Clavicipitaceae Anamorphic Endophytes in *Hordeum* germplasm

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Abstract: The incidence of clavicipitaceous anamorphic endophytes, non-choke inducing endosymbiotic fungi of the genus *Neotyphodium* that systemically infect grasses, in eighteen *Hordeum* species from the U.S. National Plant Germplasm System was examined using light and Scanning Electron Microscopy (SEM). Seventeen plant inventory accessions from only three *Hordeum* species, including *H. bogdani*, *H. brevisubulatum* subsp. *violaceum* and *H. comosum*, were found to contain significant levels of seed and seedling infection ranging from 18-99%. *Neotyphodium*-endophytes were found in *Hordeum* germplasm from one country (Argentina) in South America and four countries (Afghanistan, China, Iran and Kazakhstan) in Asia. The viability of endophytic mycelium in seeds was confirmed by culturing these fungi on potato dextrose agar medium from aleurone tissue of seeds and by direct observations of hyphae in leaf sheaths of 3-4 week-old seedlings. Morphological characteristics of these fungi were further characterized using SEM to determine similarities and differences in conidiophores and conidia produced in culture. The importance, significance and potential benefits of *Neotyphodium*-endophytes from wild *Hordeum* as sources of insect resistance in cultivated barley, other cereal grasses and in wild grasses included in the Conservation Reserve Program are discussed. Suggested strategies for the proper maintenance of this valuable germplasm also are elucidated.

Key words: *Neotyphodium* species, wild barley, biological control, Clavicipitaceae, insect resistance

INTRODUCTION

Clavicipitaceous endophytes are an important group of fungi with world-wide distribution in graminaceous hosts of the Poaceae. These fungi form relationships with grasses ranging from mutualistic to parasitic (Carroll, 1988; Clay, 1988). Consequently, they may be divided into two major groups, Clavicipitaceous Teleomorphic (CT) endophytes and Clavicipitaceous Anamorphic (CA) endophytes, based on the relationships and trophic interactions formed with their grass hosts (Wilson *et al*., 1991a; Wilson, 1996). Clavicipitaceous teleomorphic endophytes (Clavicipitaceae: tribe Balansiae) are pathogenic and induce choke diseases of grasses that abort flowering and preclude seed production due to the formation of subterminal sclerotia on the culm. This effect is different from the ergot fungi caused by *Claviceps* species that do not prevent flowering, but cause ergotised grain. Clavicipitaceous anamorphic endophytes of the genus *Neotyphodium* Glenn, Bacon, Price and Hanlin gen. nov. (formerly *Acremonium* section *Albolanosa* Morgan-Jones and Gams) (Glenn *et al*., 1996), are a closely related group of fungi, probably derivatives of the teleomorphic forms (Clay, 1990; Schardl and Phillips, 1997), that have lost the ability to produce a sexual stage (teleomorph) and to cause diseases of grasses. This group of mutualistic endophytic fungi has received considerable attention since the late 1970s when it was discovered that they are responsible for causing serious toxicoses of livestock (primarily cattle, sheep and horses) that consume endophyte-infected forage grasses (Bacon *et al*., 1997), but also provide important sources of resistance in grasses to a wide variety of insects (Clement *et al*., 1994; Rowan and Latch, 1994; Anderson *et al*., 2006) and diseases (Schmidt, 1990). The biologically active compounds responsible for the toxic syndromes in livestock and insect resistance were later discovered to be ergot alkaloids (clavine, lysergic acid and ergopeptine) (Lyons *et al*., 1986; Yates and Powell, 1988), loline (pyrrolizidine) alkaloids (Yates *et al*., 1990; Siegel *et al*., 1990; Bush *et al*., 1993), lolitrem (Miles *et al*., 1992, 1993) and peramine (pyrolopyrazine) alkaloids (Siegel *et al*., 1990, Rowan *et al*., 1986). Economic losses in animal production caused by endophyte toxicoses in cattle and sheep annually exceed $100s millions in U.S. dollars (Siegel *et al*., 1985). Endophyte-infected grass cultivars have been utilized for the biocontrol of insect and disease
pests primarily in the lawn or turf-grass industry (Gwinn and Gavin, 1992; Greulich et al., 1999; Clarke et al., 2006).

