Research paper

Arbuscular Mycorrhizal Fungus *Diversispora spurcum* Improved the Growth and Freeze Tolerance of Mongolian Crested Wheatgrass (*Agropyron cristatum*)

Burenjargal Otgonsuren,[1,2] Ming-Jen Lee[3,4]

【Summary】

Agropyron cristatum (L.) Gaertn. (crested wheatgrass) is an endemic grass species, which dominates the Mongolian steppe. In this study, spores of arbuscular mycorrhizal fungi (AMF) in the rhizosphere soil of crested wheatgrass were isolated with wet-sieving/decanting methods and sucrose density gradient centrifugation, and the associated species was identified as *Diversispora spurcum* C. Walker & Schuessler. An arbuscular-mycorrhizal resynthesis experiment showed that *D. spurcum* formed arbuscular mycorrhizae with crested wheatgrass seedlings, and promoted their growth and biomass. The dependency of the crested wheatgrass on arbuscular mycorrhizae (AMs) with *D. spurcum* was 292%. *Diversispora spurcum* inoculation also significantly increased the nitrogen and mineral (P, K, Ca, Mg, and Na) contents in roots, stems, and leaves of crested wheatgrass. Inoculated and non-inoculated crested wheatgrass seedlings were cold-acclimated and subsequently subjected to freeze tolerance tests at -8, -11, -14, -15, -16, and -17℃, respectively. The leaf lethal temperatures for 50% mortality (LT_{50}) of non-inoculated and inoculated crested wheatgrass were -8 and -14℃, respectively, while the whole plant LT_{50} values of non-inoculated and inoculated crested wheatgrass were -11 and -15.5℃, respectively. These results demonstrated that *D. spurcum* could effectively form arbuscular mycorrhizae with crested wheatgrass and improve its growth, presumably through enhanced nutrition acquisition, and freeze tolerance.

Key words: *Agropyron cristatum*, Arbuscular mycorrhiza, *Diversispora spurcum*, freeze tolerance, growth.

Otgonsuren B, Lee MJ. 2011. Arbuscular mycorrhizal fungus *Diversispora spurcum* improved the growth and freeze tolerance of Mongolian crested wheatgrass (*Agropyron cristatum*). Taiwan J For Sci 26(2):179-92.

[1] Graduate Institute of Agriculture, National Chiayi University, 300 University Rd., Chiayi 60004, Taiwan. 國立嘉義大學農學研究所，60004嘉義市學府路300號。

[2] Department of Ecology, Mongolian State University of Agriculture, Zaisan 153, Khan-Uul District, Ulaanbaatar 210153, Mongolia. 蒙古國立農業大學生態學系，210153蒙古烏蘭巴托市汗烏拉區Zaisan街153號。

[3] Department of Forestry and Natural Resources, National Chiayi University, 300 University Rd., Chiayi 60004, Taiwan. 國立嘉義大學森林暨自然資源學系，60004嘉義市學府路300號。

[4] Corresponding author, e-mail:mjlee@mail.ncyu.edu.tw 通訊作者。

Received February 2011, Accepted April 2011. 2011年2月送審 2011年4月通過。

研究報告

叢枝菌根菌沾屑多樣孢囊菌(*Diversispora spurcum*)增進蒙古扁穗冰草(*Agropyron cristatum*)的生長和耐凍性

奧特根蘇仁布倫[1,2)] 李明仁[3,4)]

摘 要

蒙古扁穗冰草(*Agropyron cristatum* (L.) Gaertn., crested wheatgrass)為蒙古草原的優勢原生草種。本研究以濕篩傾倒法和蔗糖密度梯度離心技術分離蒙古扁穗冰草根圈土壤的叢枝菌根菌孢子，並鑑定為沾屑多樣孢囊菌(*Diversispora spurcum* C. Walker & Schuessler)。叢枝菌根再合成的結果顯示，沾屑多樣孢囊菌與蒙古扁穗冰草苗形成叢枝菌根，並增進其生長與生物量。蒙古扁穗冰草對叢枝菌根的菌根依賴度為292%。接種沾屑多樣孢囊菌也顯著提高蒙古扁穗冰草根莖葉的氮與礦物質(磷、鉀、鈣、鎂、鈉)含量。接種與未接種的蒙古扁穗冰草苗經冷馴化後，再分別以凍結溫度-8、-11、-14、-15、-16及-17℃進行耐凍試驗；結果顯示，對照組與接種組的蒙古扁穗冰草苗葉部的半致死溫度，分別為-8和-14℃，而對照組與接種組植株的半致死溫度，分別為-11和-15.5℃。本研究結果證實，沾屑多樣孢囊菌能與蒙古扁穗冰草有效形成叢枝菌根，並增進其養分吸收、生長及耐凍性。

關鍵詞：蒙古扁穗冰草、叢枝菌根、沾屑多樣孢囊菌、耐凍性、生長。

奧特根蘇仁布倫、李明仁。2011。叢枝菌根菌沾屑多樣孢囊菌(*Diversispora spurcum*)增進蒙古扁穗冰草(*Agropyron cristatum*)的生長和耐凍性。台灣林業科學26(2):179-92。

INTRODUCTION

Agropyron cristatum (L.) Gaertn. (crested wheatgrass) is a dominant species of indigenous grass in the Mongolian steppe. The major desirable forage species in this area consist of *Stipa krylovii*, crested wheatgrass, and *Allium polyrrhizum*, accounting for 80% of the available phytomass (Retzer 2007). Crested wheatgrass is widely used to restore the Mongolian grassland.

