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Distribution of epichloë endophytes in Chinese populations of *Elymus dahuricus* and variation in peramine levels

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Abstract

We examined the occurrence and vertical transmission of epichloë endophytes (fungal genus *Epichloë* or *Neotyphodium*) in natural populations of the grass, *Elymus dahuricus*, in China. Six of 21 sites examined had fungal endophytes in the seeds with an incidence of 4.4–100%. Conidial measurements of agar cultures derived from infected populations were used to characterize these endophytes as belonging to genus *Neotyphodium*, and to assess their diversity. The colony diameter of most isolates was less than 30 mm after four weeks growth at 22°C. Mean conidiophore lengths ranged from 14.54 to 20.76 μm, gradually tapering from 2.53 μm at the base to 0.9 μm at the tip. The conidia were semiglobose, elliptical or subspherical, 5.21 (4.75–5.45) × 3.15 (2.87–3.43) μm. Peramine was the only alkaloid produced by endophyte-infected (E+) *Elymus dahuricus* plants (except for the Wutaishan population). Peramine levels differed between plant populations. Peramine was only detected in the leaves of infected plants from the Baiwangshan population. The concentration of peramine in the infected plant of *Elymus dahuricus* was highest in October but below the detectable level in June.

Keywords: *Elymus dahuricus*, endophytic fungi, isolate, China, peramine

1. Introduction

Fungal endophytes have a world-wide distribution in the Poaceae. Endophytic fungi belonging to the related genera *Epichloë* and *Neotyphodium* (epichloë endophytes) have been found in many cool-season grasses (subfamily Pooideae), including *Festuca, Lolium, Dactylis, Agrostis, Stipa, Melica* (White and Morgan-Jones, 1987b), *Poa* (Moy et al., 2000), *Achnatherum* (Li et al., 2004), *Bromus* (Mirlohi et al., 2006; Iannone and Cabral, 2006), *Elymus* (Schardl and Leuchtmann, 1999), *Hordeum* (Nan, 1996a) and *Triticum* (Marshall et al., 1999) species. Studies have mainly focused on the endophytes of *Lolium* and *Festuca* species. Endophytes from other grasses could be a valuable source of germplasm for biotechnological applications (Clay, 1990).

The mutualism enhances ecological fitness for both host and microbe and sustains the association against a myriad of abiotic and biotic stresses (Clay, 1990). Peramine is an unusual pyrrolopyrazine alkaloid which occurs in many endophyte/grass associations and is a highly active feeding deterrent, e.g. to the Argentine stem weevil (*Listronotus bonariensis*), a major pest of New Zealand pasture (Rowan, 1993). Peramine has been reported from *L. perenne* (Tapper et al., 1989), *F. arizonica* (Faeth et al., 2002), *F. arundinacea, Bromus benekenii, B. ramosus* and *F. rubra* (Leuchtmann et al., 2000). However, not all hosts infected by epichloë are found to contain peramine (Rowan, 1993).

Elymus dahuricus is a perennial caespitose grass with wide geographical distribution in most arid and semi-arid regions of China and neighboring countries. In the 1970's, it was cultivated in north and northeast China and in Inner Mongolia (Chen and Jia, 2000). This grass grows on native rangelands in China, mixed with other grasses and legumes. It is an important grass for rangeland rehabilitation in degraded grassland zones of northern China; reducing wind erosion and land desertification (Shao et al., 2006). *El. dahuricus* is also added to seed mixtures of saline-tolerant grasses to promote rapid cover and establishment (Jefferson et al., 2005; Shao et al., 2006).

The antiherbivore effects of the endophyte alkaloids, particularly peramine and lolines, are among the factors mediating mutualism between host and epichloë (Siegel et

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al., 1990). Peramine and lolines are important antiinsect alkaloids, and ergot alkaloids and indole-diterpenes are associated with livestock toxicosis (Schardl et al., 2004). There have been no reports of toxicosis in animals grazing on pastures containing *El. dahuricus* in China. The objectives of this study were to evaluate endophyte infection in *El. dahuricus* samples collected from native rangelands of China, to characterize the fungal isolates obtained from them, and to determine the peramine levels at different harvest dates.