Clavicipitaceae anamorphic endophytes of the genus Neotyphodium are known only from the subfamily Pooideae that dominate temperate areas of the world and have the C3 photosynthetic mechanism (Clay, 1994, 1997). Neotyphodium-endophytes are very host specific (Clay, 1992) and have been found in many cool-season grasses such as Achnatherum (Stipa) (Bruehl et al., 1994; Jones et al., 2000), Alopecurus (Zabalagoecza et al., 1999), Bromus (White et al., 2001; Brem and Leuchtmann, 2003; Mirlho et al., 2006), Dactylis (Zabalagoecza et al., 1999), Echinopogon (Miles et al., 1998), Elymus (Vinton et al., 2001; Nan and Li, 2001), Festuca (Hahn et al., 2003; Koh et al., 2006), Holcus (Zabalagoecza et al., 1999), Hordeum (Wilson et al., 1991a, 1991b; 1991c), Lolium (Wilson et al., 1992; Lewis et al., 1997), Poa (Cabral et al., 1999) and diploid Triticeum (Marshall et al., 1999). The discoveries of Neotyphodium-endophytes in goatgrass species, Aegilops mutica (Boiss.) Eig and A. uniaristata Vis., represent the first diploid cereal grass species with endophytes that could be hybridized with cultivated wheat (Triticum aestivum L.) (Marshall, 1993). All Neotyphodium-endophytes form predominantly asymptomatic interactions with their hosts, systemically infect only aboveground parts within intercellular spaces, overwinter in meristematic tissues, are maternally transmitted via the seed and often impart superior performance to their grass hosts (Wilson, 1996; Clay, 1997; Malinowski and Belesky, 2000).

The National Plant Germplasm System (NPGS), administered by the United States Department of Agriculture, Agricultural Research Service (ARS), is the largest source of plant germplasm maintained in the United States. A large number of Plant Inventory (PI) accessions of numerous grass genera are available in the NPGS which potentially could be endophyte infected (Wilson, 1996). Surveys of this germplasm, especially in the cool-season grasses, have proven useful for discovering many new sources of endophyte-infected PI accessions in Festuca (Springer and Kindler, 1990), Lolium (Wilson et al., 1991d) and Poa species (Wilson, 1996). The objectives of this research were to conduct a microscopic survey of plant inventory accessions of a portion of the Hordeum species found in the NPGS to determine the incidence and rates of infection of seeds and seedlings by CA-endophytes, particularly endosymbiotic fungi of the genus Neotyphodium and to culture and characterize isolates from infected seeds and seedlings using scanning electron microscopy. Supporting information about Hordeum germplasm characteristics, collection data and some preliminary results of this research were reported previously (Wilson et al., 1991a-c).

MATERIALS AND METHODS

Seed source: Seed samples of 142 PI accession lines from a portion of the Hordeum sp. germplasm collection, previously held at the Western Regional Plant Introduction Station (WRPIS) prior to 22 November 1989, were obtained from the Small Grains Germplasm Research Facility in Aberdeen, Idaho that presently maintains the U.S. Hordeum collection in the NSGC. Seeds were previously stored at 4-5°C and 30-35% relative humidity at the WRPIS and subsequently at 6-7°C and 36-38% relative humidity since 22 November 1989 at the NSGC. Most accessions had been increased one or two times since original seeds were received into the collection. The oldest seeds of each accession were selected since original seeds often were unavailable.

Endophyte seed and seedling assays: Seeds of 96 PI accessions of seventeen Hordeum species including H. agriocrichon Aberg, H. bogdani Wilensky, H. brachyantherum Nevski, H. brevisubulatum (Trin.) Link, H. bulbosum L., H. californicum Covas and Stebbins (= H. brachyantherum subsp. californicum (Covas and Stebbins) Bothmer et al.), H. capense Thunb., H. chilense Roem. and Schult., H. comosum J. Presl, H. jubatum L., H. lechleri (Steud.) Schenck, H. marinum Huds., H. marinum L., H. procerum Nevski, H. secalinum Schreb., H. stenostachys Godr. and H. vulgare L. were examined microscopically for clavicipitaceae anamorphic endophyte, specific endosymbiotic fungi of grasses as defined by Wilson (1996). This survey represented 67.6% of available accessions that were examined. At least 40% of available PI accessions of H. brevisubulatum subsp. violaceum (Boiss. and Hohen.) Tzvelev, H. marinum subsp. gussoneanum (Parl.) Thell., H. marinum subsp. glaucum (Steud.) Tzvelev and H. marinum subsp. leporinum (Link) Arcang. also were examined. The Hordeum germplasm examined here originated from many countries within five continents of the world, including North America (NA), South America (SA), Europe (EU), Africa (AF) and Asia (AS).

Seed samples, 0.5-1.3 g depending on seed size, were soaked overnight in 5% sodium hydroxide at 22°C, rinsed with tap water and stained for several days at 22°C in 0.07% aniline blue as described previously (Wilson et al., 1991d). Seeds were then rinsed, squashed and mounted on slides in 1:1 v/v glycerol-distilled water and examined with a Zeiss compound microscope at 100-400x.
magnification within tissues of the aleurone layer and outer integument. Seed infection rates were based on examinations of 50 seeds in endophyte-free accessions and 100 seeds in endophyte-infected accessions. Seedlings of some infected accessions were grown in a greenhouse under natural light in 15 cm pots containing 55% peatmoss, 35% pumice and 10% sand. Leaf sheaths from 30-100 7-9 week old seedlings were examined microscopically at 400x to determine endophyte viability.