Arbuscular mycorrhizae (AMs) are important elements of virtually all terrestrial ecosystems (Brundrett 1991, Smith and Read 2008). Arbuscular mycorrhizal fungi (AMF) are some of the most important soil microbes as they form symbiotic associations with > 80% of land plants (Ulrich et al. 2002). AMF are widespread in semi-arid grasslands, and their association with grasses is of vital importance in biomes grazed by large ungulates (Trappe 1981). The major benefits of plants from these relationships include improved uptake of water and inorganic nutrients, especially phosphorus (Baon et al. 1992, Sanders and Koide 1994, Clark and Zeto 2000, Singh 2004, Javot et al. 2007), and increased tolerance to adverse environmental conditions such as nutrient deficiencies (Raju et al. 1990), diseases (Benhamou et al. 1994), drought, and salinity (Gupta and Kumar 2000, Smith and Read 2008).

Low temperatures are one of the most important stress factors that reduce plant growth by affecting physiological and metabolic processes (Charest and Phan 1990, Guy

1990). Low temperatures impair plant-water relationships through reduced hydraulic conductance and loss of stomatal control (Aroca et al. 2003). Also, over a long period of time, low temperatures decrease the capacity and efficiency of photosynthesis through changes in pigment compositions, and impaired chloroplastic development and electron transport efficiency (Farooq et al. 2009). Mycorrhizae are known to confer improved protection against low temperature stress, via access to a better nutritional status and modification of plant physiology, e.g., maintenance of photosynthetic pigments and photosynthesis (Wu and Xia 2006, Wu and Zou 2010, Zhu et al. 2010). Charest et al. (1993) reported that mycorrhizae counteract chilling injury of 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 leaves caused by late-spring and early-autumn frosts significantly limits the growing season length, and exerts a strong influence over plant production and distribution (Woodward 1987). Late-spring frosts are particularly damaging, because they occur at a time when most plants have broken dormancy, and result in significant costs for leaf replacement. Freezing injury is primarily caused by the physical disruption of cellular structures by ice crystals 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.

Mongolian grasslands are encountering rapid desertification due to global warming and extreme temperatures. Freezing events are critical to the ecology of the Mongolian steppe during springtime. The growing season of Mongolian crested wheatgrass begins in late April or early May. Late-spring frosts generally occur in May. Freezing injury can cause serious mortality of crested wheatgrass. Therefore, cold tolerance and survival of leaves and whole plants play an important role in the growth and survival of crested wheatgrass. A better understanding of the interaction between mycorrhizae and the cold tolerance of dominant grass species on the Mongolian steppe should help Mongolia maintain its grasslands. Thus, the aims of this study were to isolate, identify, and propagate AMF and to assess the effects of the native AMF on growth and freeze tolerance of Mongolian crested wheatgrass seedlings through mycorrhizal resynthesis and freezing tests. It is hoped that the findings from this study can contribute to the application of mycorrhizal techniques in restoring Mongolian grasslands.

MATERIALS AND METHODS

Sample collection

Root samples and rhizosphere soil were collected from a crested wheatgrass grassland at Bogd Mountain in the vicinity of Ulaanbaatar City (107º08'31"E, 47º45'767"N, at an elevation of 1597 m), Mongolia. Feeder roots, and soil and root samples were collected from 0 to 20 cm depth of 8 individual plants, kept in polyethylene bags, and stored at 5~10℃ until analyzed. Seeds of crested wheatgrass were also collected from the same site.

Extraction, identification, and propagation of AMF spores

Spores of AMF in soil samples of crested wheatgrass were isolated by a 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). The identified spores were subsequently propagated in corn seeds germinated in sterilized sand pot culture for an AM fungal inoculum preparation. Spores in the sand were quantified for a further inoculation test.

Seedling culture

Seeds of crested wheatgrass were surface-cleaned with running tap water, sterilized with a 10% sodium hypochlorite solution for 15 min, rinsed 3 times with sterile distilled water, and then germinated in a sterilized mixture of peat moss, vermiculite, and perlite (1: 1: 1, v/v) in a growth chamber at 20±2℃ in the day, and 16±2℃ at night, with 60% relative humidity, and 10,000-lux light intensity with a 16-h photoperiod. After seedlings had reached 4 cm in height, they were transferred to pots filled with sterilized sand for AM resynthesis.

Mycorrhizal resynthesis

Grass seedlings (3 mo old) were inoculated with 10 g of spore-containing sand (*D. spurcum*, 12±2 spores g^{-1} sand). Non-inoculated seedlings were treated with the filtrate of sand-spores as a control. All seedlings were cultured in a greenhouse at 20±3℃ with 1000±200 μ mole photons m^{-2} s^{-1} photosynthetic photon flux density (PPFD), and watered with deionized water as needed without supplemental fertilization.

Morphology of AM

Two months after treatments, the roots of seedlings 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 a 0.05% trypan blue solution. The morphology of the AM was observed with a stereomicroscope (Abbott 1982, Brundrett et al. 1996). The percentage of root colonization was accordingly calculated (Phillips and Hayman 1970).

For the ultrastructural study, root samples were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde fixative in a phosphate-buffered solution (PBS; 0.1 M, pH 7.0) for 4 h at room temperature, then rinsed with the PBS 3 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 under a scanning electron microscope (SEM, Brundrett et al. 1996).

Mycorrhizal colonization in roots was assessed by the grid line intersection method (Giovannetti and Mosse 1980).

