

## A Native Arbuscular Mycorrhizal Fungus, *Acaulospora scrobiculata* Stimulated Growth of Mongolian Crested Wheatgrass (*Agropyron cristatum* (L.) Gaertn.)

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### Abstract

*Agropyron cristatum* (L.) Gaertn. (crested wheatgrass) is an endemic plant species, which dominates most area of the Mongolian steppe and forest steppe. In the present study, spores of arbuscular mycorrhizal fungi in the rhizosphere soil of crested wheatgrass were isolated with wet-sieving/decanting methods, and the major species was identified as *Acaulospora scrobiculata* Trappe. For arbuscular-mycorrhizal resynthesis, the spores of *A. scrobiculata* were propagated with corn pot-culture technique and inoculated onto the roots of crested wheatgrass seedlings. The inoculated crested wheatgrass seedlings exhibited vigor in growth, and examination of the root structure revealed the occurrence of arbuscules and vesicles in the cortical cells. These results demonstrated that *A. scrobiculata* could effectively form arbuscular mycorrhizas with crested wheatgrass and promote its growth, which can be used to restore Mongolian grassland.

**Key words:** *Acaulospora scrobiculata*, *Agropyron cristatum*, arbuscular mycorrhizas, biomass, crested wheatgrass, growth, mineral, mycorrhizal dependency

### Introduction

*Agropyron cristatum* (L.) Gaertn. (crested wheatgrass) is a dominant native plant 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 prairie.

Arbuscular mycorrhiza (AM) is important components of virtually all terrestrial ecosystems (Brundrett, 1991; Smith & Read, 2008). Arbuscular mycorrhizal fungi (AMF) are one of the most important soil microbes as they form mutualistic association with more than 80% of land plants (Ulrich *et al.*, 2002). AMF are known to be widespread in semi-arid grasslands plants, and their association with grasses is important in biomes grazed by large ungulates (Trappe, 1981). It has been well documented that the major benefits of plants from these relationships are improvement of uptakes in water and inorganic nutrients, especially phosphorus (Sanders

& Koide, 1994). Additional benefits include increased tolerance to the environmental stresses such as nutrient deficiency, diseases, drought and salinity (Smith & Read, 2008; Gupta & Kumar, 2000).

AMF may further influence plant community structure (Marler *et al.*, 1999), biodiversity (Hartnett *et al.*, 1993), primary production (Hedlund, 2002), ecosystem dynamics (Van Der Heijden *et al.*, 1998), and survival of tree seedlings (Hetrick *et al.*, 1988; Smith & Read, 2008; Stinson *et al.*, 2006). Previous research has examined the distribution of AMF in sandy area (Błaszczowski *et al.*, 2002), in agricultural soils (Oehl *et al.*, 2009), and in certain natural ecosystems (Guadarrama & Alvarez-Sanchez, 1999), but few of them have looked into grasslands (Smith & Read, 2008), especially in arid and semiarid areas (Lugo *et al.*, 2002).

Mongolian grasslands are facing rapid desertification due to the uncontrolled growth of animal herding, mining and global warming. The grassland has been thinning out in 75 percent of the Mongolian land area, while 7 percent has completely turned into deserts.

Some actions must be taken to halt the process to help Mongolia keep its grasslands. Thus, the aims of this study were to isolate, identify and propagate the AMF and to assess the effect of this native AMF on growth of Mongolian crested wheatgrass seedlings through mycorrhizal resynthesis. It is hoped that the finding from this study will contribute to the use of mycorrhizal technique in grassland restoration.

### Materials and Methods

**Sample collection.** Root samples of crested wheatgrass and their rhizosphere soil were collected from grassland at Bogd Mountain in the vicinity of Ulaanbaatar city (E 107°08'31", N 47°45'767" 1597 m) of Mongolia in October 2008. Feeder roots, soil and root samples were collected from 0 to 20 cm depth of eight individual plants, put in polyethylene bags and stored at 5-10°C until analyzed. Seeds of crested wheatgrass were also collected from natural grassland at the same site.