**Isolation and culture:** Clavicipitaceous endosymbiotic fungi from endophyte-infected accessions were isolated on Potato Dextrose Agar (PDA) from aleurone tissue of surface-sterilized seeds and from infected leaf sheaths of seedlings. For seed isolations, seed were soaked in 10% Clorox for 3-5 min, rinsed several times in sterile distilled water and plated on 3.5% PDA with or without 0.2% streptomycin sulfate to reduce bacterial contamination. For leaf sheath isolations, seedlings were generated from seed taken from NSGC storage by placing seeds on moist filter papers under 12 h fluorescent lights until germination. Germinated seeds were then transferred to a 55% peat moss 35% pumice and 10% sand soil mixture and grown in the greenhouse under conditions described previously (Wilson et al., 1991d). Seedlings were allowed to grow for several weeks to allow the endophytic fungi to grow out of the seed into leaf sheath tissue. Leaf sheaths were then collected from the base of stems, surface sterilized in 5% Clorox for 1-5 min. and plated onto 3.5% PDA using aseptic techniques. Colonies generated on plant tissues were further purified by transferring small amounts of clean inoculum to new plates.

**Scanning electron microscopy:** Mycelial plugs taken from cultures in exponential growth were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1M Pipes buffer, rinsed in 0.1M potassium phosphate buffer, postfixed in 1.0% osmium tetroxide and rinsed again in phosphate buffer. The mycelial plugs were dehydrated in an ethanol series, critical point dried and sputter coated with gold. Morphological characteristics of mycelia, conidiopores and conidia from each isolate were examined and compared at 5-15,000x magnification using a Hitachi 5571 scanning electron microscope. Data from SEM observations of CA-endophytes from each *Hordeum* species were taken including conidiophore grouping, conidiophore shape and taper, presence or absence of basal septum, conidia shape, conidial orientation on conidiophores and spore dimensions. A minimum of fifty conidial measurements were taken from SEM photographs for determining the range of dimensions for endophytes of each *Hordeum* species.

**RESULTS**

**Endophyte seed and seedling assays:** Endophytic hyphae in seeds of infected *Hordeum* species were concentrated primarily in the aleurone tissue and to a lesser extent, in the outer integument tissue of the seed. A microscopic survey of CA-endophytes in aleurone seed tissue of 96 PI accession lines, representing seventeen species of *Hordeum* germplasm from the NSGC, indicated that the majority of accessions of most *Hordeum* species examined were CA-endophyte free (Table 1). Only 17 (18%) of the 96 accessions examined contained hyphae of *Neotyphodium* species within aleurone and outer integument seed tissues. These *Neotyphodium* endophyte-infected accessions were found in only three *Hordeum* species (17.6%) of the species examined in this survey. Most of the endophyte-infected accessions were found in *H. bogdani* (70.6%) and *H. brevisubulatum* subsp. *violaceum* (11.8%) germplasm that originated from Asia. The remainder of *Neotyphodium* endophyte-infected accessions was found in *H. comosum* seeds originating from South America. None of the endophyte-infected *Hordeum* germplasm originated from North America, Europe, Africa, or Australia. All source countries of infected germplasm were in temperate regions either north or south of the equator (Table 2). *Neotyphodium*-infected accessions of *H. bogdani* originated primarily from Kazakhstan and China, although one infected accession was found from Afghanistan. The *H. brevisubulatum* subsp. *violaceum* infected accessions originated from Kazakhstan and Iran whereas all *Neotyphodium*-infected accessions of *H. comosum* originated from Argentina.

Endophyte infection rates in *Hordeum* seeds and seedlings germplasm varied widely among individual accessions (Table 2). Seed infection rates within individual accessions ranged from 18-99% infection in *H. bogdani*, 68-98% infection in *H. brevisubulatum* subsp. *violaceum* and 74-92% infection in *H. comosum*. Infection rates determined from growth of endophytes up into leaf sheafs of infected seedlings were usually similar or higher than seed infection rates from the same accession, indicating that most inoculum in aleurone seed tissue was viable (Table 2). Higher rates of infection in seedlings relative to seeds indicated that small amounts of inoculum not detected in seeds were detectable in seedlings. Seeding infection rates lower than apparent seed infection rates in the same accession generally indicated that inoculum in some individual seeds was not viable. Plant inventory accessions collected from wet habitats on uncultivated lands tended to have higher seed-infection rates, whereas accessions from dry habitats on cultivated or grazed lands were often endophyte-free.
Table 1: Incidence of clavicipitaceous anamorphic endophytes in seeds of the NSGC Hordeum germplasm collection

