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Control of Clavicipitaceous Anamorphic Endophytes with Fungicides, Aerated Steam and Supercritical Fluid CO_2-Seed Extraction

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Abstract: The effects of soil drenches with systemic fungicides on viability of clavicipitaceous anamorphic endophytes, non-choke inducing endosymbiotic fungi of the genus *Neotyphodium* that systemically infect grasses, were tested in endophyte-infected seedlings of *Hordeum brevisubulatum* subsp. *violaceum*, *Lolium perenne* and *Festuca arundinacea* germplasm. None of the endophytes of these grasses were sensitive to benomyl at 3-5 ppt. The endophyte of *H. brevisubulatum* was sensitive only to propiconazole. The viability of the *F. arundinacea* endophyte (*N. coenophialum*) was reduced, but not completed eliminated by prochloraz, imazalil and propiconazole in the 2-3 ppt range. Aerated-steam seed treatments at 60 C for 5 min were effective in reducing viability of the perennial ryegrass endophyte (*N. lolii*) by 83%, although some reduction in seed germination and negative growth effects were observed. A novel disease-control method, supercritical fluid carbon dioxide extraction (SFE-CO_2), for the elimination of *Neotyphodium*-endophytes in grass seeds is reported here for the first time. The endophyte *N. lolii* was completely controlled in seeds of *L. perenne* cv. Ellett by extracting the seeds at 400 atmospheres for 10 or 20 min. Only minor reductions in seed germination resulted from the SFE-CO_2 seed-extraction treatment and no appreciable effects on growth of seedlings or mature plants were observed. Several potential applications of these disease-control technologies for seed-borne fungal pathogens are discussed.

Key words: Aerated steam, chemical control, clavicipitaceae, seed extraction, wild barley

INTRODUCTION

Clavicipitaceous endophytes are biologically and ecologically important fungi that form various trophic interactions, ranging from parasitic to symbiotic associations, with numerous temperate grasses of the Poaceae world-wide (Clay, 1988, 1990, 1997; Spyreas *et al*., 2001; Rudgers *et al*., 2004; Malinowski and Belesky, 2006; Wilson, 2007). Clavicipitaceous anamorphic endophytes (CA-endophytes), are imperfect forms (lacking a sexual stage) that develop usually mutualistic, but occasionally commensalistic symbioses (rarely parasitic) with their grass hosts (Wilson, 1996, 2007; Faeth, 2002). CA-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 seed-transmitted fungi that produce mycotoxins responsible for causing serious toxicoses of livestock (cattle, sheep and horses) that consume endophyte-infected forage grasses (Bacon *et al*., 1977; Fletcher and Havey, 1981; Siegel *et al*., 1985, 1987; Clay, 1992; Wilson *et al*., 1992). These fungi also confer beneficial effects to their grass hosts such as resistance to a wide variety of insect and disease pests (Clay, 1989; Schmidt, 1990; Clement *et al*., 1994; Rowan and Latch, 1994; Anderson *et al*., 2006), tolerance to drought stress (Ravel *et al*., 1997; Hesse *et al*., 2004), increased growth rate and competitive ability (Clay, 1994) and improved physiological efficiencies (Siegel *et al*., 1987; Lewis *et al*., 1996). The beneficial attributes expressed in endophyte-infected grass cultivars have been utilized particularly in the turf-grass and more recently, forage-grass industries (Gwinn and Gavin, 1992; Greulich *et al*., 1999; Hill *et al*., 2002; Rolston *et al*., 2002; Bouton and Hopkins, 2003; Clarke *et al*., 2006). The toxic metabolites responsible for causing syndromes in livestock and insect resistance are ergot and related alkaloids (clavine, lysergic acid and ergopeptine) (Lyons *et al*., 1986; Yates and Powell, 1988),

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loline (pyrrolizidine) alkaloids (Yates *et al*., 1990; Siegel *et al*., 1990; Bush *et al*., 1993), lolitrems (Miles *et al*., 1992, 1993) and peramine (pyrrolopyrazine) alkaloids (Siegel *et al*., 1990; Rowan *et al*., 1986).