2. Materials and Methods

Detection of endophyte in El. dahuricus *populations*

El. dahuricus germplasm was collected from Inner Mongolia to Xinjiang, with 9 provinces being represented: Inner Mongolia, Hebei, Shanxi, Shanxi, Beijing, Xinjiang, Gansu, Qinghai, and Sichuan (Table 1). These localities covered the main distribution range of *El. dahuricus* in China. A total of 26 samples were collected from 21 sites and all seed samples were stored at 4°C. At least 100 seeds were examined from each population for fungal endophytes by examining the aleurone layer for fungal hyphae using an aniline blue staining method (Dapprich et al., 1994). Seeds were soaked in 5% NaOH for 16 h at room temperature, washed thoroughly in distilled water then boiled in 0.4% aqueous aniline blue for 20 min. After staining, individual seeds were placed on a microscope slide in a drop of distilled water, squashed under a cover slip, and observed with a light microscope at 400× magnification.

Collection, cultivation and screening

Seeds of the Wulingshan population were collected in September 2002. Those of Baiwangshan, Qinyuan, Guyuan, Shihezi and Wutaishan populations were collected in September 2003. Pots (30 cm × 25 cm × 8 cm) were filled with silt loam soil (1 kg) which had been sterilized in an oven at 160°C for 6 h. Only well-filled, healthy-looking seeds were used. Five rows of 10 seeds each were planted per pot at a depth of 0.5 cm. Pots were placed in a temperature-controlled greenhouse (18–24°C) with 10 h illumination per day. Pots were watered each day with 90 ml water. After four weeks, the percentage infection by endophyte was tested by staining with aniline blue (Nan, 1996a,b). A leaf sheath was removed, and an epidermal strip from the inside surface of the sheath was placed in a drop of 0.2% aniline blue on a microscope slide, covered with a slip, passed briefly over a flame, and observed microscopically. Leaf sheaths were examined from a minimum of 30 plants from each population.

On 23 July 2004, germinated seedlings were transplanted into 2.4 m × 2.5 m field plots at a spacing of 20 cm per plant and 20 cm between rows. The field plots were located at the Lanzhou University campus, Lanzhou city, China at an elevation of 1517 m above sea level. Endophyte infected (E+) and endophyte free (E–) plants of approximately the same size (four to six tillers each) were paired. Approximately 50 uninfected and 50 infected plants were selected. Twenty E+ plants and 20 E– plants from each population were then labeled and watered daily for the first week after being transplanted, but there was no watering thereafter.

Table 1. Incidence of endophyte in seeds of *Elymus dahuricus* in China.

No. shown on Fig. 1	Province	Collection site	Incidence (%)
1	Inner Mongolia	Xilinguolemeng	0
2		Wulanchabumeng	0
3		Chifeng	0
4		Duolun	0
5	Hebei	Guyuan	4.4
6	Shanxi	Wutaishan	31.4
7		Qinyuan	73.3
8	Beijing	Baiwangshan	100.0
9		Wulingshan	100.0
10	Shanxi	Yulin	0
11	Xinjiang	Shihezi	86.0
12		Nileke	0
13	Gansu	Longzhong	0
14		Maqu	0
15		Jiuquan	0
16		Gannan1	0
16		Gannan2	0
17	Qinghai	Huangyuan1	0
17		Huangyuan2	0
18		Gonghe1	0
18		Gonghe2	0
19		Gangcha1	0
19		Gangcha2	0
20		Pingan1	0
20		Pingan2	0
21	Sichuan	Hongyuan	0

Table 2. Incidence of endophytes in seedlings grown from *Elymus dahuricus* seeds containing endophytes.

Populations	No. of plants examined	No. of plants with endophytes	Percent of tiller infection (%)
Wulingshan	162	62	38.3
Baiwangshan	36	27	75.0
Qinyuan	93	56	60.2
Wutaishan	145	33	22.8
Shihezi	90	0	0.0
Guyuan	100	0	0.0

Figure 1. Areas surveyed for *Elymus dahuricus* endophyte in China. Sites are given by numbers (see Table 1). The shaded areas show the main provinces in China where *Elymus dahuricus* is found.