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

**Seedling raising.** After surface cleaning with running tap water, seeds of crested wheatgrass were sterilized with a 10% sodium hypochlorite solution for 15 min, rinsed three times with sterile distilled water, and then germinated in sterilized mixture of peat moss, vermiculite and perlite (1:1:1, v/v). As the seedlings attained 4 cm in height, they were transferred to pots filled with sterilized sand for AM resynthesis.

**Resynthesis.** The grass seedlings were inoculated with 10 g sand spore (containing 15±5 spores/g sand). Non-inoculated seedlings treated with the sand-spore filtrate served as the control. All seedlings were cultured in greenhouse at

20±3°C, and 1000±200  $\mu\text{mole photons m}^{-2} \text{sec}^{-1}$  photosynthetic photon flux density, and watered with deionized water as needed without supplemental fertilization. The seedlings were examined after 6 months of cultivation.

**Morphology of mycorrhiza.** After 6 months, 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<sub>2</sub>O<sub>2</sub> and 1% HCl, and then stained with 0.05% trypan blue. The morphology of mycorrhiza was observed with a stereomicroscope (Abbott, 1982; Brundrett *et al.*, 1996). The percentage of root colonization was calculated accordingly (Phillips & Hayman, 1970).

For ultrastructural study, root samples were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde fixative in a phosphate-buffered solution (0.1 M, pH 7.0) for 4 hr at room temperature, then rinsed with the phosphate-buffered solution three times each time for 15 min, followed by serial dehydration in 30, 50, 70, 80, 95, and 100% ethanol and 100% acetone, and finally dried in a critical-point dryer using liquid carbon dioxide. Dried materials were mounted on an aluminum stub with adhesives, coated with gold, and observed with a scanning electron microscope (Brundrett *et al.*, 1996).

Mycorrhizal root colonization was assessed by grid line intersection method (Giovannetti & Mosse, 1980).

**Mineral content analysis.** For mineral content analysis, root, shoot, and leaf samples were oven-dried at 70±2°C and digested with concentrated H<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub>. Nitrogen contents of root, shoot, and leaf were estimated by microkjeldahl method (MacDonald, 1977). Phosphorus, potassium, calcium, sodium, and magnesium contents 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 & Syvertsen, 1985).

**Statistical analysis.** After 6 months, non-inoculated and inoculated seedlings were harvested. Growth characters, such as plant height, root length, leaf and root fresh weights

were recorded. Dry weight was determined after drying in an oven at  $70\pm 2^\circ\text{C}$  for 48 hr. Statistical analysis was performed using the software Statistical Package for the Social Science (SPSS 12.0, IL, USA) for windows program. All data represent means of 4 separate experiments  $\pm$  standard error ( $n = 4$ ). Differences in growth rates among treatments were analyzed by Tukey's multiple range test at  $P < 0.05$  significant level.

## Results

**Spore morphology and identification.** After extraction, spores of AMF were collected. The dominant species was identified as *Acaulospora scrobiculata*. Spores were formed singly in soil and distributed abundantly in sampling sites. Spores of *A. scrobiculata* were orange yellow to orange brown, and globose to subglobose (Figs. 1, 2). The morphology of the spore clearly showed that its cell wall is consisted of 3 layers (L1, L2 and L3). Also, germinal wall, hypha remnant and cicatrix were found (Fig. 2a-c). The ultrastructure showed that the spore surface is smooth with little pits (Fig. 2b-d).

**Morphology of arbuscular mycorrhiza.** Vesicles, intracellular and intercellular hyphae were observed in the root cortical cells of crested wheatgrass collected from the sampling sites in Mongolian grassland (Fig. 3). Root colonization rate in crested wheatgrass collected from its natural habitat was 25.5%.