Cold acclimation

Eight-month-old plants of crested wheatgrass were used for the freeze-tolerance test, and each treatment included 40 pots of plants (6 plants pot^{-1}). For non-stressed treatments, plants were inoculated with AMF (*D. spurcum*) or non-inoculated (control). Plants were grown in a greenhouse at 20±3℃ until being harvested. For cold acclimation treatments, plants were inoculated with AMF (*D. spurcum*) or non-inoculated (control), hardened for 2 wk at 12℃ under a 10-h photoperiod, and then cold-acclimated at 2℃ under an 8-h photoperiod for 2 wk, 0℃ for 24 h, and 2℃ for 24 h (Pociecha et al. 2009). The cold-acclimated plants were subsequently used for the freezing test.

Assessment of freeze tolerance

After hardening and cold acclimation, all

dry leaves were removed and 6 plants in each treatment were subjected to freezing tests at -8, -11, -14, -15, -16, and -17℃ for 2 h (Rapacz et al. 2004). Then, the plants were transferred to a greenhouse at 12±2℃ with 1000±200 μmole photons m^{-2} s^{-1} PPFD, and watered with deionized water. The extent of leaf injury was assessed for all plants exposed to freezing treatments. Total numbers of dry and green leaves were counted in the following 3~7 d, and used to calculate the leaf LT_{50} as the number of dead leaves over the total number of leaves. The LT_{50} (lethal temperature causing 50% mortality) was determined by controlled freezing treatment followed by visual rating of plant recovery after 10 d at 12 ±2℃ (Palonen and Buszard 1998).

Growth and yield measurements

After assessment of freeze tolerance, 4 plants per treatment of non-inoculated and inoculated seedlings were harvested. Growth parameters including plant height, root length, number of leaves, areas of leaves, and fresh and dry weights of leaves, stems, and roots were determined. The dry weight was measured after drying the samples in an oven at 70±2℃ for 48 h. Leaf area was measured using a Li-3100 leaf area meter (Li-COR, Lincoln, NE, USA). The specific leaf area (SLA) was calculated according to the following equation: SLA (cm^2 g^{-1}) = leaf area (cm^2)/leaf dry weight (g) (Ibrahim et al. 1998).

Mineral content analysis

For the mineral content analysis, root, shoot, and leaf samples were oven-dried at 70±2℃ for 48 h and digested with concentrated H_2SO_4 and H_2O_2. Nitrogen contents of roots, shoots, and leaves were estimated by the micro-Kjeldahl method (MacDonald 1977). Phosphorus, potassium, calcium, sodium, and magnesium contents were estimated by inductively coupled plasma atomic emission spectrometry (ICP-AES P4010 Hitachi, Tokyo, Japan).

Quantification of mycorrhizal dependency

Mycorrhizal dependency was defined as the percentage 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, Chicago, IL, USA) for Windows. All data are presented as the mean of 4 separate experiments±standard error (n = 4). Differences in growth rates among treatments were analyzed by Tukey's multiple-range test at $p \leqq 0.05$ significance level.

RESULTS AND DISCUSSION

Spore morphology and identification

After isolation and extraction, spores of AMF were collected. The AMF species was identified as *D. spurcum* using the synoptic keys and species morphology of the INVAM website (http://www.invam.caf.wvu.edu). Spores of *D. spurcum* were usually found singly in soil, or sometimes coupled in clusters (Fig. 1A, C). Spores of *D. spurcum* were yellow to yellowish, usually spherical, occasionally subglobose to pyriform. The spore cell wall consist of 2 layers (L1 and L2), with 2 layers of the germinal wall (gw1 and gw2) subtending hypha and septa (Fig. 1A-C). Other AMF, such as *Glomus macrocarpum* and *G. macrocarpum* var. *macrocarpum*, were also found to form AM with *Agropyron smithii* in Colorado, USA (Singh 2004).

Morphology of AM

Staining of root samples revealed that

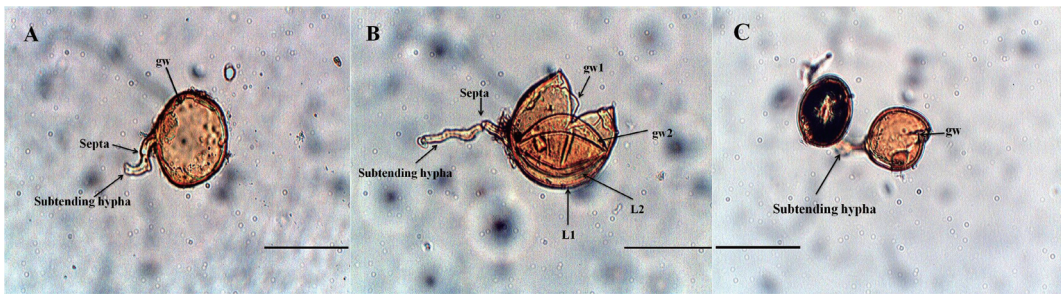

Fig. 1. Morphology of spores of *Diversispora spurcum*. (A) Spore structure of *D. spurcum*, gw: germinal wall (bar, 100 µm); (B) squashed spore of *D. spurcum*, L1, L2: inner wall layers (bar, 100 µm); (C) a pair of *D. spurcum* spores (bar, 100 µm).

