reported by Dixon et al. (1997). Furthermore, Bhoopander and Mukerji (2004) reported that under salt stress condition, the mycorrhizal dependency of *Sesbania aegyptiaca* and *S. grandiflora* increased with the age of the plants.

Table 4 Water content of inoculated and control seedlings under normal growth and cold acclimation conditions

Treatment	Water content (%)		
	Root	Stem	Leaf
As	67.3 ± 0.2^{b}	50.2 ± 0.2^{d}	66.1 ± 0.1^{c}
AsC	66.0 ± 0.3^{c}	53.5 ± 0.6^{c}	67.7 ± 0.1^{b}
C	74.7 ± 0.8^{a}	59.3 ± 0.3^{b}	66.0 ± 0.1^{c}
CC	75.0 ± 0.1^{a}	64.5 ± 0.3^{a}	79.0 ± 0.1^{a}

All values were means± standard error of four replicates, and analyzed after arcsine transformation.
Values in the same column with different superscript letters are significantly different at 5% significant level. The treatments were the same as those specified in Table 1.

Chlorophyll concentration

In general, the concentrations of chlorophyll (a, b, and a+b) in all treatments were significantly reduced by low temperatures (Table 5). However, the concentrations of photosynthetic pigments (chlorophylls a, b and a+b in leaves) of crested wheatgrass inoculated with *A. scrobiculata* were significantly greater than those of non-inoculated ones under normal growth, hardening and cold acclimation conditions (Table 5). For example, after cold acclimation the chlorophyll concentrations (a, b, and a+b) of inoculated crested wheatgrass (AsC) increased two fold compared to non-inoculated ones (CC) (Table 5).These results showed that AMF inoculation significantly increase the chlorophyll concentration of crested wheatgrass leaves at low temperatures, in agreement with the results of wheat and maize under cold stress by Paradis et al. (1995) and Zhu et al. (2010). Clearly, mycorrhizae improved the nutritional status and support higher chlorophyll concentration (Rachel et al. 1992), which would subsequently lead to a higher photosynthesis.

Table 5 Chlorophyll concentrations of seedlings under normal growth, hardening and cold acclimation conditions

Treatment	Concentration(mg g^{-1})		
	Chlorophyll a	Chlorophyll b	Chlorophyll a+b
As	1.98 ± 0.16^{a}	1.04 ± 0.19^{a}	3.03 ± 0.30^{a}
AsH	1.31 ± 0.76^{b}	0.80 ± 0.05^{a}	2.11 ± 0.10^{b}
AsC	0.88 ± 0.11^{c}	0.39 ± 0.07^{bc}	1.27 ± 0.16^{c}
C	1.25 ± 0.17^{b}	0.51 ± 0.07^{b}	1.76 ± 0.13^{b}
CH	0.71 ± 0.18^{cd}	0.44 ± 0.18^{b}	1.15 ± 0.32^{c}
CC	0.45 ± 0.09^{d}	0.14 ± 0.08^{d}	0.58 ± 0.06^{d}

All values were means± standard error of four replicates.
Values in the same column with different superscript letters are significantly different at 5% significant level.
As *A. scrobiculata* + normal growth condition. **AsH** *A.scrobiculata* + hardening.**AsC** *A. scrobiculata* +cold acclimation. **C** control + normal growth condition.***CH*** control + hardening.**CC** control + cold acclimation

Proline concentration

Proline is an osmoprotectant, which has been shown to accumulate in response to abiotic stresses (Janda et al. 2003; Naidu et al. 1991). Proline accumulation was more pronounced in low temperature treated plants of tobacco than in non-treated control plants (Konstantinova et al. 2002).In our study, proline concentration was significantly higher after cold acclimation in both AMF inoculated and non-inoculated crested wheatgrass, as compared to hardened and cold acclimated plants (Table 6).However, under normal growth condition there were no significant differences in proline concentration between AM-inoculated and non-inoculated grasses. Cold acclimation and hardening increased the proline concentration of crested wheat grasses inoculated with *A. scrobiculata* by 53.9% and 14.7%, respectively, in comparison with non-inoculated crested wheatgrasses. Abdel Latef and Chaoxing (2010) reported that proline concentration in the leaves of mycorrhizal tomato plants was lower than in non-mycorrhizal plants at 8°C.On the contrary, our results indicated that the accumulation of proline in crested wheatgrass leaves is increased by AMF inoculation under both hardening and cold acclimation conditions and higher leaf proline concentrations were found in AMF inoculated crested wheatgrass compared with non-inoculated seedlings. Our results clearly showed that *A. scrobiculata* inoculation significantly stimulates proline accumulation at low temperatures, which may contribute to cold tolerance of crested wheatgrass.