The crested wheatgrass seedlings inoculated with *A. scrobiculata* were found to produce more leaves and roots than the control seedlings (Fig. 4a-b). After six months of cultivation, AM

fungal colonization was as high as 100 percent in crested wheatgrass seedlings inoculated with *A. scrobiculata* (Fig. 5). Roots of seedlings inoculated with *A. scrobiculata* produced a network of external hyphae (Fig. 5a-b). Staining of root samples revealed that AM developed well in the roots of inoculated seedlings. Arbuscules and vesicles were present abundantly in root tissues of inoculated seedlings (Fig. 5c, e-f).

In roots of inoculated seedlings, hyphae extended intercellularly and formed arbuscules, which showed *Arum*-type morphology (Fig. 5e-f). The number of vesicles was very high in roots, where an average of 12-15 vesicles per cm root was observed (Fig. 5c). However, no hyphae, arbuscules, and vesicles were found in the roots of the non-inoculated seedlings (Fig. 6).

**Seedling growth.** In the first two weeks, relatively uniform growth of inoculated and non-inoculated seedlings was observed. However, after three weeks, the influence of inoculation on seedling growth was evident as the growth of inoculated seedlings increased sharply. After eight weeks, there were significant differences in growth between inoculated and non-inoculated seedlings (Fig. 4). After six months, seedling height and root length of inoculated seedlings were significantly higher (175% and 191%, respectively) than the control (Fig. 4a-b; Table 1). Meanwhile, fresh and dry weights of leaves and roots of inoculated seedlings were also significantly higher than the control (Table 2). After six months of cultivation, the nitrogen, phosphorus, potassium, calcium, sodium, and magnesium concentrations of roots, stems, and leaves of inoculated crested were also higher than those of the control (Tables

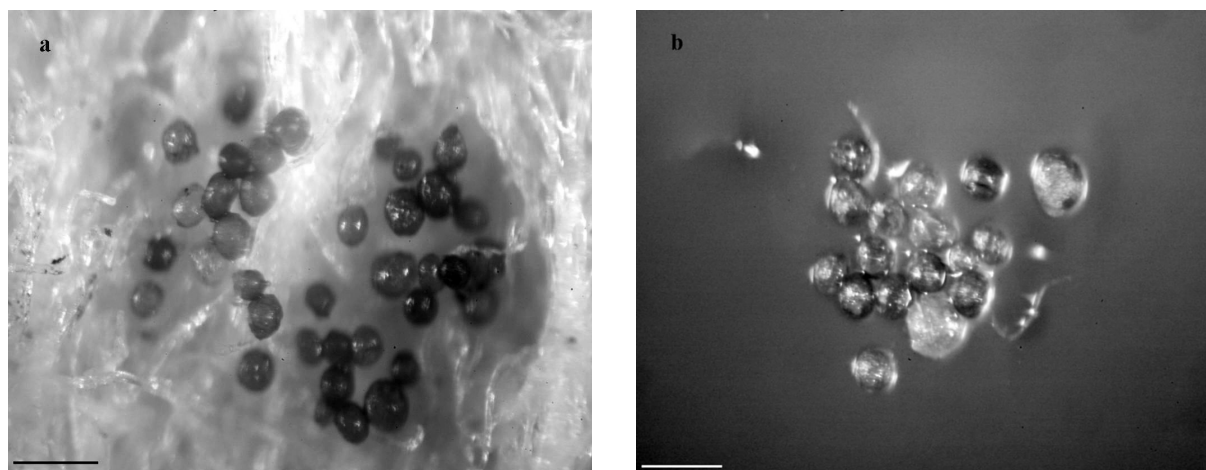


Figure 1. Spores of *Acaulospora scrobiculata*. a: natural spores; b: cleaned spores. (bars = 1 mm)