AM developed well in the roots of inoculated seedlings. Arbuscules and vesicles were abundantly present in root tissues of inoculated seedlings (Fig. 2). In roots of inoculated seedlings, hyphae extended intercellularly and formed arbuscules, showing an *Arum*-type morphology (Fig. 2C-E). There were numerous vesicles in the roots; an average of 22~26 vesicles (cm of root)$^{-1}$ was observed (Fig. 2A). In contrast, no hyphae, arbuscules, or vesicles were present in roots of non-inoculated seedlings (Fig. 3). In nature, > 90% of all higher plants are associated with mycorrhizae, and > 80% of these plants form

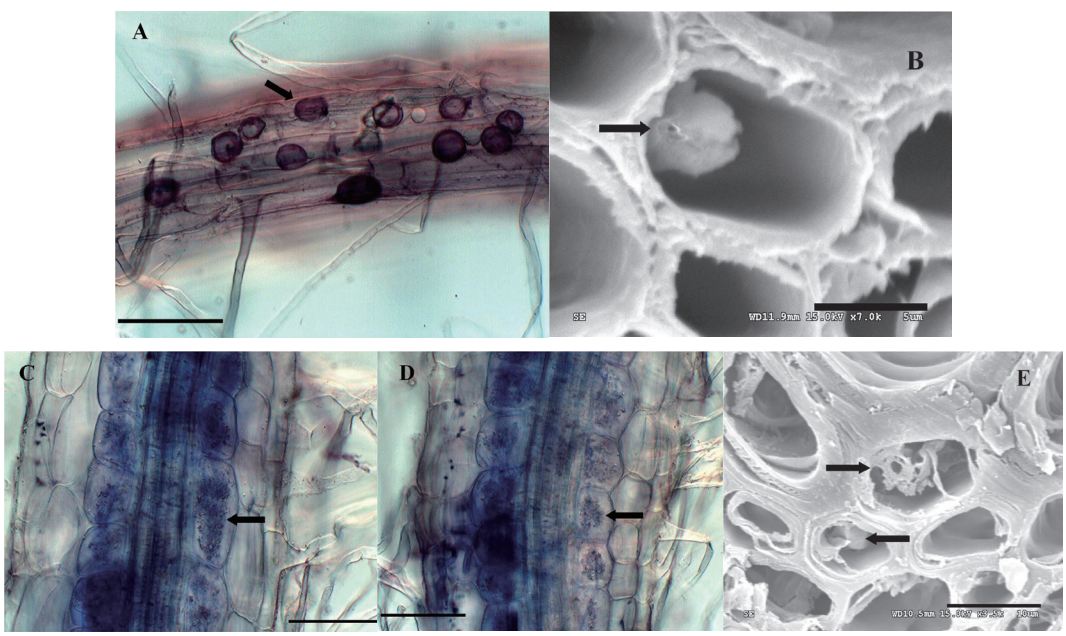

Fig. 2. Arbuscular mycorrhiza of crested wheatgrass. (A) Structure of a root with vesicles (arrowhead, bar, 100 µm); (B) ultrastructure of a root with a vesicle (arrowhead, bar, 5 µm); (C, D) *Arum*-type arbuscules (arrowhead, bar, 100 µm) in root cortical cells; (E) ultrastructure of a root with arbuscules and vesicle (arrowhead, bar, 20 µm).

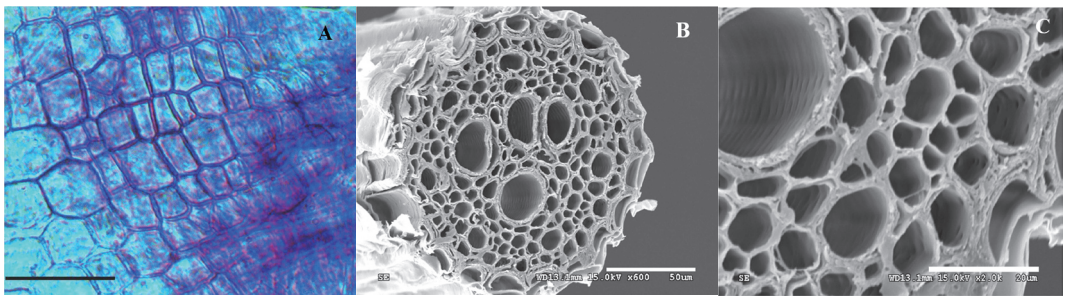

Fig. 3. Morphology of a root of non-inoculated crested wheatgrass seedlings. (A) Structure of the root (bar, 100 μm); (B, C) ultrastructure of the root (bars, 50 and 20 μm).

AM relationships (Smith and Read 2008). O'Connor et al. (2001) also indicated that *Arum*-type structures are associated with all of the 21 herbaceous AM plants in an Australian desert.

Plant growth

The height and root length of *D. spurcum*-inoculated plants were significantly higher than these of non-inoculated plants (Table 1). The enhancements in plant height and root length were 96 and 69%, respectively (Table 1). Meanwhile, fresh and dry weights of roots, stems, and leaves of inoculated plants were also significantly higher than the controls (Tables 2, 3). The enhancements in root, stem and leaf fresh weights were 416, 138, and 145%, respectively (Table 2), and the corresponding values in dry weight were 547, 220, and 176%, respectively (Table 3).

AMF efficiency can be measured in terms of host plant growth under different environmental conditions (Ruiz-Lozano et al. 1996). Our study revealed that *D. spurcum* inoculation largely promoted the growth and biomass of crested wheatgrass seedlings (Tables 1-3). The mycorrhizal influence was more pronounced in the root biomass than in aerial biomass (Table 2), which may have been due to a proportionally greater allocation of carbohydrates to the roots than to shoot tissues after AMF colonization, which is contrary to the findings of Schwab et al. (1982). This is reasonable because AM enhances root growth and nutrient acquisition. In this study, the beneficial effects of AM symbiosis on plant growth in terms of fresh and dry weight were in agreement with previous studies on other plant species (Anderson et al. 1987, Volkmar and Woodbury 1989, Charest et al. 1993, Gavito et al. 2003, Wu and Xia 2006, Wu and Zou 2010, Zhu et al. 2010).