Neotyphodium-endophytes are economically important because of their variable effects on invertebrate and mammalian herbivores, plant growth and their ability to modify the expression of agronomic plant traits. Consequently, the presence of these fungi often make it difficult to evaluate and distinguish between the agronomic traits of endophyte-infected grasses that may be attributed to plant genomic expression from those expressed as a result of the modifying effects of the endophyte. It is usually necessary to established endophyte-free cultivars of new grass varieties in order to evaluate their true agronomic values and characteristics in the absence of any endophytic symbionts. For research purposes of experimental design, the production of endophyte-infected and endophyte-free clonal lines of each grass variety is most useful for controlling the effects of genotype variation in both the plant and endophyte.

The beneficial effects of endophyte infections in certain European grasses have not been obvious because plant responses varied with the genotypes of the plant-endophyte combination (Lewis, 2004). Endophyte infected grasses are considered to be of low benefit in some European countries because of the absence of pest resistance and because cases of livestock toxicoses are only occasionally observed (Raynal, 1991; Bony *et al*., 1998; Benkhelil *et al*., 2004). For example, the presence of *Neotyphodium*-endophytes in some indigenous grasses of Europe do not appear to deter herbivory of major insect pests, such as the frit fly (*Oscinella* sp.) and leatherjackets (*Tipula* sp.), of northern European grasses (Lewis and Vaughan, 1997). In France, legal regulations require that varieties submitted for official registry may be evaluated for agronomic values only when the grass variety has less than 20% endophyte infection, owing to existing risks of toxicoses and low benefits for controlling insect pests (Leyronas *et al*., 2006). All of these cases indicate the need to develop new methods for producing grass germplasm with reduced levels of *Neotyphodium*-endophyte infections and endophyte-free lines for various applications.

Most research studies involved in the development of methods for the control of *Neotyphodium*-endophytes have utilized seed treatments with various fungicides. Fungicide treatments applied to foliage of mature field plants have been largely ineffective in controlling these fungi (Latch and Christensen, 1988; Dernoeden *et al*., 1990), although soaking treatments of seedlings with benomyl, triforine and thiophanate methyl have proven effective in eliminating certain *Neotyphodium*-endophytes in specific grasses (Saiga *et al*., 2003). Seed treatments have proved to be the best approach for reducing endophyte infections of grass germplasm because these fungi are seed borne and require seed dissemination and transmission in order to propagate inoculum of the fungus from plant to plant to subsequent generations. The relatively small amount of endophyte inoculum found in predominantly aleurone tissue of grass seeds, compared with augmented amounts in grass tillers, offers an advantage for the control of seed inoculum, but also runs the risk of reducing seed viability if treatments are too rigorous. The fungicides found most effective in seed treatments against *Neotyphodium*-endophytes have been specific to particular grass-endophyte combinations. The tall fescue endophyte, *N. coenophialum* Morgan-Jones and Gams, was most sensitive to various triazole fungicides (Williams *et al*., 1984; Bilotti *et al*., 1989; Maddaloni *et al*., 1989), whereas the ryegrass endophyte, *N. lolii* Latch, Christensen and Samuels, was best controlled with prochloraz and propiconazole (Harvey *et al*., 1982; Latch and Christensen, 1982). All of these fungicide treatments may cause various types and levels of phytotoxic effects at variable dosages, most commonly including chlorosis, leaf distortion and stunting.