Table 6 Proline concentrations of seedlings under normal growth, hardening and cold acclimation treatments

Treatment	Proline concentration (ppm)
As	2.95 ± 0.51^{d}
AsH	6.07 ± 0.31^{c}
AsC	18.71 ± 2.42^{a}
C	2.83 ± 0.66^{d}
CH	5.29 ± 0.47^{cd}
CC	12.16 ± 0.88^{b}

All values were means± standard error of four replicates.
Values in the same column with different superscript letters are significantly different at 5% significant level.
The treatments were the same as those specified in Table 5.

Concentrations of mineral elements

AMF inoculation significantly increased the nitrogen and mineral (P, K, Ca, Mg, and Na) concentrations in roots, stems and leaves of crested wheatgrass seedlings under both normal growth condition and cold acclimation conditions (Tables7 and 8).The concentrations of elements (Ca, K, Mg, Na, P and N) in roots, stems and leaves of AMF inoculated and non-inoculated crested wheatgrass seedlings reduced significantly when subjected to low temperature stress. However, the mineral concentrations were higher in AMF inoculated crested wheatgrass than the controls. In this study, *A. scrobiculata* inoculation was found to significantly increase the acquisition of nitrogen and mineral (P, K, Ca, Mg, and Na) in roots, stems and leaves of crested wheatgrass seedlings under normal and cold acclimation *(*Tables7 and 8), that presumably stimulated its growth productivity (Tables 1-3). Consistent to our results, previous studies also have shown that growth and mineral nutrition of plants are commonly enhanced by AMF inoculation (Clark and Zeto2000; Javot et al. 2007; Wu and Zou 2009). Smith and Read (2008) reported that AMF increases plant growth mainly by increasing nutrient acquisition and thus enhances the plant's resistance to both biotic and abiotic stresses. Thus, our results also demonstrated the significant effect of *A. scrobiculata* inoculation on the nutrition and growth of crested wheatgrass.

Freezing tolerance

The freezing tolerance test revealed that leaf mortalities of non-inoculated crested wheatgrass were higher than those of the inoculated plants. The leaf LT_{50} of the non-inoculated crested wheatgrass was -8°C, whereas the leaf LT_{50} of the inoculated ones was lowered to -12.5°C (Table 9, Fig. 2a). Similarly, the plant LT_{50}ofnon-inoculated crested wheatgrass was -11°C, while that of the inoculated ones was lowered to -15.5°C (Table 10, Fig. 2b). In both analyses, the freezing tolerance was enhanced by 4.5°C.

Table 7 Mineral concentrations of root, stem and leaf of inoculated and control seedlings after cold acclimation

	Treatments	Ca (mg g^{-1})	K (mg g^{-1})	Mg (mg g^{-1})	Na (mg g^{-1})	P (mg g^{-1})
Root	As	1529±185^{a}	1774±298^{a}	281±76^{a}	736±46^{a}	7534±3803^{a}
	AsC	874±155^{b}	1016±123^{b}	151±11^{b}	352±77^{b}	3626±234^{b}
	C	434±50^{c}	509±93^{c}	92±34^{bc}	187±19^{c}	1293±306^{bc}
	CC	218±50^{d}	299±52^{d}	48±3^{c}	98±29^{d}	693±39^{c}
Stem	As	1849±563^{a}	3099±1166^{a}	872±351^{a}	803±62^{a}	5616±906^{a}
	AsC	877±74^{b}	1753±357^{b}	462±21^{b}	493±65^{b}	3073±1016^{b}
	C	601±37^{bc}	726±52^{bc}	114±11^{c}	187±5^{c}	724±135^{c}
	CC	273±14^{c}	332±41^{c}	74±10^{d}	102±16^{d}	498±13^{d}
Leaf	As	1233±196^{a}	5071±215^{a}	302±92^{a}	805±82^{a}	5369±268^{a}
	AsC	906±151^{b}	3254±489^{b}	127±8^{b}	502±114^{b}	3164±161^{b}
	C	527±92^{c}	1171±117^{c}	103±16^{bc}	250±112^{c}	1225±88^{c}
	CC	247±51^{d}	602±37^{d}	55±10^{c}	140±15^{d}	859±66^{d}

All values were means ± standard error of four replicates.
Values in the same column with different superscript letters are significantly different at 5% significant level.
The treatments were the same as those specified in Table 1.