On 3 June and 8 October 2005, all labeled mature tillers were harvested. Leaf and sheath components were separated for peramine determination. Plant tissues were plunged for 2 min in liquid nitrogen before freeze drying in –60°C and grinding to a fine powder in a mortar, the powder stored in –80°C .

Endophyte isolation

To test for endophyte fungi in seeds or plants developing from collected seeds, the seeds were surface sterilized by a dip in 70% ethanol for 1 min and then 5% sodium hypochlorite solution 10 min. Seeds were rinsed three times in sterile water and parts of them planted in pots containing sterilized soil. To isolated endophytes from tillers, Samples were surface sterilized by a dip in 70% ethanol for 1 min and soaked in 1% sodium hypochlorite solution for 5 min, rinsed three times in sterile distilled water and allowed to air dry on sterilized filter paper and cut 5 mm long.

Seeds or tillers were placed on agar in Petri dishes (90 mm diam.) containing Potato Dextrose Agar (PDA) plus penicillin and streptomycin (0.10 g of each per 1 l). Approximately 10 tissue pieces were placed in each dish. The Petri dishes were sealed with adhesive tape, placed on their sides and incubated in the dark at 22±2°C. Colony diameters were measured and morphological characteristics observed daily. This isolation procedure was used for colonies for light micrographs of conidia. For each isolate, 50 measurements of hyphae, conidia, and conidiophores were made with a light microscope (Olympus BX51, Japan) at 1000× magnification.

Peramine

The freeze-dried and ground grass material (100 mg) was extracted in two stages with a two-phase solvent system. The first stage employed 3 ml of methanol-chloroform (1:1, v/v) for 30 min at about 18°C in a centrifugation tube with mixing by continuous gentle inversion. For the second stage, 3 ml of hexane and 3 ml of water were added with a further 30 min of mixing before centrifugation at 10,000 g to separate the two phases and sediment the plant residues. A 1 ml aliquot of the lower aqueous phase of the extract was passed through a single CBA column (Waters, USA) containing 100 mg absorbent, in the ammonium ion form. The sample was washed with 1 ml of water and the bound peramine eluted with 0.5 ml of 10% (v/v) aqueous formic acid.

Peramine was measured by reversed-phase HPLC using an Agilent Liquid Chromatograph fitted with an Agilent 250 × 4.6 mm C_{18} column with 5 µl particle size. Detection was by a fixed-wavelength UV detector at 280 nm (close to the peak of peramine UV absorption). The isocratic mobile phase was 33% aq. acetonitrile containing guanidinium formate and excess formic acid to give a solution of approximately pH 3.7. This buffer was prepared by dissolving guanidinium carbonate (1.8 g/l) in water, adding reagent grade (98–100%) formic acid (1.6 ml/l), chromatography grade acetonitrile (200 ml/l), and making up to volume before degassing. The flow-rate was 1 ml/min. The quantity of peramine in 25µl-injection samples was determined by comparing the peak heights with those of peramine (1 mg/ml) in a peramine (1 mg/ml) and homoperamine (1 mg/ml) mixed standard. The limit of peramine detection was approximately 0.5 mg/g.

Statistical analysis

Variation in peramine content between populations and harvest dates was analyzed using analysis of variance (ANOVA) procedures with SPSS 11.5 software.

3. Results

Distribution of endophyte in El. dahuricus *populations*

Endophytes were detected in 26 collections from different regions (Table 1). Serpentine hyaline hyphae of the endophytes were seen in the leaf sheathes and aleurone layers of the seeds. Hyphae were 2–4 mm wide, parallel and unbranched in the leaf sheaths. Of the 26 samples analyzed, 6 were associated with epichloë endophytes (Table 1). Infection frequencies within populations ranged from 4.4–100% (Table 1).

The viability of endophytes in seeds was assessed by growing seedlings from the seeds. Seeds from the Shihezi, Xinjiang population and the Guyuan, Hebei population had 86.0% and 4.4% endophyte infection, respectively, but no endophyte was found in the seedlings. In the Wulingshan population from Beijing, seedling infection was only 38.3% even though 100% of the seeds contained endophytes. In the other three populations, endophytes in the seeds were more viable as incidence of seedling infection was 22.8 to 75% (Table 2). Choke symptoms were not seen in any rangeland plants of *El. dahuricus*.