The leaf area, leaf blade number, and specific leaf area of *D. spurcum*-inoculated

Table 1. Growth of inoculated and non-inoculated crested wheatgrass seedlings after 8 mo of cultivation

Inoculation treatment	Net height growth (cm)	Net root length growth (cm)
Diversispora spurcum	54.0 ± 5.89^{a}	24.5 ± 2.38^{a}
Control (non-inoculated)	27.5 ± 2.08^{b}	14.5 ± 2.65^{b}

All values are the mean ± standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

Table 2. Fresh biomass of inoculated and non-inoculated crested wheatgrass seedlings after 8 mo of cultivation

Inoculation treatment	Root (g)	Stem (g)	Leaf (g)
Diversispora spurcum	3.15±0.66[a]	2.00±0.28[a]	3.53±1.12[a]
Control (non-inoculated)	0.61±0.09[b]	0.84±0.11[b]	1.44±0.33[b]

All values are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

Table 3. Dry biomass of inoculated and non-inoculated crested wheatgrass seedlings after 8 mo of cultivation

Inoculation treatment	Root (g)	Stem (g)	Leaf (g)
Diversispora spurcum	0.97±0.28[a]	1.09±0.26[a]	1.35±0.34[a]
Control (non-inoculated)	0.15±0.03[b]	0.34±0.01[b]	0.49±0.11[b]

All values are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

crested wheatgrass were significantly higher than those of non-inoculated plants, with respective enhancements of 169, 100, and 26% (Table 4). These results suggested that *D. spurcum* could effectively stimulate leaf growth of crested wheatgrass seedlings. Berta et al. (1995) found that inoculated AM colonization with either of the AM fungi *G. mosseae* or *G. 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 *G. intraradices* produced more leaves than the control. Kothari et al. (1990) reported that inoculation with the AMF, *G. mosseae*, increased the leaf blade area and dry weight in maize by about 30%. A study on the combined effects of AM (with *G. mosseae*) and low temperature (5℃) on 2 wheat (*Triticum aestivum*) cultivars revealed that the dry biomass was higher in the spring cultivar (Glenlea) than the winter cultivar (AC Ron) after 5 wk of culture (Paradis et al. 1995).

Mycorrhizal dependency of crested wheatgrass on AM with *D. spurcum* was estimated to be 292% based on biomass accumulation. This indicates a high degree of responsiveness of crested wheatgrass growth to mycorrhizal colonization. Consistently, a similar effectiveness of AM fungi in different plant species was also reported by Dixon

Table 4. Total leaf area, number of leaf blades and specific leaf area of inoculated and non-inoculated crested wheatgrass seedlings after 8 mo of cultivation

Inoculation treatment	Total leaf area (cm^2)	Leaf blade (no.)	Specific leaf area ($cm^2\ g^{-1}$)
Diversispora spurcum	105±18.7[a]	44±8.4[a]	122±10[a]
Control (non-inoculated)	39±2.8[b]	22±3.9[b]	97±10.2[b]

All values are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

et al. (1997). Furthermore, Bhoopander and Mukerji (2004) reported that under a salt stress condition, the mycorrhizal dependency of *Sesbania aegyptiaca* and *S. grandiflora* increased with the age of the plants.

Mineral contents

Mineral contents in the roots, stems, and leaves of crested wheatgrass inoculated with *D. spurcum* were significantly higher than those of non-inoculated plants (Tables 5-7). Enhancements in root Ca, K, Mg, Na, and P contents were 217, 209, 216, 279, and 510%, respectively (Table 5). The corresponding values in stem mineral contents were 188, 332, 674, 334, and 682% (Table 6), while the values in leaf mineral content were 128, 326, 224, 222, and 350%, respectively (Table 7). Clearly, the most significant increases were in phosphate, especially in roots and stems. Also, the nitrogen contents in roots, stems, and leaves were higher in AM-inoculated than control plants, with respective enhancements of 168, 179, and 181% (Table 8).

Diversispora spurcum inoculation significantly increased the acquisition of nitrogen and minerals (P, K, Ca, Mg, and Na) in roots, stems, and leaves of crested wheatgrass seedlings (Tables 5-8) that presumably stimulated its growth (Tables 1-4). Consistent with our results, previous studies also showed that growth and mineral nutrition of plants are

Table 5. Mineral contents of roots of inoculated and non-inoculated crested wheatgrass seedlings after 8 mo of cultivation

Inoculation treatment	Ca (mg g^{-1})	K (mg g^{-1})	Mg (mg g^{-1})	Na (mg g^{-1})	P (mg g^{-1})
Diversispora spurcum	1376 ± 275^{a}	1575 ± 411^{a}	291 ± 87^{a}	709 ± 58^{a}	7882 ± 2003^{a}
Control (non-inoculated)	434 ± 50^{b}	509 ± 93^{b}	92 ± 34^{b}	187 ± 19^{b}	1293 ± 306^{b}

All values are the mean ± standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

Table 6. Mineral contents of stems of inoculated and non-inoculated crested wheatgrass seedlings after 8 mo of cultivation

Inoculation treatment	Ca (mg g^{-1})	K (mg g^{-1})	Mg (mg g^{-1})	Na (mg g^{-1})	P (mg g^{-1})
Diversispora spurcum	1730 ± 491^{a}	3138 ± 809^{a}	882 ± 285^{a}	812 ± 39^{a}	5662 ± 449^{a}
Control (non-inoculated)	601 ± 37^{b}	726 ± 52^{b}	114 ± 11^{b}	187 ± 5^{b}	724 ± 135^{b}

All values are the mean ± standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