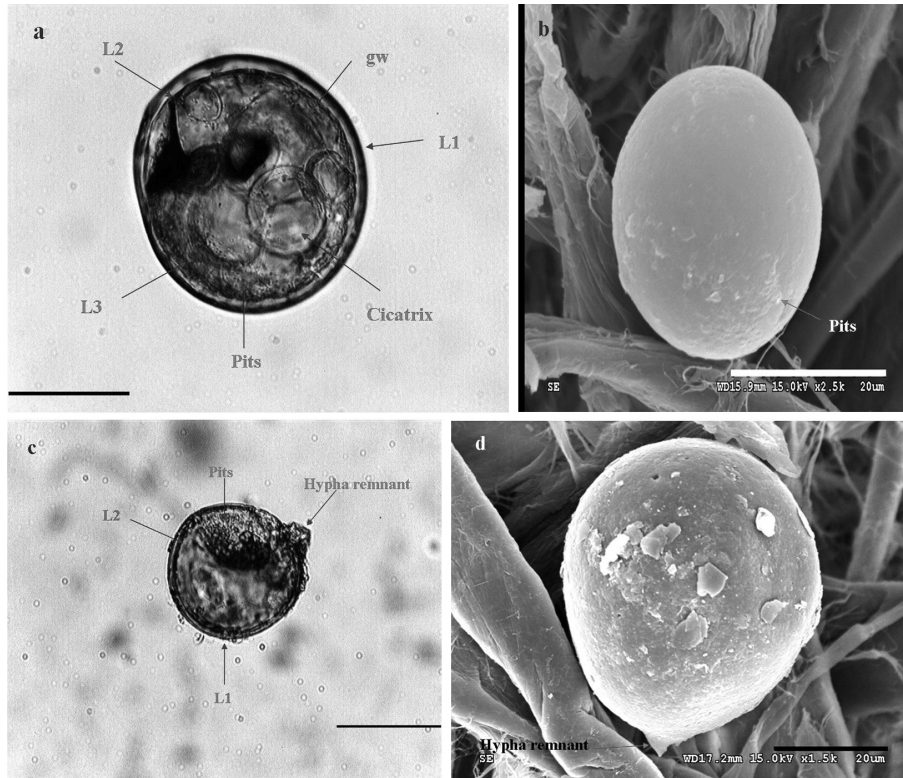


Figure 2. Morphology of spores of *Acaulospora scrobiculata*. a, c - structure of *Acaulospora scrobiculata*, gw: germinal wall. (bar = 100  $\mu$ m); b, d - ultrastructure of spore surface texture. (bar = 20  $\mu$ m)

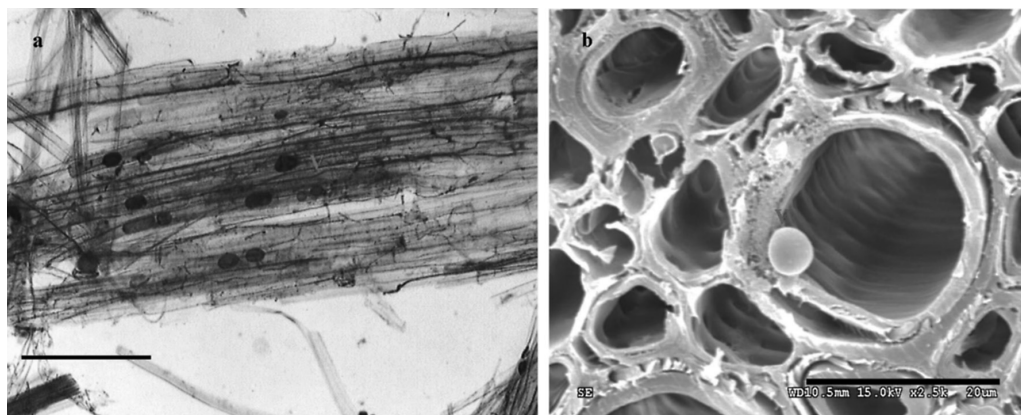


Figure 3. Arbuscular mycorrhiza of crested wheatgrass. a - structure of root with vesicles (V) and hyphae (arrowhead) (bar = 100  $\mu$ m); b - ultrastructure of root with vesicles (V) (bar = 20  $\mu$ m).