The objectives of this research were to determine the efficacy of seed treatments, including four classes of fungicides, aerated-steam and supercritical fluid CO_2-extraction, in controlling or reducing *Neotyphodium*-endophyte viability in accessions of wild barley (*Hordeum brevisubulatum* subsp. *violaceum* (Trin.) Link, perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinaceae* Schreber) and to determine the effects of these treatments on plant growth responses. The adverse effects of these control treatments on CA-endophytes in seedlings of wild barley (*Neotyphodium* sp.), *N. lolii* of perennial ryegrass and *N. coenophialum* of tall fescue also were assessed to determine the variability of resistance or tolerance to specific control treatments.

MATERIALS AND METHODS

Seed source: Seed samples of wild barley (*Hordeum brevisubulatum* subsp. *violaceum*) PI accession 440420 and perennial ryegrass (*Lolium perenne* cv. Ellett) PI accession 462339 were obtained from the USDA-ARS, National Small Grains Collection (NSGC) Research Facility in Aberdeen, Idaho; part of the US National Plant Germplasm System (NPGS). Seeds of tall fescue (*Festuca arundinacea* cv. Tribute) were obtained from Oregon

State University, Department of Plant Pathology. Seeds were stored previously at 4-5°C and 30-35% relative humidity prior to this study.

Endophyte seed and seedling assays: Seeds were examined microscopically for CA-endophytes of the genus *Neotyphodium*, specific endosymbiotic fungi of grasses as defined by Wilson (1996). 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*., 1991). 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 per accessions. Seedlings of each accession were grown in a greenhouse under natural light in 15.2 cm pots containing 55% peatmoss, 35% pumice and 10% sand. Leaf sheaths from 50-100 7-9 week-old seedlings were examined microscopically at 400x to determine endophyte viability prior to treatments.

Fungicide tests: The effects of representative fungicides from four chemical classes (benzimidazoles, pyrimidines, imidazoles and triazoles) on *Neotyphodium*-endophyte viability were tested using soil-drench treatments at concentrations ranging from 2-4 parts per thousand (ppt) active ingredient. The fungicides tested included benomyl (Benlate, 50% WP), fenarimol (Rubigan, 12% EC), prochloraz (Sportak, 50 WP), imazalil (Fungiflor, 100 SL) and propiconazole (Tilt, 43.2% EC). Seven week-old 10 cm seedlings of wild barley (*H. brevisubulatum* subsp. violaceum) PI accession 440420, perennial ryegrass (*L. perenne* cv. Ellett) and tall fescue (*F. arundinaceae* cv. Tribute) were transplanted into separate 25.5×34×8 cm galvanized metal flats at appropriate spacing to obtain approximately 56 plants per flat. A minimum of 15-20 replicate plants were prepared per treatment. Plants were maintained in a greenhouse at a diurnal temperature of 20±5°C throughout the duration of the study. Two weeks after transplanting, diluted fungicide solutions of each treatment were applied to soil until runoff (complete saturation) in separate flats with drain holes. Not all combinations of fungicide rates and plant species were tested. Plants were not watered for 5 days following fungicide applications, but thereafter plants were watered to saturation twice weekly until the study was completed. In addition to effects on endophyte viability, fungicide effects on plant height and leaf dry weight were determined and compared with untreated controls. Fungicide phytotoxic effects causing chlorosis were determined by measuring leaf total chlorophyll (a+b) content (μmoles g^{-1} dry wt.) for the wild barley treatments only. All above-ground leaf parts were cut up, extracted with N,N-dimethyl formamide and total chlorophyll content measured using a Beckman DU650 spectrophotometer (647 and 664.5 nm wavelengths) according to methods modified from Inskeep and Bloom (1985).