Table 7. Mineral contents of leaves of inoculated and non-inoculated crested wheatgrass seedlings after 8 mo of cultivation

Inoculation treatment	Ca (mg g^{-1})	K (mg g^{-1})	Mg (mg g^{-1})	Na (mg g^{-1})	P (mg g^{-1})
Diversispora spurcum	1203 ± 78^{a}	4988 ± 302^{a}	334 ± 63^{a}	805 ± 85^{a}	5515 ± 181^{a}
Control (non-inoculated)	527 ± 92^{b}	1171 ± 117^{b}	103 ± 16^{b}	250 ± 112^{b}	1225 ± 88^{b}

All values are the mean ± standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

Table 8. Nitrogen contents of inoculated and non-inoculated crested wheatgrass seedlings after 8 mo of cultivation

Inoculation treatment	N (%)		
	Root	Stem	Leaf
Diversispora spurcum	1.26±0.16[a]	2.12±0.83[a]	3.29±1.64[a]
Control (non-inoculated)	0.47±0.08[b]	0.76±0.39[b]	1.17±0.30[b]

All values are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

commonly enhanced by AMF inoculation (Clark and Zeto 2000, Javot H et al. 2007, Wu and Zou 2009). Smith and Read (2008) reported that AMF increased plant growth mainly by increasing nutrient acquisition and thus enhanced the plant's resistance to biotic and abiotic stresses. Thus, our results confirm the significant effect of *D. spurcum* inoculation on the nutrition of crested wheatgrass.

Freeze tolerance

Analysis of the freeze tolerance revealed that the leaf mortality of non-inoculated crested wheatgrass was significantly higher than that of the inoculated ones; the leaf LT_{50} of the non-inoculated crested wheatgrass was -8℃, whereas the leaf LT_{50} of the inoculated ones was down to -14℃ (Fig. 4A, Table 9). For the whole plant, the mortality of non-inoculated crested wheatgrass was also significantly higher than that of inoculated ones; the plant LT_{50} of non-inoculated crested wheatgrass was -11℃, while that of the control was -15.5℃ (Fig. 4B, Table 10).

Judging from plant recovery after the freezing treatment, crested wheatgrass inoculated with *D. spurcum* also exhibited a higher freeze tolerance than the control (Tables 9, 10). For example, at -11℃, which occurs frequently in the winter in Mongolia, rates for inoculated and non-inoculated plants were 0.0±0.0 and 50.0±5.5%, respectively, representing a 2-fold increase in cold tolerance. Clearly, AM inoculation largely improved survival rates of plants, with a 100% survival rate at temperatures as low as -11℃. Taken

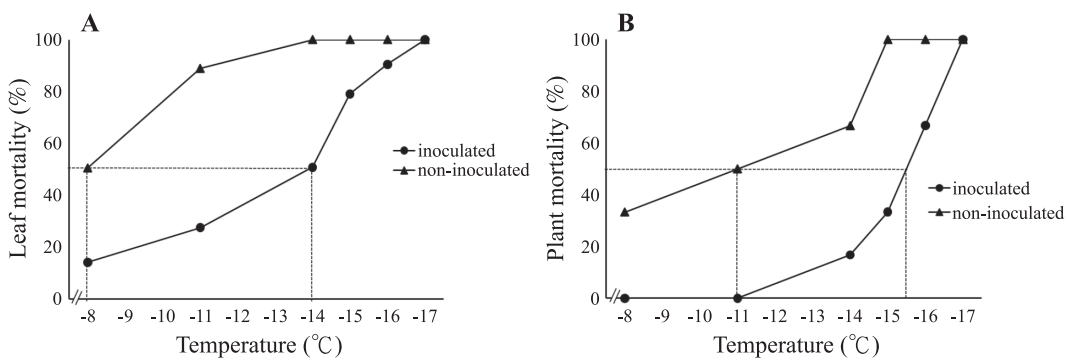

Fig. 4. Leaf and plant mortality as a function of freezing temperatures and the leaf (A) and whole plant (B) lethal temperature for 50% mortality of inoculated and non-inoculated crested wheatgrass.

Table 9. Leaf mortality of crested wheatgrass after cold acclimation and freezing treatments

Inoculation treatment	Leaf mortality (%)						
	2℃	-8℃	-11℃	-14℃	-15℃	-16℃	-17℃
Diversispora spurcum	8±4.6[b]	14.1±4.5[b]	27.5±5.6[b]	50.8±4.5[b]	79.1±4.8[b]	90.5±2.4[b]	100±0.0[a]
Control (non-inoculated)	20±3.7[a]	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 are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

Table 10. Whole plant mortality of crested wheatgrass inoculated with *Diversispora spurcum* and non-inoculated ones under different freezing temperatures

Inoculation treatment	Plant mortality (%)					
	-8℃	-11℃	-14℃	-15℃	-16℃	-17℃
Diversispora spurcum	0.0±0.0[b]	0.0±0.0[b]	16.7±4.1[b]	33.3±5.2[b]	66.7±5.2[b]	100±0.0[a]
Control (non-inoculated)	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 are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.

together, these results demonstrate that inoculation of AMF *D. spurcum* improved the freeze tolerance of crested wheatgrass.