<table>
<thead>
<tr>
<th>Species</th>
<th>Available</th>
<th>Examined</th>
<th>Infected</th>
<th>NA</th>
<th>SA</th>
<th>EU</th>
<th>AF</th>
<th>AS</th>
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<td>16</td>
<td>16</td>
<td>12</td>
<td>0</td>
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<td>5</td>
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<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>H. brevisubalatum</em></td>
<td>2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>4</td>
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<tr>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td>1</td>
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<td>96</td>
<td>17</td>
<td>9</td>
<td>11</td>
<td>9</td>
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1Incidence is based on examinations of 100 seeds in endophyte-infected accessions and 50 seeds in endophyte-free accessions of the National Small Grains Collection (NSGC) of Hordeum species. 2Accessions from the National Small Grains Collection (NSGC) in Aberdeen, Idaho. 3Abbreviations for continents of origin of all accessions examined: North America (NA), South America (SA), Europe (EU), Africa (AF), Asia (AS). Information on specific endophyte-free accessions is available from the Germplasm Resources Information Network (GRIN) database, USDA-ARS

Table 2: Neotyphodium endophyte-infected accessions of the NSGC Hordeum germplasm collection

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession (PI)</th>
<th>Country of origin</th>
<th>Year</th>
<th>Mean % endophyte</th>
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<td><em>H. bogdani</em></td>
<td>264406</td>
<td>Afghanistan</td>
<td>1960</td>
<td>47 (53)</td>
</tr>
<tr>
<td></td>
<td>314696</td>
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<td>1966</td>
<td>62 (47)</td>
</tr>
<tr>
<td></td>
<td>440413</td>
<td>Kazakhstan</td>
<td>1978</td>
<td>88 (95)</td>
</tr>
<tr>
<td></td>
<td>440414</td>
<td>Kazakhstan</td>
<td>1978</td>
<td>80 (80)</td>
</tr>
<tr>
<td></td>
<td>499499</td>
<td>China</td>
<td>1984</td>
<td>18</td>
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<td>499500</td>
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<td>1984</td>
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<td></td>
<td>499501</td>
<td>China</td>
<td>1984</td>
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<td>531761</td>
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<td>401386</td>
<td>Iran</td>
<td>1975</td>
<td>68 (63)</td>
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<td>Kazakhstan</td>
<td>1978</td>
<td>98 (100)</td>
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<tr>
<td></td>
<td>264404</td>
<td>Argentina</td>
<td>1960</td>
<td>86</td>
</tr>
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<td></td>
<td>264405</td>
<td>Argentina</td>
<td>1960</td>
<td>92</td>
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<tr>
<td></td>
<td>269648</td>
<td>Argentina</td>
<td>1960</td>
<td>74</td>
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1Plant Inventory (PI) accessions of the National Small Grains Collection (NSGC) in Aberdeen, Idaho, were held at the Western Regional Plant Introduction Station (WRPIS) in Pullman, WA prior to 22 Nov 1989. 2Date the accession was received into the WRPIS Hordeum germ plasm collection. 3Infection rates were based on examinations of 100 seeds. Values in parentheses indicate viable endophyte as determined from examinations of 50-100 leaf sheaths of 7-9 week-old seedlings

Microscopic examinations of *Neotyphodium*-endophyte hyphae in aleurone seed tissues and leaf sheaths of 7- to 9-week old seedlings revealed a diversity of morphological types in accessions of endophyte-infected *Hordeum* species. *Neotyphodium* hyphae in *H. bogdani* PI accession 440413 was highly torulose with little branching in aleurone seed tissue (Fig. 1A), but relatively straight between leaf sheaf cells of seedlings (Fig. 1B). Somatic hyphae of the *Neotyphodium* endophyte in *H. brevisubalatum* subsp. violaceum aleurone tissue of PI accession 440420 was sinuous, of finer diameter and contained some hyphal segments that were straighter and less curvilinear (Fig. 2A). By contrast, endophytic hyphae in leaf sheafs of PI accession 440420 were much thicker, straight and with occasional branching between cells across the long axis of the leaf sheaf (Fig. 2B). Hyphae of the *Neotyphodium* endophyte in aleurone tissue of *H. comosum* PI accession 264405 were much less sinuous, but straighter, with abundant branching (Fig. 3A). Hyphae within the outer integument of the seed tissue in PI accession 264405 also exhibited frequent branching perpendicular to the long axis of the cells (Fig. 3B). All *Neotyphodium* hyphae within aleurone and outer integument seed tissues and in leaf sheaf tissues of seedlings grew only intercellularly and never penetrated cell walls, typical of true endophytes.