Aerated-steam seed tests: Seeds of *L. perenne* cv. Ellett (PI accession 462339) with 100% viable endophyte infection (*Neotyphodium lolii*) were prepared in 0.5 g aliquots that were placed into 7.0 cm^2 plastic mesh screen packets. All seeds were hydrated by pretreatment in a 100% relative humidity glass-jar for 12 h at 21-22°C prior to aerated-steam treatments. Preconditioned seeds in screen packets were placed individually into a sterilization chamber fed with aerated steam from a SG10 steam generator and blower apparatus (Siebring Manufacturing, George, IA 51237) at temperatures and times of either 56°C for 5, 10, or 15 min 58°C for 5 or 10 min and 60°C at 3 or 5 min. Immediately after aerated-steam treatments, seed packets were immersed in 15°C sterile water to cool 2-3 min. Seeds were then plated onto five stacked No. 1 Whatman filter papers moistened with distilled water in separate 9.0 cm Petri dishes for each treatment. Seed germination counts were taken 12 days after plating and counted at 7 day intervals thereafter until no new germination was observed. Germinated seedlings were transplanted into potting soil in 25.5×34×8 cm galvanized metal flats and the endophyte-infection status was determined after 8 weeks growth by visual examinations of stained leaf sheaths as described by Wilson *et al.* (1991). Aerated-steam seed treatment effects on plant height, leaf dry weight and plant mortality after germination also were determined.

Supercritical fluid CO_2-seed extraction tests: Separate aliquots of fifty *L. perenne* cv. Ellett seeds were placed into the 5 mL extraction chamber of an online model 601 SFE-supercritical fluid chromatograph (Lee Scientific, Salt Lake City, UT) and critical point CO_2-extracted for either 10, 15, or 20 min at 100 to 400 atmospheres (ATMs) of pressure and 21°C during routine chromatographic runs for detection of ergot and related alkaloids. Seeds treated with supercritical fluid CO_2-extraction (SFE-CO_2) and controls were then planted and grown in a greenhouse under natural light in 15.2 cm pots containing 55% peatmoss, 35% pumice and 10% sand. Leaf sheaths from 50-100 7-9 week-old seedlings were examined microscopically at 400x to determine endophyte viability

and relative abundance of growth within leaf sheaths following treatments. Seed germination and growth following SFE-treatments also were recorded to determine the effects of treatments on seed viability.

RESULTS

Endophyte seed and seedling assays: The viability of endophytic mycelium in aleurone seed tissue of each grass species was confirmed by direct examinations of *Neotyphodium* endophytic hyphae within leaf sheath tissue of 50-100 7-9 week-old seedlings. In all cases, light microscopy revealed new hyphal growth of endophytes in leaf sheaf tissue that originated from inoculum in aleurone seed tissue. Hyphal growth of *Neotyphodium*-endophytes found in the intercellular spaces of leaf sheaf tissue occurred parallel to the longitudinal axis of plant cells and appeared relatively thin (usually <3 μm in diameter), straight with few convolutions and rarely branching. Septa stained poorly or not at all. *Neotyphodium*-endophyte viability observed in control seedlings derived from pretreated seed was 100% in *Hordeum brevisubulatum* subsp. *violaceum*, 98.7-100.0% in *Lolium perenne* cv. Ellett and 44.6% in *Festuca arundinacea* cv. Tribute. *Neotyphodium*-endophyte viabilities determined for seedlings derived from post-treated seeds are reported individually in the following sections of each respective seed-treatment experiment.

Fungicide tests: Fungicide-treatment effects on *Neotyphodium*-endophyte viability in *H. brevisubulatum* subsp. *violaceum* and on plant responses were highly significant ($p<0.0001$). However, fungicide soil-drench treatments, applied at concentrations ranges of 3-5 ppt to *H. brevisubulatum* subsp. violaceum seedlings, were mostly ineffective in reducing *Neotyphodium*-endophyte viability relative to controls (Table 1). Only minor control of 13% reduction in endophyte viability occurred with imazalil at 4 ppt, but no control occurred at 3 ppt. However, propiconazole was extremely effective at 2 and 3 ppt in killing endophyte inoculum in the seedlings. This 100% control was achieved without killing seedlings, although significant phytotoxicity was observed with this treatment.