CONCLUSIONS

In this study, a native AMF species of Mongolian crested wheatgrass was isolated and identified as *Diversispora spurcum*. Mycorrhizal resynthesis experiment revealed that *D. spurcum* could effectively form AM in roots of crested wheatgrass seedlings. *Diversispora spurcum* inoculation significantly promoted the growth and biomass accumulation of crested wheatgrass seedlings. The mycorrhizal influence was more pronounced in the root biomass than in the aerial biomass. Mycorrhizal dependency of crested wheatgrass on AM with *D. spurcum* was 292% based on biomass accumulation. The enhancement in growth was reflected in an increased leaf blade number, leaf area, and specific leaf area of *D. spurcum*-inoculated crested wheatgrass. *Diversispora spurcum* inoculation significantly increased the nitrogen and mineral (P, K, Ca, Mg, and Na) contents in all tissues of crested wheatgrass. The increase in the phosphorus content was of particular significance. Clearly, enhanced acquisition of P through AM can stimulate root growth and subsequently promote absorption of other minerals and nitrogen for higher growth rates. Furthermore, leaf and plant LT_{50} values of inoculated crested wheatgrass were much higher than those of non-inoculated plants. Inoculated crested wheatgrass plants exhibited total survival at temperatures of as low as -11℃, while only 50% of non-inoculated plants could survive this freezing temperature. The increased freeze tolerance of inoculated crested wheatgrasses may have been due to higher metabolic activities for synthesis of osmoregulation compounds. These results clearly demonstrate that *D. spurcum* could effectively form AM with crested wheatgrass and improved its growth and freeze tolerance. This information can be applied to efforts to restore Mongolian grasslands.

ACKNOWLEDGEMENTS

We wish to thank Prof. Maurice S. B. Ku of the Department of Bioagricultural Science of National Chiayi Univ., Chiayi, Taiwan for reviewing the manuscript. Financial support from National Chiayi Univ. is gratefully acknowledged.

LITERATURE CITED

Abbott LK. 1982. Comparative anatomy of vesicular-arbuscular mycorrhizas formed on subterranean clover. Aust J Bot 30:485-99.

Anderson CP, Sucoff EI, Dixon RK. 1987. The influence of low soil temperature on the growth of vesicular-arbuscular mycorrhizal *Fraxinus pennsylvanica*. Can J For Res 17:951-6.

Aroca R, Vernieri P, Irigoyen JJ, Sancher-Diaz M, Tognoni F, Pardosso A. 2003. Involvement of abscisic acid in leaf and root of maize (*Zea mays* L.) in avoiding chilling-induced water stress. Plant Sci 165:671-9.

Baon JB, Smith SE, Alston AM, Wheeler RD. 1992. Phosphorus efficiency of three cereals as related to indigenous mycorrhizal infection. Aust J Agric Res 43:479-91.

Benhamou N, Fortin JA, Hamel C, St-Arnaud M, Shatilla A. 1994. Resistance responses of mycorrhizal Ri T-DNA transformed carrot roots to infection by *Fusarium oxysporum* f. sp. *chrysanthemi*. Phytopathology 84:958-68.

Berta G, Trotta A, Fusconi A, Hooker JE, Munro M, Atkinson D, Giovannetti M, Morini S, Fortuna P, Tisserant B, Gianinazzi-Pearson V, Gianinazzi G. 1995. Arbuscular mycorrhizal induced changes to plant growth and root system morphology in *Prunus cerasifera*. Tree Physiol 15:281-93.

Bhoopander G, Mukerji KG. 2004. Mycorrhizal inoculant alleviates salt stress in *Sesbania aegyptiaca* and *Sesbania grandiflora* under field conditions: evidence for reduced sodium and improved magnesium uptake. Mycorrhiza 14:307-12.

Brundrett MC. 1991. Mycorrhizas in natural ecosystems. Adv Ecol Res 21:171-313.

Brundrett MC, Bougher N, Dell B, Grove T, Malajczuk N. 1996. Working with mycorrhizas in forestry and agriculture. *Aust* Centre *Intl* Agric Res Monogr 32:1-374.

Busquets D, Calvet C, Victoria A, Estaún V. 2010. Differential effects of two species of arbuscular mycorrhiza on the growth and water relations of *Spartium junceum* and *Anthyllis cytisoides*. Symbiosis 52:95-101.

Charest C, Dalpé Y, Brown A. 1993. The effect of vesicular arbuscular mycorrhizae and chilling on two hybrids of *Zea mays* L. Mycorrhiza 4:89-92.

Charest C, Phan CT. 1990. Cold acclimation of wheat *Triticum aestivum*: properties of enzymes involved in proline metabolism. Physiol Plant 80:159-68.

Clark RB, Zeto SK. 2000. Mineral acquisition by arbuscular mycorrhizal plants. J Plant Nutr 23:867-902.

Daniels BA, Skipper HD. 1982. Methods for the recovery and quantitative estimation of propagules from soil. In: Schenck NC, editor. Methods and principles of mycorrhizal research. St. Paul, MN: The American Phytopathological Society. p 29-36.

Dixon RK, Mukerji KG, Chamola BP, Kaushik A. 1997. Vesicular arbuscular mycorrhizal symbiosis in relationship to forestation in arid lands. Ann For 5:1-9.

Farooq M, Aziz T, Wahid A, Lee DJ, Siddique KHM. 2009. Chilling tolerance in maize: agronomic and physiological approaches. Crop Pasture Sci 60:501-16.

Gavito ME, Schweiger P, Jakobsen I. 2003. P uptake by arbuscular mycorrhizal hyphae: effect of soil temperature and atmospheric CO_2 enrichment. Global Change Biol 9:106-16.

Gerdemann JW, Nicolson TH. 1963. Spores of mycorrhizal *Endogone* species extracted from soils by wet sieving and decanting methods. Trans Br Mycol Soc 46:235-44.

Giovannetti M, Mosse B. 1980. An evaluation technique for measuring vesicular and arbuscular mycorrhizal infection in roots. New Phytol 84:489-500.