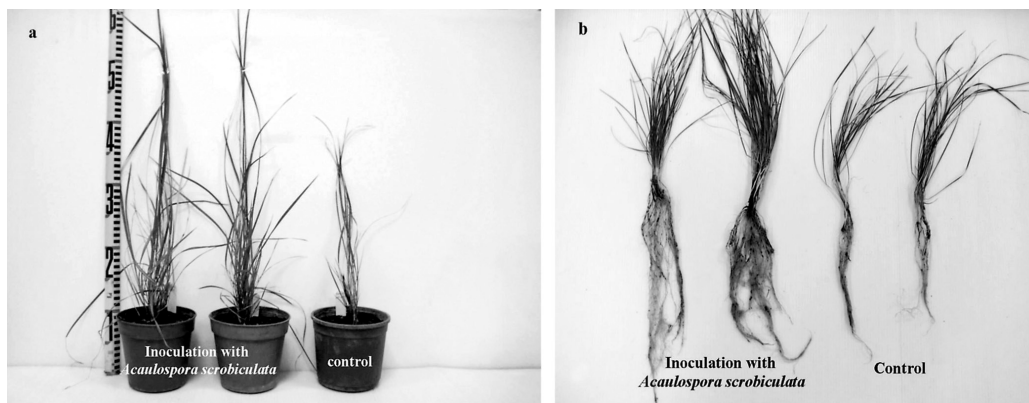


Figure 4. Morphology of inoculated and non-inoculated (control) crested wheatgrass seedlings.

3-6). Mycorrhizal dependency of the crested wheatgrass on arbuscular mycorrhiza with *A.*

*scrobiculata* was estimated to be 839.5% based on biomass accumulation.