Cultural characteristics of endophytes: The CA-endophytes isolated from infected aleurone seed tissue were identified as *Neotyphodium* species in all cases based on cultural characteristics, somatic hyphae and...
Fig. 1: *Neotyphodium*-endophyte in *H. bogdanii* tissue. (A) Torulose hyphae within the aleurone layer of seed, PI accession 499499. (B) Straight hyphae within leaf sheath at the base of stem, PI accession 440413

Fig. 2: *Neotyphodium*-endophyte in *H. brevisubulatum* subsp. *violaceum* tissue of PI accession 440420. (A) Convoluted hyphae within the aleurone layer of seed. (B) Straight hyphae with a branch between two cells in the leaf sheath, parallel to the long-axis of the stem

Fig. 3: *Neotyphodium*-endophyte in *H. comosum* tissue of PI accession 264405. (A) Sinuous hyphae within the aleurone layer of seed. (B) Straight and branched hyphae in the outer integument of seed

conidiophore morphology. These identifications were confirmed by subsequent observations of endophytic hyphae in the leaf sheafts of infected *Hordeum* seedlings and in SEM observations. Cultures of *Neotyphodium*-endophytes from *Hordeum* germplasm were usually slow-growing (<20 mm/week), white to tan in color, with oppressed or raised growth, waxy to cottony, often puckering the agar as moisture was removed and producing seed colonies from germinating conidia as they were formed and released from conidiophores. Somatic hyphae of *Neotyphodium*-endophytes in *Hordeum* germplasm were typical of CA-endophytes in that they tended to be fine (<5 μm diameter), convoluted, torulose, or sinuous, highly vacuolated cells, rarely or infrequently branching, occasionally constricted at the septa and having septa that stained poorly or not at all. Sporulation was abundant with most strains. Conidiophores were produced abundantly, usually perpendicular to hyphal
strands and singly or grouped into synnemata. Crescent-shaped and elliptical or allantoid conidia formed singly at the ends of determinate conidiophores via holoblastic conidiogenesis.

Species of Neotyphodium endophytes isolated from Hordeum germplasm were not determined because of the common overlap in spore and conidiophore measurements between Neotyphodium species and the limited number of morphological and cultural characteristics that are available to differentiate species. Consequently, reliable identifications of individual Neotyphodium endophytes to species generally require the use of DNA-sequencing data. Such identifications were beyond the scope of this study.

Hyphal growth of Neotyphodium endophytes on the agar surface generally resulted in highly wrinkled colonies as growth continued and moisture was removed from the surface layers of the agar (Fig. 4A). Mycelium observed on the edge of colonies grew radially in dense, parallel masses of convoluted hyphae that tended to form appressed growth, particularly in the endophyte of H. brevisubulatum subsp. violaceum from PI accession 440420 (Fig. 4B). Convoluted hyphae that grew radially, relative to the center of the colony, maintained a parallel growth pattern and rarely crossed over adjacent hyphae.

By contrast, endophyte cultures that tended to form synnemata, such as those from certain H. bogdanii accessions, produced ropelike strands of synnemata that frequently crossed adjacent strands and produced raised aerial cottony growth (Fig. 4C). The torulose somatic growth form, characteristic of appressed colonies in exponential growth, continued until the colony was sufficiently old to initiate sporulation. Sporulation from determinate conidiophores was initiated in 2- to 4-week old cultures. Seed colonies often formed later from germinating conidia released from conidiophores.

**Scanning electron microscopy:** Analysis of Neotyphodium-endophyte conidiophores and conidia morphology using SEM revealed differences in groupings, shape and taper and occurrence of basal septa with conidiophores, as well as variations in shape, orientation and dimensions of conidia of endophytes from different Hordeum species and accessions. Conidiophores of the H. bogdanii endophyte in PI accession 440413 were densely compacted to form synnemata composed of long, thin-tapering conidiophores that contained a basal septum (Table 3). By contrast, conidiophores of the H. bogdanii endophyte isolated from PI accession 314696 occurred singly and

![Fig. 4: Growth and culture of Neotyphodium-endophytes on 3.5% PDA medium. (A) Culture of endophyte isolated from H. brevisubulatum subsp. violaceum (PI accession 440420) with seed colonies arising from the mother colony at the center of the Petri dish. (B) SEM of convoluted hyphae of H. bogdanii endophyte from PI accession 314696 on the agar surface. (C) SEM of raised synnematal strands of H. bogdanii endophyte from PI accession 269406.](image)