Table 8 Nitrogen concentrations of inoculated and control seedlings after cold acclimation

Treatment	N (% dry weight)		
	Root	Stem	Leaf
As	1.19±0.25^{a}	2.42±0.57^{a}	2.94±1.41^{a}
AsC	0.73±0.18^{b}	1.37±0.19^{b}	1.21±0.15^{b}
C	0.47±0.08^{bc}	0.76±0.39^{bc}	1.17±0.30^{b}
CC	0.30±0.14^{c}	0.33±0.05^{c}	0.69±0.07^{c}

All values were means ± standard error of four replicates, and analyzed after arcsine transformation.
Values in the same column with different superscript letters are significantly different at 5% significant level.
The treatments were the same as those specified in Table 1.

Table 9 Leaf mortality percentage of seedlings after cold acclimation and freezing treatments

Treatment	Leaf mortality (%)					
	-8°C	-11°C	-14°C	-15°C	-16°C	-17°C
A. scrobiculata	18.8±5.7^{b}	34.7±7.3^{b}	67±3.9^{b}	81.9±2.5^{b}	93.1±3.4^{a}	100±0.0^{a}
Control	50.6±5.6^{a}	88.9±12.9^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}

All values were means ± standard error of four replicates, and analyzed after arcsine transformation.
Values in the same column with different superscript letters are significantly different at 5% significant level.
Control: non-inoculated seedlings.

Examination of plant recovery after the freezing treatment revealed that crested wheatgrass inoculated with *A. scrobiculata* had better freezing tolerance than the non-inoculated ones as well. For example, at -11°C, a temperature occurred frequently in the winter in Mongolia, the plant mortality rates for inoculated and non-inoculated plants were 16.7±4.1 and 50.0±5.5%, respectively, representing a 3fold increase in cold tolerance (Table 10). Clearly, AMF inoculation could largely improve the survival rate of crested wheatgrass, with a 100% survival rate at temperature as low as -8°C. These results, taken together, demonstrated that inoculation of AMF *A. scrobiculata* significantly improves the freezing tolerance of crested wheatgrass, by as much as 4.5°C (Fig. 2b).

Table 10 Whole plant mortality percentage of seedlings inoculated with *A. scrobiculata* and control under different freezing temperatures

Treatment	Plant mortality (%)					
	-8°C	-11°C	-14°C	-15°C	-16°C	-17°C
A. scrobiculata	0.0±0.0[b]	16.7±4.1[b]	16.7±4.1[b]	33.3±5.2[b]	66.7±5.2[b]	100±0.0[a]
Control	33.3±5.0[a]	50.0±5.5[a]	66.7±5.1[a]	100±0.0[a]	100±0.0[a]	100±0.0[a]

All values were means ± standard error of four replicates, and analyzed after arcsine transformation.
Values in the same column with different superscript letters are significantly different at 5% significant level. Control: non-inoculated seedlings

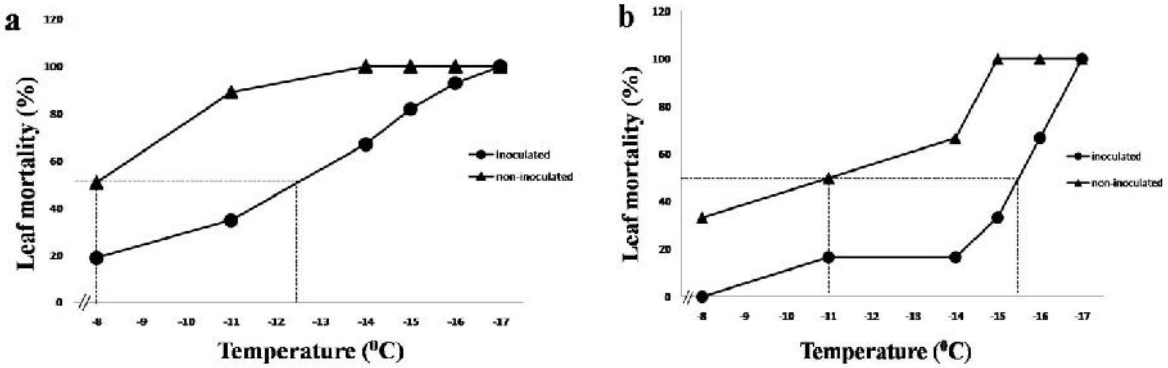

Fig. 2 Leaf (a) and seedling (b) mortality LT_{50} of crested wheatgrass inoculated with *A. scrobiculata* and the non-inoculated control.