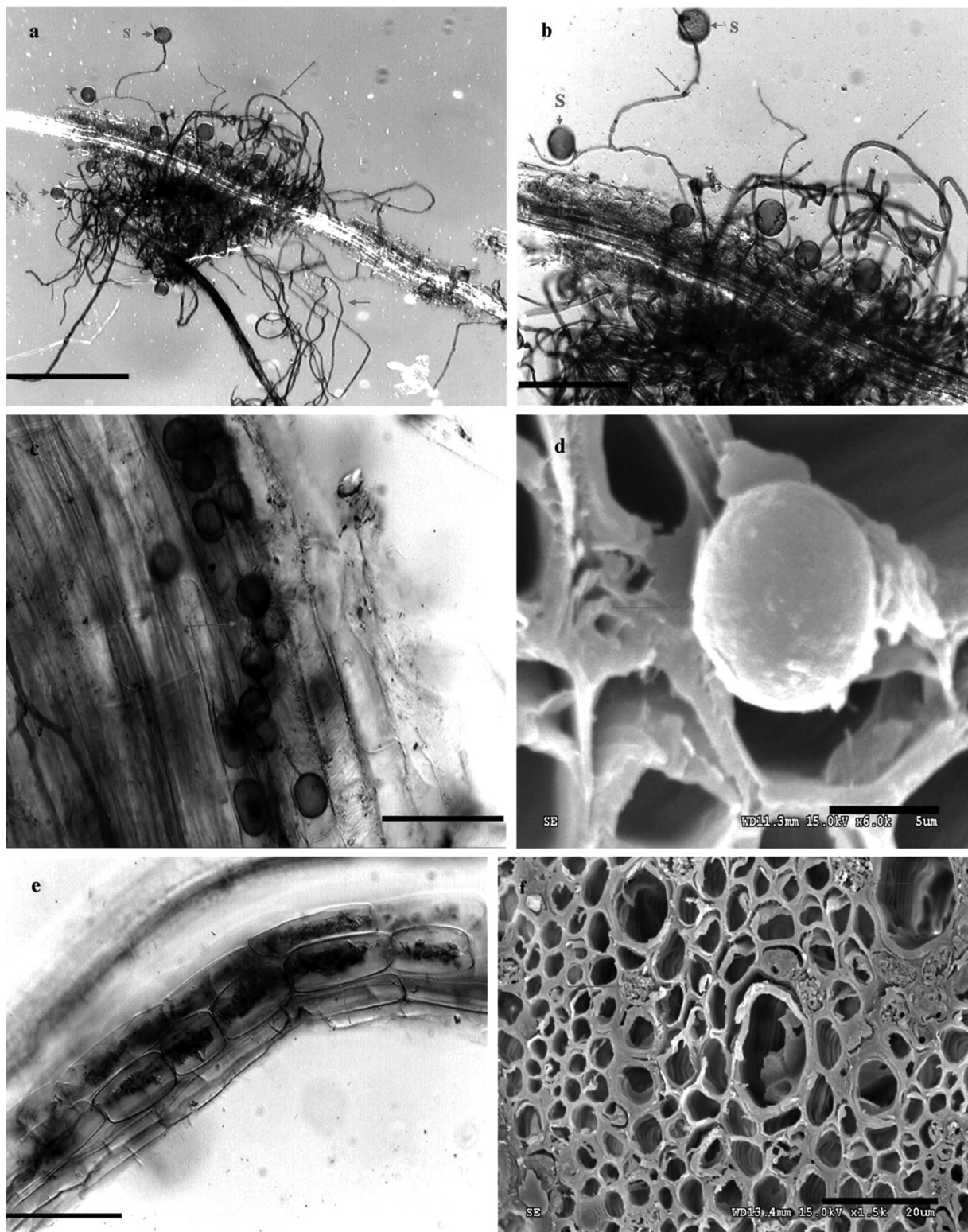


Figure 5. Morphology of root of crested wheatgrass inoculated with *A. scrobiculata*. a, b - external hyphae (arrowhead) and chlamydospores (s) produced by *A. scrobiculata* (bar = 100  $\mu$ m); c - structure of root with vesicles (arrowhead) and intercellular hyphae (H) (bar = 100  $\mu$ m); d - ultrastructure of root with vesicle (arrowhead) (bar = 5  $\mu$ m); e - structure of root with arbusculus (arrowhead) (bar = 100  $\mu$ m); f - ultrastructure of root with arbusculus (arrowhead) (bar = 20  $\mu$ m).

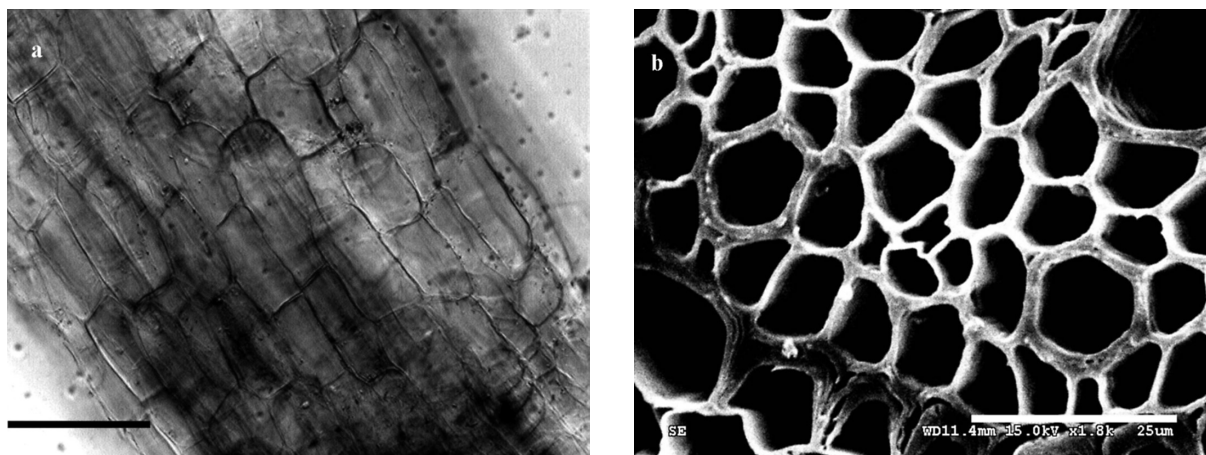


Figure 6. Morphology of root of non-inoculated crested wheatgrass seedling. a: structure of root (bar = 100  $\mu\text{m}$ ); b, ultrastructure of root (bar = 25  $\mu\text{m}$ ).