<table>
<thead>
<tr>
<th>Species</th>
<th>Accessions (PI)</th>
<th>Grouping</th>
<th>Shape and taper</th>
<th>Basal septum</th>
<th>Shape</th>
<th>Orientation</th>
<th>Dimensions</th>
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<td>Synnemata</td>
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<td>Ellipsoid</td>
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<td></td>
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<td>Single</td>
<td>Short, thick-tapering</td>
<td>Absent</td>
<td>Ellipsoid</td>
<td>Perpendicular</td>
<td>2.6-4.4 x 1.5-2.4</td>
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<tr>
<td></td>
<td>269406</td>
<td>Single or clusters</td>
<td>Long, slight-tapering</td>
<td>Present</td>
<td>Allantoid</td>
<td>Vertical</td>
<td>4.1-6.9 x 1.1-3.9</td>
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<td>440420</td>
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<td>Short, thick-tapering</td>
<td>Absent</td>
<td>Ellipsoid</td>
<td>Vertical</td>
<td>3.3-5.2 x 1.4-1.8</td>
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<td>401386</td>
<td>Single</td>
<td>Short, thick-tapering</td>
<td>Absent</td>
<td>Ellipsoid</td>
<td>Vertical</td>
<td>2.8-4.3 x 1.0-1.6</td>
</tr>
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</table>

1 Conidiophores occurring singly (ungrouped), loosely clustered, or organized into synnemata (bundles). A single septum was either absent or present at the base of conidiophores. 2 Conidia shape indicates the predominant form for the majority of spores. Conidial orientation is relative to the central axis of conidiophores in scanning electron micrographs. Dimensions (length x width, in μm) are mean values from 50 spores determined using measurements from scanning electron micrographs.
were short and stout, thick-tapering and lacked a basal septum, very similar to the conidiophores of the endophyte from two accessions of *H. brevisubulatum* subsp. *violaceum*.

Conidia shape was ellipsoid for most *Neotyphodium*-endophytes from *Hordeum* species, but conidia of the *H. boganii* endophyte (PI accession 269406) were distinctly allantoid. Orientation of conidia on conidiophores was often vertical in early stages of conidiogenesis, but mature detached spores were usually oriented perpendicular to the apex of the conidiophores. Thus, orientation of conidia on conidiophores may be more a function of conidial age rather than morphological specificity that can be associated with specific endophytes. Conidiogenesis was holoblastic for all strains of *Neotyphodium*-endophytes isolated here from *Hordeum* species. Conidia dimensions varied with endophyte strains isolated from different *Hordeum* species and accessions, but there were considerable overlaps in measurements that may be affected by growth.

**Fig. 5:** Scanning electron micrographs of conidiophores and crescent-shaped conidia of *Neotyphodium*-endophyte from different *H. boganii* PI accessions. (A) Long thin-tapering conidiophores (PI accession 440413), (B) Short thick-tapering conidiophores (PI accession 314696), (C) Immature conidium forming by holoblastic conidiogenesis (PI accession 314696) (D) Two detached ellipsoid conidia with conidiophore-attachment scars (PI accession 440413) (E) Conidiophores with swollen apices and perpendicular conidial orientation (PI accession 269406) (F) Closeup of swollen conidiophore apex and perpendicular conidial orientation (PI accession 269406) (G) Elongated swelling of conidiophore apex with perpendicular conidial orientation (PI accession 269406) (H) Bulbous swelling of conidiophore apex with perpendicular orientation (PI accession 269406) (I) Germinating conidium on a swollen conidiophore apex (PI accession 269406)
conditions and genetic differences in individual strains. Consequently, conidia dimensions were not useful for distinguishing species of *Neotyphodium*-endophytes from wild *Hordeum* species.

The diversity of conidiophore and conidial morphologies found among endophytes isolated from *H. bogdani* accesses were revealed by SEM analyses of conidiogenesis. The long, thin-tapering conidiophores of the endophyte from PI accession 440413 with perpendicular conidial configuration and unswollen conidiophore apices (Fig. 5A) were distinguished from the short thick-tapering conidiophores of the endophyte from PI accession 314696 (Fig. 5B) having conidia formed from swollen conidiophore apices (Fig. 5C). Conidia from the endophyte from PI accession 440413 were broadly ellipsoid (Fig. 5D), whereas the conidia of the endophyte isolated from PI accession 269406 were allantoid to broadly lunate (crescent shaped) and formed on swollen conidiophore apices, either elongated or bulbous (Fig. 5E-H). Conidia occasionally germinated while still on the apex of the conidiophore prior to release (Fig. 5I).