Observations of the inoculated and non-inoculated control plants showed that leaves rolled up during freezing. Under sunlight, the leaves of crested wheatgrass exhibited a freeze-injury symptom of browning, followed by leaf desiccation. Freezing injury of leaves in the control crested wheatgrass seedlings was higher than the inoculated seedlings. For example, -14°C freezing stress caused serious damage of many leaf cells in the leaves of control seedlings including chloroplasts, vascular bundle and epidermis, while the freezing temperature caused less freezing injury in the epidermis and mesophyll cells in leaves of the inoculated seedlings (Fig. 3). In this study, comparison of the cross section of leaves of AM inoculated and the control seedlings showed that the sizes of bulliform and bundle sheath cells were increased by freezing stress (Fig.3). These enlarged bulliform cells are very crucial in adaption to freezing temperatures as these cells are responsible for leaf folding and rolling movement to reduce water loss (Abernethy et al. 1998). Ball et al. (2004) also found that frost-freezing in unacclimated leaf tissues of Eucalyptus pauciflora caused irreversible tissue damage consistent with tissue death as intracellular ice formed in the cambium and phloem, killing the cells and leaving persistent gaps between xylem and phloem.

Examination of leaf cross sections showed that the number of mesophyll cells of AM inoculated and non-inoculated crested wheatgrass decreased after freezing stress, as compared with non-stressed plants, presumably due to cell lysis, whilst the numbers of mesophyll cell of AM inoculated crested wheatgrass were higher than non-inoculated ones after freezing stress (Fig. 3). Furthermore, our results showed that the leaf thickness was significantly increased by freezing temperature with the expansion of cells compared with those of normal condition treatments (Fig. 3, Table 11). The increase in leaf thickness of the inoculated crested wheatgrass was 31.5%, while that of non-inoculated crested wheatgrass was 36.2% after freezing test. However, the differences in leaf thickness were not significantly different between AM inoculated and non-inoculated crested wheatgrass (Table 11).Other studies also showed a similar cold-induced increase in leaf thickness and cell dimensions in oilseed rape (*Brassicanapus* L. *var*. *oleifera*) (Maciejewska and Kacperska 1987; Stefanowska et al. 1999).