Isolation of fungal endophyte

Seeds and tillers were placed in potato dextrose agar for 5 weeks and observed daily. Endophytic fungi hyphae grew from the tiller pieces after 10 days but two weeks were required before they emerged from the ends of seeds. Hyphae were subcultured to fresh plates of PDA and after four weeks incubation at 22°C, the fungal colonies were 15–35 mm diam., white and cottony on top but brown when were viewed from underneath. Examination of the fungi by light microscopy showed solitary phialides that appeared to have basal septa. Few conidia were produced on PDA. The conidia were semiglobose, elliptical or subspherical, 5.21 (4.75–5.45) × 3.15 (2.87–3.43) µm, the conidiophores were 17.98 (14.54–20.76) µm long, gradually tapering from 2.53 (2.43–2.69) µm at the base to 0.9 (0.79–0.99) µm at the tip (Table 3). These characteristics were consistent with the genus *Neotyphodium*.

Temporal and spatial variation in peramine levels in El. dahuricus

Peramine was not detected in leaves or sheaths of any E– plants of the four populations examined. Peramine concentrations did not vary significantly between populations (Table 4). Peramine was detected in the sheaths of all E+ plants except those of the Wutaishan population. The presence and amount of peramine was dependant on infection and also on the time of harvest (Table 4). Concentrations of peramine in sheaths of E+ plants of the Wulingshan, Qinyuan and Baiwangshan populations were higher in October than in June, but the difference was not significant ($p<0.05$). In leaves, peramine was only found in the Baiwangshan population.

4. Discussion

The extent of endophyte infections in natural populations has been studied in other species of *Elymus* (*El. canadensis, El. virginicus, El. repens, El. cylindricus, El. nutans* and *El. tangutorum*). For example, Vinton et al. (2001) found that higher incidence of infection present in *El. canadensis* of the central grassland region of the United States. White and Bultman (1987) estimated that 61% of *El. canadensis* and 47% of *El. virginicus* plants were infected in Texas. White and Morgan-Jones (1987a) identified the endophyte as *Neotyphodium typhinum*, and later determined to be *Epichloë elymi* (Schardl and Leuchtmann, 1999). Clay and Leuchtmann (1989) detected epichloë in *El. viriginicus* and found that 72% of the seeds were infected with these endophytes. Nan and Li (2000)

Table 3. Taxonomic characteristics of *Neotyphodium* spp. isolated from *Elymus dahuricus* after four weeks growth at 22°C on PDA media.

Isolated from	Hyphae width (μm)	Conidia (μm)			Conidiophores (μm)			Colony		Colony diameter (mm)
		Shape	Length	Width	Length	Base	Top	Upper	Reverse	
WLP	1.97	Subspherical	5.45	2.87	20.76	2.51	0.79	White, cottony	Tan to brown	23.5
QYP	2.07	Fusiform	5.20	3.43	16.97	2.69	0.99	White, cottony	Tan to brown	15.0
BWP	1.92	Semiglobular	5.43	3.02	19.66	2.48	0.86	White, cottony	Tan to brown	28.0
WTP	1.89	Ellipsoid	4.75	3.30	14.54	2.43	0.96	White, cottony	Tan to brown	30.5

WLP: Wulingshan; QYP: Qinyuan; BWP: Baiwangshan; WTP: Wutaishan.

Table 4. Peramine concentrations (μg/g dry weight tissue) of four populations of *Elymus dahuricus* at two harvest times in 2005.