The effects of fungicide treatments on plant growth and physiological responses generally were not correlated with effects on the endophyte. Plant response varied considerably with type and concentration of fungicide treatments. Significant reductions in plant height (stunting) occurred in association with all fungicide treatments except for imazalil at 3 and 4 ppt and for benomyl and prochloraz at 3 ppt (Table 1). Stunting was most prominent in all fenarimol and propiconazole treatments. Drenching treatments had no effect on plant dry weight for benomyl, prochloraz and imazalil at 3 ppt, but all other fungicide drenches significantly reduced plant dry weight relative to controls. Seedling dry weight was most significantly reduced in those exposed to fenarimol and propiconazole treatments at all concentrations and those receiving benomyl drenches at 4 ppt. Overall, propiconazole at 3 ppt had the greatest combined effect in reducing plant height and dry weight of *H. brevisubulatum* seedlings. The 3 ppt benomyl treatment had no post-treatment effect on total chlorophyll content of leaves, although the same fungicide at 4 ppt did reduce total chlorophyll content (Table 1). Fenarimol treatments at all concentrations tested caused the high levels of chlorosis and the greatest reduction in leaf total chlorophyll content. Prochloraz treatments at 3 and 4 ppt also cause significant leaf chlorosis and reduction in total chlorophyll content. However, imazalil and propiconazole treatments caused only minor chlorosis and reductions in leaf chlorophyll content.

Fungicide-treatment effects on *Neotyphodium*-endophyte viability in *L. perenne* and *F. arundinacea* and on plant responses were highly significant ($p<0.001$). Benomyl drenches at 3-5 ppt had no significant effect on *N. lolii* endophyte viability, plant height, or dry weight in *L. perenne* cv. Ellett (Table 2). Similar results were observed with benomyl at 3 ppt for the endophyte in *F. arundinacea* cv. Tribune. However, *N. coenophialum* viability in *F. arundinacea* was significantly reduced by propiconazole at 2 ppt and imazalil and prochloraz at 3 ppt and plant heights and dry weights also were significantly reduced by these treatments. The effects of imazalil, prochloraz and propiconazole on endophyte viability and plant responses were not measurable due to very high levels of phytotoxicity and mortality resulting from these treatments that precluded acquisition of sufficient data for analysis.

Aerated-steam seed tests: The effects of aerated-steam seed treatments on *Neotyphodium*-endophyte viability in *L. perenne* and on plant responses were highly significant ($p<0.0001$). Aerated-steam treatments of *L. perenne* cv. Ellett seeds with gradual increases in temperature and time of exposure reduced *Neotyphodium*-endophyte viability in proportion to the severity of the treatment (Table 3). Nevertheless, endophyte viability was significantly reduced when aerated steam was applied at 58°C for 10 min. Endophyte viability dropped off rapidly as the temperature was increased to 60° and the duration of exposure of 3 to 5 min.

Table 1: Effects of fungicide drenches on viability of *Neotyphodium*-endophyte and plant responses of *Hordeum brevisubulatum* subsp. *violaceum*

			Plant responses[3]		
Fungicide[1] treatment	Rate (ppt)	Endophyte[2] viability (%)	Plant height (cm)	Dry weight (g)	Total chlorophyll (μmoles g^{-1} dry wt.)
Control		100^{a}	12.4^{b}	1.3^{ab}	4.0^{ab}
Benomyl	3	100^{a}	13.3^{ab}	1.4^{a}	4.3^{a}
	4	100^{a}	9.8^{c}	0.6^{de}	3.3^{bcd}
Prochloraz	3	100^{a}	13.0^{ab}	1.4^{a}	2.6^{d}
	4	100^{a}	9.2^{cd}	0.8^{cd}	1.8^{e}
Fenarimol	3	100^{a}	8.1^{def}	0.7^{cde}	1.6^{e}
	4	100^{a}	7.7^{ef}	0.8^{cd}	1.5^{e}
	5	100^{a}	6.7^{f}	0.4^{ef}	1.6^{e}
Imazalil	3	100^{a}	14.2^{a}	1.2^{ab}	3.6^{abc}
	4	87^{b}	13.2^{ab}	1.0^{bc}	3.1^{cd}
Propiconazole	2	0^{c}	6.8^{f}	0.5^{de}	3.6^{abc}
	3	0^{c}	9.1^{cde}	0.2^{f}	3.6^{abc}