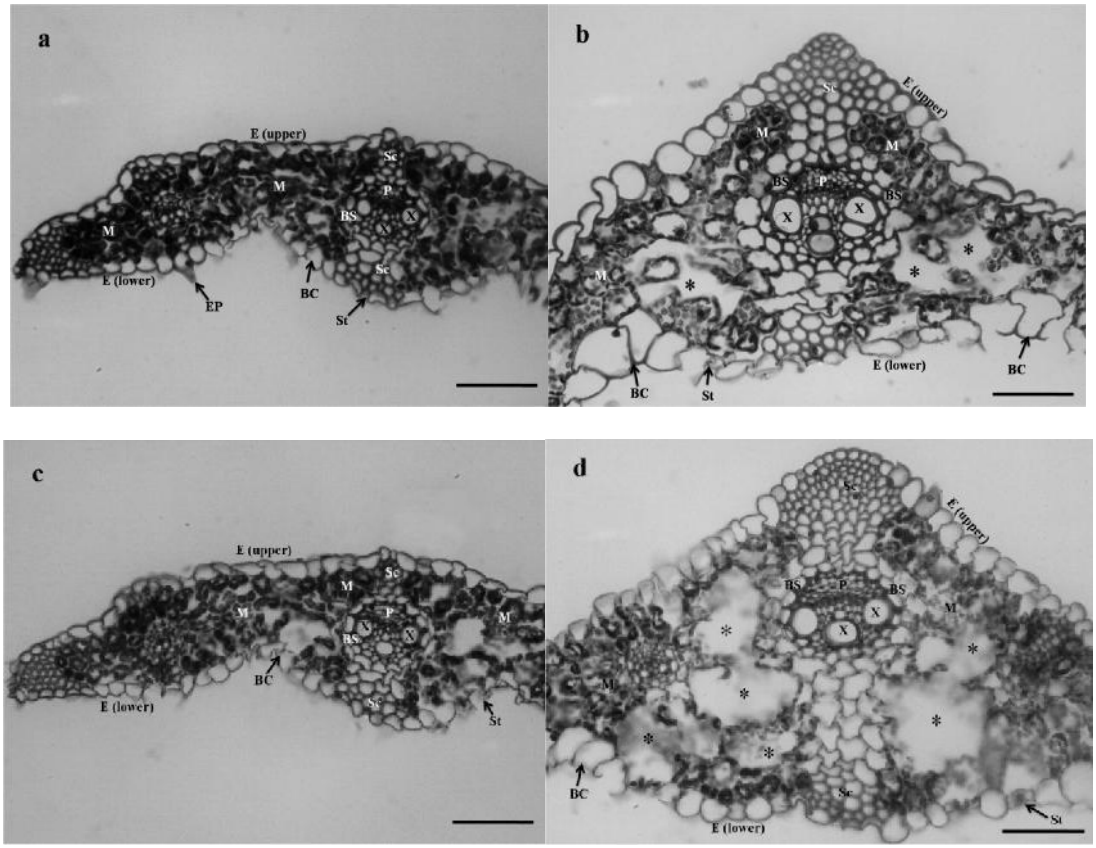

Fig. 3 Cross section of leaves of crested wheatgrass inoculated with *A. scrobiculata* and control. ***a*** Crested wheatgrass inoculated with *A. scrobiculata* under normal condition. ***b*** Crested wheatgrass inoculated with *A. scrobiculata* after 2 hrs of -14°C freezing stress. ***c*** Non-inoculated crested wheatgrass under normal condition. **d** Non-inoculated crested wheatgrass after2 hrs of -14°C freezing stress. Stars indicated *(*)*severe freezing injury in the mesophyll cells and vascular bundle of crested wheatgrass inoculated with *A. scrobiculata* and control, respectively; *Bars* =50 µm.
E (upper)Upper epidermis; **E(lower**)Lower epidermis. **EP -**Epidermal papilla. **Sc-** Schlerenchyma. **P**-Phloem. **X**-Xylem. **M-**Mesophyll cells. **BS**-Bundle sheath cells. **BC**- Bulliform cells. **St- S**toma.

Table 11 Leaf thickness of crested wheatgrasses after freezing test

Treatment	Leaf thickness(mm)
As	0.074 ±0.002[b]
AsF	0.111 ±0.001[a]
C	0.076 ±0.002[b]
CF	0.116 ±0.003[a]

All values were means ± standard error of four replicates.
Values in the same column with different superscript letters are significantly different at 5% significant level. **As***A. scrobiculata* + normal condition. **AsF** *A.scrobiculata* + *freezing.* **C** control + normal condition.**CF** *control* + *freezing* (2h, -14°C).

These results collectively showed that leaves of non-inoculated grasses were more sensitive to freeze temperatures, whereas leaves of AMF inoculated grasses were relatively freezing tolerant. Thus, AMF inoculation can improve the freezing tolerance of crested wheatgrass leaves, presumably by enhanced proline accumulation (Table 6).

Conclusion

Our study showed that *A. scrobiculata* could effectively form arbuscular mycorrhizae in the roots of crested wheatgrass seedlings (Fig. 1). *A. scrobiculata* inoculation significantly promoted the growth and biomass accumulation of crested wheatgrass seedlings.

The enhancement in growth was reflected in increased plant height, root length, leaf blade number, leaf area and specific leaf area of *A. scrobiculata* inoculated crested wheatgrass. *A. scrobiculata* inoculation also significantly increased the chlorophyll, mineral (P, K, Ca, Mg, and Na) and nitrogen concentrations in all tissues of crested wheatgrass (Tables5, 7 and 8). Furthermore, our results showed that low temperature and *A. scrobiculata* inoculation increase the freezing tolerance of crested wheatgrass (Tables 9 and 10), presumably due to the accumulation of the compatible solute proline (Table 6). Most significantly, the leaf and plant LT_{50} of the inoculated crested wheatgrass were4.5°Clowerthan non-inoculated plants (Fig. 2) with a lower cellular damage (Fig. 3). Inoculated crested wheatgrass plants exhibited a total survival at temperatures as low as -11°C, while only 50% of the non-inoculated crested wheatgrass could survive this freezing temperature. Taken together, these results demonstrate that *A. scrobiculata* could effectively form arbuscular mycorrhizae with crested wheatgrass and improve its growth and freezing tolerance, which will be very useful for the restoration of Mongolian steppe.

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