Populations /Harvest dates	E+ Leaf	E− Leaf	E+ Sheath	E− Sheath
Wulingshan				
June 3	0	0	23.0*	0
October 8	0	0	26.3*	0
Qinyuan				
June 3	0	0	25.8*	0
October 8	0	0	27.5*	0
Wutaishan				
June 3	0	0	0	0
October 8	0	0	0	0
Baiwangshan				
June 3	26.4*	0	29.7*	0
October 8	30.0*	0	33.3*	0

*Significance at 0.05.

examined wild populations of *El. cylindricus*, *El. dahuricus,* and *El. tangutorum* in China and found only 33% of the samples *El. dahuricus* infected with endophytes. We detected 100 percent infection in two populations, it indicated that endophytes may provide a wide range of benefits to their host plants in some populations; however, the frequencies of epichloë endophyte on each *El. dahuricus* population was always not higher (Table 1). The results maybe which plant-endophyte interactions in native grass populations are much more complex and variable compared with agronomic grass (Saikkonen et al., 1998, 2000); because cultivar grasses genetic background maybe more homogenous and grown in uniform abiotic condition. So, environmental conditions (e.g. soil water) may be importance factor to the frequencies of endophyte infected native grasses (West, 1994). In the Guyuan, Hebei population, the infection frequency only was 4.4 percent (Table 1). The reasons may be due to either rapid spread of the fungus in grassland via the production of fruiting fungal stromata, or increased numbers of endophyte-infected plants as a result of their greater fitness due to enhanced herbivore resistance from the symbiosis. The former is unlikely since we did not find fungal stromata in any of the natural *El. dahuricus* populations during field work when seeds were collected. The endophytes in this grass would appear to be transmitted through successive generations via the seeds. So, it may be increases in the frequency of fungal endophytes in plant populations with time and the similar result also has been found in wild populations of Lolium perenne in Denmark (Jensen and Roulund, 2004), in turfgrass accessions in Italy (Romani et al., 2002), in *Festuca arundinacea* from Morocco, Tunisia and Sardinia (Clement et al., 2001) and in *El. canadensis* in natural grasslands (Vinton et al., 2001).

After emergence, seedlings grown from our collected seeds exhibited a lower incidence of seedling infection than in the seeds from which they were grown (Table 2). Perhaps the endophytes lose their viability with time in storage causing the incidence of seedling infection to decline sharply. The Shihezi population seeds were colleted in September 2000, whereas the Wulingshan population seeds were collected in September 2002. Both were stored at 4°C. Endophytes in stored seed can lose viability even though the seed itself retains its germinability. Seed held at 5°C and 60% relative humidity will suffer a loss of its viable endophyte infection in a few months (Rolston et al., 1986). Similar results were found by Nan (1996a, b) for *Hordium bogdanii* and by Marshall et al. (1999) for wild *Triticum* species. In contrast, seed stored at close to 0°C and 30% relative humidity maintains endophyte viability (Rolston et al., 1986). Under natural conditions where seed falls to the ground and does not germinating for several months, the loss of viable endophytes may also occur.

Light micrographs of conidia illustrate the morphological diversity of *Neotyphodium* strains in *El. dahuricus* populations from Wulingshan (Fig. 2H), Baiwangshan (Fig. 2E), Qinyuan (Fig. 2F) and Wutaishan (Fig. 2G). There is a variation in the mean conidia length and width in isolates from each of the four plant populations (Table 3). A similar variation has been observed in *Neotyphodium typhinum* isolated from *El.*