Table 1. Growth of inoculated and non-inoculated crested wheatgrass seedlings after six months of cultivation

Inoculation treatments	Net height growth (cm)	Net root length growth (cm)
<i>Acaulospora</i> scrobiculata	37.36 $\pm$ 4.90a	30.63 $\pm$ 4.24a
Control (non-inoculated)	21.38 $\pm$ 4.00b	16.00 $\pm$ 2.98b

All values were means  $\pm$  standard error of four replicates ( $P < 0.05$ ). Values in the same column with different superscript letters are significantly different at 5% significant level.

Table 2. Biomass of inoculated and non-inoculated crested wheatgrass seedlings after six months of cultivation

Inoculation treatments	Leaf & shoot fresh biomass (g)	Leaf & shoot dry biomass (g)	Root fresh biomass (g)	Root dry biomass (g)
<i>Acaulospora</i> scrobiculata	4.63 $\pm$ 1.25a	2.25 $\pm$ 0.66a	3.27 $\pm$ 1.30a	1.66 $\pm$ 0.72a
Control (non-inoculated)	1.00 $\pm$ 0.40b	0.34 $\pm$ 0.07b	0.26 $\pm$ 0.24b	0.09 $\pm$ 0.06b

All values were means  $\pm$  standard error of four replicates ( $P < 0.05$ ). Values in the same column with different superscript letters are significantly different at 5% significant level.

Table 3. Nitrogen contents of inoculated and non-inoculated crested wheatgrass seedlings after six months of cultivation.

Inoculation treatments	N (%)		
	Root	Stem	Leaf
<i>Acaulospora</i> scrobiculata	1.19 $\pm$ 0.19a	2.42 $\pm$ 0.53a	2.94 $\pm$ 0.41a
Control (non-inoculated)	0.47 $\pm$ 0.76b	0.51 $\pm$ 0.06b	1.30 $\pm$ 0.44b

All values were means  $\pm$  standard error of four replicates ( $P < 0.05$ ). Values in the same column with different superscript letters are significantly different at 5% significant level.

Table 4. Mineral contents of root of inoculated and non-inoculated crested wheatgrass seedlings after six months cultivation

Inoculation treatments	Ca (mg g <sup>-1</sup> )	K (mg g <sup>-1</sup> )	Mg (mg g <sup>-1</sup> )	Na (mg g <sup>-1</sup> )	P (mg g <sup>-1</sup> )
<i>Acaulospora</i> scrobiculata	1529 $\pm$ 185a	1774 $\pm$ 298a	281 $\pm$ 76a	736 $\pm$ 46a	7534 $\pm$ 3804a
Control (non-inoculated)	434 $\pm$ 50b	509 $\pm$ 93b	92 $\pm$ 34b	187 $\pm$ 19b	1293 $\pm$ 306b

All values were means  $\pm$  standard error of four replicates ( $p < 0.05$ ). Values in the same column with different superscript letters are significantly different at 5% significant level.

Table 5. Mineral contents of stem of inoculated and non-inoculated crested wheatgrass seedlings after six months of cultivation

Inoculation treatments	Ca (mg g <sup>-1</sup> )	K (mg g <sup>-1</sup> )	Mg (mg g <sup>-1</sup> )	Na (mg g <sup>-1</sup> )	P (mg g <sup>-1</sup> )
<i>Acaulospora scrobiculata</i>	1849±563a	3099±1166a	872±351a	803±62a	5617±906a
Control (non-inoculated)	601±37b	726±52b	114±11b	187±5b	724±136b

All values were means ± standard error of four replicates ( $P < 0.05$ ). Values in the same column with different superscript letters are significantly different at 5% significant level.