Graham JH, Syvertsen JP. 1985. Host determinants of mycorrhizal dependency of citrus rootstock seedlings. New Phytol 101:667-76.

Gupta R, Kumar P. 2000. Mycorrhizal plants in response to adverse environmental conditions. In: Mukerji K, editor. Mycorrhizal biology. Delhi, India: Plenum Publisher. p 67-84.

Guy CL. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Ann Rev Plant Physiol Plant Mol Biol 41:187-223.

Ibrahim L, Proe MF, Cameron AD. 1998. Interactive effects of nitrogen and water availabilities on gas exchange and whole-plant carbon allocation in poplar. Tree Physiol 18:481-7.

Javot H, Pumplin N, Harrison MJ. 2007. Phosphate in the arbuscular mycorrhizal symbiosis: transport properties and regulatory roles. Plant Cell Environ 30(3):310-22.

Kothari SK, Marschner H, George E. 1990. Effect of VA mycorrhizal fungi and rhizosphere microorganisms on root and shoot morphology, growth and water relations in maize. New Phytol 116:303-11.

MacDonald DC. 1977. Methods of soil and tissue analysis used in the analytical laboratory. Ottawa, Canada: Canadian Forestry Service Information Report MM-X-78.

O'Connor PJ, Smith SE, Smith FA. 2001. Arbuscular mycorrhizal associations in the southern Simpson Desert. *Aust J* Bot 49:493-9.

Palonen P, Buszard D. 1998. In vitro screening for cold hardiness of raspberry cultivars. Plant Cell Tiss Org 53:213-6.

Paradis R, Dalpé Y, Charest C. 1995. The combined effect of arbuscular mycorrhizas and short-term cold exposure on wheat. New Phytol 129:637-42.

Pearce RS. 2001. Plant freezing and damage. Ann Bot 87:417-24.

Phillips JM, Hayman DS. 1970. Improved procedures for clearing and staining parasitic and arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158-61.

Pociecha E, Plazek A, Janowiak F, Zwierzykowski Z. 2009. ABA level, proline and phenolic concentration, and PAL activity induced during cold acclimation in androgenic *Festulolium* forms with contrasting resistance to frost and pink snow mould (*Microdochium nivale*). Physiol Mol Plant Pathol 73:126-32.

Raju PS, Clark RB, Ellis JR, Maranville JW. 1990. Effects of species of VA-mycorrhizal fungi on growth and mineral uptake of sorghum at different temperatures. Plant Soil 121:165-70.

Rapacz M, Gasior D, Zwierzykowski Z, Lėsniewska-Bocianowska A, Humphreys MW, Gay AP. 2004. Changes in cold tolerance and the mechanisms of acclimation of photosystem II to cold hardening generated by anther culture of *Festuca pratensis* x *Lolium multiflorum* cultivars. New Phytol 161:105-14.

Retzer V. 2007. Forage competition between livestock and Mongolian pika (*Ochotona pallasi*) in southern Mongolian mountain steppes. Basic Appl Ecol 8:147-57.

Ruiz-Lozano JM, Azcon R, Gomez M. 1996. Alleviation of salt stress by arbuscular mycorrhizal *Glomus* species in *Lactuca sativa* plants. Physiol Plant 98:767-72.

Sanders IR, Koide RT. 1994. Nutrient acquisition and community structure in co-occurring mycotrophic and nonmycotrophic old-field annuals. Funct Ecol 8:77-84.

Schwab SM, Johnson ELV, Meng JA. 1982. The influence of Simazine on formation of

vesicular-arbuscular mycorrhizae in *Chenopodium qunona* Willd. Plant Soil 64:283-7.

Singh S. 2004. Effect of soil moisture on arbuscular mycorrhizal development in plants. Part 1. In grasses, cereals, and fodder crops. Mycorrh News 15:2-19.

Smith SE, Read DJ. 2008. Mycorrhizal symbiosis. 3rd ed. London: Academic Press. 787 p.

Tommerup IC. 1992. Methods for the study of the population biology of vesicular-arbuscular mycorrhizal fungi. Meth Microbiol 24:23-51.

Trappe JM. 1981. Mycorrhizae and productivity of arid and semiarid rangelands. In: Manasah JT, Briskey EJ, editors. Advances in food producing systems for arid and semiarid lands. New York: Academic Press. p. 581-99.

Ulrich H, Katharina J, Hermann B. 2002. Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl Environ Microbiol 68:1919-24.

Upson R, Read DJ, Newsham KK. 2007. Microscopy analyses of field-collected *Cephaloziella varians*. New Phytol 176:460-71.

Volkmar KM, Woodbury W. 1989. Effects of soil temperature and depth on colonization and root and shoot growth of barley inoculated with vesicular-arbuscular mycorrhizae indigenous to Canadian prairie soil. Can J Bot 67:1702-7.

Woodward FI. 1987. Climate and plant distribution. Cambridge, UK: Cambridge Univ. Press. 174 p.

Wu QS, Xia RX. 2006. Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-water and water stress conditions. J Plant Physiol 163:417-25.

Wu QS, Zou YN. 2009. Arbuscular mycorrhizal symbiosis improves growth and root nutrient status of citrus subjected to salt stress. Sci Asia 35:388-91.

Wu QS, Zou YN. 2010. Beneficial roles of arbuscular mycorrhizas in citrus seedlings at temperature stress. Sci Hort 125:289-93.

Zhu XC, Song FB, Xu HW. 2010. Arbuscular mycorrhizae improve low temperature stress in maize via alterations in host water status and photosynthesis. Plant Soil 591-3.