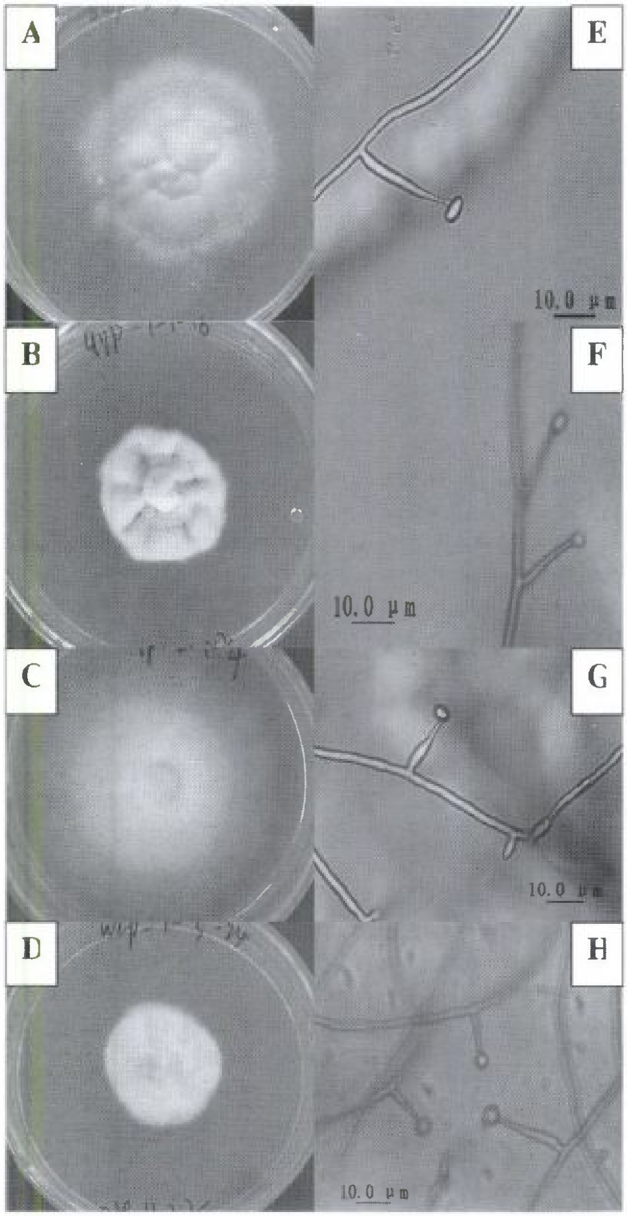

Figure 2. Endophyte cultures showed colony (A–D) and conidial morphologies (E–H). *A, E: Isolated from the Baiwangshan population; B, F: Isolated from the Qinyuan population; C, G: Isolated from the Wutaishan population; D, H: Isolated from the Wulingshan population.

canadensis plants of diverse origin (Austin and Dallas) (White and Morgan-Jones, 1987c).

The presence of an endophyte was shown to be necessary for production of the alkaloid, peramine in *El. dahuricus*. Plants without an endophyte produced no detectable peramine under field conditions. The Wutaishan population was unusual in that no peramine was detected despite the presence of endophyte. However our results suggested that the varying levels of peramine found in the four populations of endophyte-infected *El. dahuricus* are the result of infection by the endophyte. Peramine has been found in culture filtrates of *Neotyphodium lolii* cultures, which confirms that this alkaloid can be made by the fungus (Bacon and Battista, 1991). Symbioses involving grass species and endophytes result in various combinations and levels of alkaloids (Bush et al., 1997). Leuchtmann et al. (2000) found that peramine levels varied in different populations of *Bromus benekenii* and *B. ramosus*. A similar observation was made in studies of *Festuca arizonica* (Faeth et al., 2002). Seasonal variation in peramine levels occurred for endophyte-infected *Lolium perenne* (Ball et al., 1995; Belesky and Hill, 1997) and in native grasses (Leuchtmann et al., 2000). Peramine levels usually accumulate during the growing season with the highest levels occurring at the end of the growing season. Environmental factors such as temperature, photoperiod, and rainfall may be important in influencing peramine synthesis in *El. dahuricus*. This may be why peramine accumulated when temperatures decreased in autumn, in the sheath of the E+ plants of the Wulingshan, Baiwangshan, and Qinyuan populations following reproductive tiller production.

Peramine provides increased protection from insects (Tapper et al., 1989) and enhances host survival. Since insect populations increase during the growing season, protection later in the season, when reproductive parts of the plant are forming, would be particularly important. Pests such as Argentine stem weevil (*Listronotus bonariensis*) and black beetle (*Heteronychus arator*) damage the base of the plants so peramine would be a more effective deterrent in the leaf sheaths than in the leaves. From evolutionary point of view, this may be important as natural selection would favor endophyte-host associations that enhance host survival (Saikkonen et al., 2004).

Finally, fungal isolates from different *El. dahuricus* populations had different morphological characteristics. Nevertheless it was possible to identify the isolates as belonging to the genus *Neotyphodium* based on cultural characteristics, morphology and size/shape of the phialides and conidia. A better understanding of the taxonomy of these endophytes could be obtained by further cultural, biochemical and molecular studies.

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