Table 6. Mineral contents of leaf of inoculated and non-inoculated crested wheatgrass seedlings after six months of cultivation

Inoculation treatments	Ca (mg g <sup>-1</sup> )	K (mg g <sup>-1</sup> )	Mg (mg g <sup>-1</sup> )	Na (mg g <sup>-1</sup> )	P (mg g <sup>-1</sup> )
<i>Acaulospora scrobiculata</i>	1232±196a	5071±215a	302±93a	805±8a	5369±268a
Control (non-inoculated)	86±23b	584±61b	32±20b	77±18b	543±26b

All values were means ± standard error of four replicates ( $P < 0.05$ ). Values in the same column with different superscript letters are significantly different at 5% significant level.

### Discussion

In nature, more than 90% of all higher plants are associated with mycorrhiza and more than 80% of these plants form AM relationships (Smith & Read, 2008). In this study, the dominant spore isolated from rhizosphere soil of Mongolian crested wheatgrass was identified as AMF *Acaulospora scrobiculata* (Fig. 1-2). AM characteristics were observed in root cortical cells of crested wheatgrass (Fig. 3). Other fungi, such as *Glomus macrocarpum*, *G. macrocarpum* var. *macrocarpum* were also found to form AM with *Agropyron smithii* in Colorado, USA (Singh, 2004). Generally, the soils of Mongolian grassland are nutrient deficient. In this study, we found that percent root colonization was as low as 25.5% in crested wheatgrass collected from the grassland at Bogd Mountain near Ulaanbaatar, Mongolia.

AMF efficiency can be measured in terms of host plant growth under different environmental conditions (Ruiz-Lozano *et al.*, 1996). Our study showed that crested wheatgrass seedlings inoculated with *A. scrobiculata* increased the growth of leaves and roots significantly under greenhouse condition (Fig. 4, Table 1). And, six months after inoculation, 100% root colonization and high spore production were observed in crested wheatgrass seedlings (Fig. 5). Based on the frequency of occurrence, *A. scrobiculata* was clearly the dominant AM fungal species. The mycorrhizal influence was more pronounced in aerial biomass than in root biomass (Table 2)

which may be because of a proportionally greater allocation of carbohydrates to the shoot than to the root tissues after AMF colonization (Schwab *et al.*, 1982). Previous works reported that mycorrhizal fungi are able to increase the shoot height, fruit number, shoot and root biomass of plants (Andrade *et al.*, 1998; Mendeiros *et al.*, 1994; Utkhede, 2006). Smith and Read (2008) reported that AMF increase plant growth mainly by increasing nutrient acquisition and thus enhanced the plant's resistance to biotic and abiotic stresses. A possible mechanism by which AM fungi increase root size could be that AM fungi modify root function and change root architecture (Andrade *et al.*, 1998).

The *Arum*-type structures were found in roots of crested wheatgrass seedlings inoculated with *A. scrobiculata* (Fig. 5e). *Arum*-type structures have been reported to associate with cultivated plants, which are usually fast-growing with plenty of sunlight (Smith & Smith, 1997). O'Connor *et al.* (2001) also reported that the *Arum*-type structures are present in all of the 21 species of herbaceous AM plants growing in Australian desert.

AM inoculation significantly increased the nitrogen and mineral (P, K, Ca, Mg, and Na) contents in roots, stems and leaves of crested wheatgrass seedlings inoculated with *A. scrobiculata* (Table 3-6). A previous study has shown that growth and mineral nutrition of plants are commonly enhanced by AMF inoculation (Clark & Zeto, 2000). Our results confirm significant effects of *A. scrobiculata* inoculation on growth of crested wheatgrass.

The 839.5% mycorrhizal dependency of crested wheatgrass with *A. scrobiculata* indicated a high degree of responsiveness of crested wheatgrass growth to mycorrhizal colonization.

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