The morphologies of conidiophores and conidia of *Neotyphodium*-endophytes isolated from *H. brevisubulatum* subsp. violaceum also showed differences among strains isolated from different PI accesses. The endophyte from PI accession 440420 formed elliptical conidia on conidiophore apices that were not swollen (Fig. 6A), but the endophyte isolated from PI accession 401386 contained swollen conidiophore apices with slightly curved conidia (Fig. 6B, C and E) and conidiophores usually formed a basal septum (Fig. 6F). The surfaces of most conidia were glabrous (Fig. 6A, D and E), but some conidia had roughened surface architecture (Fig. 6B). Detachment scars usually remained on conidiophores after conidia were released (Fig. 6A and D). Conidia of all endophytes from *H. brevisubulatum* also were formed by holoblastic conidiogenesis.

**DISCUSSION**

The *Hordeum* species surveyed here are representative of germplasm from many different climatic regions, habitat types and land-use areas within
predominantly temperate regions from five continents of the world. The three endophyte-infected cool-season perennial species (H. bogdani, H. brevisubulatum and H. comosum) are all in the tertiary gene pool (von Bothmer et al., 1991). The habitat types and land-use areas from which these endophyte-infected Hordeum species originated were reported previously (Wilson et al., 1991c). Some Neotyphodium-infected Hordeum PI accessions have been maintained in NPGR seed storage for up to 34 years, but others for six or fewer years. Seeds of infected accessions maintained in storage for many years often had reduced endophyte viability compared with accessions stored for much shorter duration (Wilson et al., 1991d). The apparent absence of Neotyphodium-endophytes from tropical regions suggests that there is a requirement for temperate hosts or that environmental conditions are not favorable for growth of endophytic fungi in the warmer regions of the tropics. This might be explained by the sensitivity of Neotyphodium-endophytes within seeds to high temperatures and high moisture content (Welty et al., 1987). Thus, the variability in environmental conditions within areas where endophyte-infected grasses are endemic may also determine the range and variability of endophyte incidence and infection rates in individual grass species and accessions.

The relatively low incidence of CA-endophytes in Hordeum germplasm suggests that the occurrence these endosymbiotic fungi of the genus Neotyphodium are relatively rare in natural populations of wild Hordeum species from which most NPGS collections are made. This relatively low incidence of Neotyphodium-endophytes in wild barley provides evidence that endophyte-infected Hordeum germplasm is potentially very valuable. However, the diversity of Neotyphodium-morphological types in wild Hordeum species suggest that several different endophyte species may be represented in this germplasm and that wild barley may be a good source of endophyte diversity among temperate grasses. The high potential importance of this relatively rare germplasm strongly suggests the need to provide special treatment and care for this germplasm within germplasm banks to assure that it is handled properly to maintain endophyte as well as seed viability. Such special handling might include more frequent seed increases, maintaining low humidity or liquid nitrogen storage conditions and checking for endophyte viability after each seed increase prior to storage. The abundance of world germplasm banks containing CA-endophytes of grasses suggests that a large diversity of these fungi may be available for many beneficial uses in plant-protection applications.

All Neotyphodium endophytes described here produced only intercellular hyphae that did not penetrate through plant cell walls into the tonoplast. This growth habit is typical of true endosymbiotic fungi that do not appear to cause any adverse pathogenic effects on host morphology within the vicinity of endophytic hyphae. Neotyphodium species function as mild parasites which utilize nutrients that leak through cell walls into intercellular spaces of their host. Infection was symptomless with no evidence of disease, hyperplasia or hypertrophy. No adverse effects on the middle lamella were produced causing any type of separation or maceration between plant cells. Neotyphodium hyphae in all cases grew systemically through the tillers and into the floral parts by which new seed infections arose. The absence of pathogenesis associated with these Hordeum grass-endophyte associations indicates that the relationship is more mutualistic than commensalistic or parasitic because both plant and fungus derive benefit from the interaction. The fungal endophyte derives nutrients, an effective means of passive dispersal via infected Hordeum seeds and can remain in the vegetative (somatic) state indefinitely without the need to sporulate or to engage in metabolically-expensive activities such as sporulation or forming a sexual stage. However, some Hordeum endophytes do occasionally sporulate on their host. The H. brevisubulatum subsp. violaceum endophyte from PI accession 440420 was found to sporulate within various leaf tissues of its host (Youssef and Dugan, 2000). Hyphae can continue to grow in the somatic phase as long as it is associated with a grass host. The fungus overwinters in the meristem of the plant and remains viable whether or not tillers and leaves are removed by herbivores.

The first discovery of Neotyphodium-endophytes in wild Hordeum (Wilson et al., 1991b) and Lolium (Wilson et al., 1991d) NPGS germplasm stimulated a considerable amount of new endophyte research because of the great potential possibility and importance of utilizing these mutualistic endosymbiotic fungi as biocontrol agents of insect and disease pests to enhance production yields of cereal crops (Wilson et al., 1991; Wilson, 1996; Clay, 1989, 1990; Clement et al., 2001). Additional surveys of NPGS and other germplasm collections led to discoveries of Neotyphodium-endophytes in other grass species, including some that are wild relatives of crop grasses with potential for hybridizations or genetic crosses with cereal species. Subsequent research has focused on the distribution and sporulation of Neotyphodium-endophytes on their Hordeum hosts (Youssef and Dugan, 2000; Dugan et al., 2002), identifying the biologically active alkaloids.

The discovery of *Neotyphodium*-endophytes in *Hordeum* species is significant for several reasons. *Neotyphodium*-endophytes of *Hordeum* species produce secondary metabolites that are biologically active against plant pests. *Neotyphodium*-infected accessions of *H. bogdani* and *H. brevisubulatum* subsp. *violaceum* have been shown to exhibit significant resistance to several insects including the Russian wheat aphid (*Diuraphis noxia* Mordvilko) (Clement et al., 1997), Rose-grass aphid (*Metopolophium dirhodum* Walker), Bird cherry-oat aphid (*Rhopalosiphum padi* L.) and Hessian fly (*Mayetiola destructor* Say) (Clement et al., 2005). Ergocladine ergot alkaloids (ergovaline, ergosine and ergotamine) and N-formyloxoline (olone alkaloid) have been detected in *Neotyphodium*-infected seeds and forage from *H. bogdani* (PI accession 314696) and *H. brevisubulatum* subsp. *violaceum* (PI accession 440420) (TePaske et al., 1993). Tests with insect bioassays have shown that *Neotyphodium*-endophytes (microbial germplasm within plants) are extra-genomic sources of resistance against numerous insects, providing fortuitous means for controlling major insect and disease pests in grasses that lack genetic resistance (Clay, 1988; Wilson, 1996; Clement et al., 1994). The high level of resistance of *H. bogdani* accessions to *R. padi*, previously attributed to genetic resistance (Weibull, 1988), was more likely due to *Neotyphodium*-infected plants because endophyte-free accessions have little resistance to this aphid (Clement et al., 2005). Seed from these PI accessions eventually may be used in breeding programs, in genetically-modified plants through biotechnological approaches, or by artificial inoculations with CA-endophytes to produce new cultivars of commercial barley or other crop grasses with resistance to a number of insect and disease pests.

The systemic nature of CA-endophyte infections is a characteristic of great useful potential in breeding programs because these specialized fungi are seed-borne. Once the endophyte is introduced by inoculation into the female parent, the symbiotic association becomes self-replicating thereafter and is passed on to the progeny of genetic crosses (Clay, 1989). Unfortunately, interspecific crosses of *H. bogdani* (section *Stenostachys*) and *H. comosum* (section *Critesion*), both diploid species, with *H. vulgare* L. (cultivated barley, section *Hordeum*) have not produced viable plants beyond the seedling stage, but crosses of *H. brevisubulatum* (a diploid, tetraploid, or hexaploid species of section *Stenostachys*) with *H. vulgare* have produced viable adult plants (von Bothmer et al., 1983). Crosses were more successful when *H. vulgare* was used as a pollen donor rather than as the female parent. Another potential benefit of *Neotyphodium*-endophytes in infected wild barley, fescue and ryegrass species is their potential for use in the Conservation Reserve Program (CRP) to significantly reduce the buildup of insect and disease pests in endophyte-infected reservoir plants, particularly wild alternate hosts of agronomic grass pests that are planted on CRP lands (fallow crop fields) between years of crop cultivations. Endophyte-infected CRP grasses growing adjacent to or surrounding grass crops also should significantly reduce the influx of insect pests into these crop fields much like barrier crops.

Endophyte-infected grasses receive many potential benefits from the fungus including increased water use efficiency, improved growth and vigor in associated with endophyte-associated auxin production, tolerance to drought and water stress, increased competitive advantage, resistance or tolerance to herbivory and protection against certain pathogenic fungi, such as *Rhizoctonia cerealis* Van der Hoeven and *Phoma sorghina* (Sacc.) Boerema, Dorenbosch, and Van Kesteren) (White and Cole, 1985), nematodes *Heterodera glycines* Ichinohe and *Meloidogyne incognita* (Kofoid and White) Chit. (Petersen et al., 1988) and viruses (Barley yellow dwarf virus) (Latch et al., 1985). New applications of CA-endophytes for the biocontrol of insects and diseases of turf grasses and other nonpasture situations are being discovered continuously (Clement et al., 1994; Rowan and Latch, 1994; Clay, 1989; Anderson et al. 2006; Clarke et al., 2006).

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**REFERENCES**