[1]Fungicide soil-drench treatments, at the indicated rates in parts per thousand (ppt) active ingredient, were tested on the *Neotyphodium*-endophyte of *Hordeum brevisubulatum* subsp. *violaceum* PI accession 440420 derived from the USDA-ARS, National Small Grains Collection (NSGC). [2]Endophyte-viability percentages (%) followed by different letter(s) were significantly different at (p = 0.001), according to Fisher's protected LSD tests following arcsin transformations.[3]Plant response values followed by different letter(s) were significantly different according to Fisher's protected LSD tests (p = 0.0001)

Table 2: Effects of fungicide drenches on viability of *Neotyphodium*-endophytes and plant responses of *Lolium perenne* cv. Ellett and *Festuca arundinacea* cv. Tribute

		L. perenne[2]			*F. arundinacea*[3]		
Fungicide[1] treatment	Rate (ppt)	Endophyte viability (%)	Plant ht. (cm)	Dry wt. (g)	Endophyte viability (%)	Plant ht. (cm)	Dry wt. (g)
Control		98.7^{a}	18.3^{a}	2.2^{a}	44.6^{a}	12.7^{a}	0.4^{a}
Benomyl	3	100.0^{a}	16.6^{a}	1.9^{a}	40.0^{a}	13.8^{a}	0.4^{a}
	4	100.0^{a}	17.0^{a}	1.7^{a}	–	–	–
	5	95.2^{a}	16.6^{a}	1.8^{a}	–	–	–
Imazalil	3	–	–	–	10.7^{b}	13.4^{a}	0.4^{a}
Prochloraz	3	–	–	–	8.9^{b}	9.8^{b}	0.2^{c}
Propiconazole	2	–	–	–	14.3^{b}	10.3^{b}	0.3^{b}

[1]Fungicide soil-drench treatments, at the indicated rates in parts per thousand (ppt) active ingredient, were tested on the *N. lolii* endophyte of *Lolium perenne* cv. Ellett (PI accession 462339) derived from the USDA-ARS, National Small Grains Collection (NSGC) and on the *N. coenophialum* endophyte of *Festuca arundinacea* cv. Tribute obtained from Oregon State University. [2]Height and weight measures followed by the same letter were not significantly different (p>0.10) according to Fisher's protected LSD tests. [3]Endophyte-viability percentages (%) and plant response values followed by different letter(s) were significantly different according to Fisher's protected LSD tests (p = 0.0001)

Table 3: Effects of aerated-steam seed treatments on viability of *Neotyphodium lolii* and plant responses of *Lolium perenne* cv. Ellett

			Plant responses[3]				
			Seed germination delay				
Treatment[1] (°C)	Min	Endophyte[2] viability (%)	(Days)	(%)	Plant height (cm)	Dry weight (g)	Mortality[4] (%)
Control		100.0^{a}	0	100^{a}	30.8^{a}	0.45^{a}	0.0
56	5	98.2^{a}	0	96^{a}	29.4^{ab}	0.38^{ab}	0.0
	10	100.0^{a}	0	97^{a}	29.6^{ab}	0.40^{ab}	0.0
	15	92.9^{a}	0	94^{ab}	28.8^{ab}	0.35^{ab}	0.0
58	5	92.9^{a}	0	90^{b}	28.0^{b}	0.38^{ab}	0.0
	10	73.2^{b}	40	89^{b}	26.0^{c}	0.33^{b}	0.0
60	3	62.5^{b}	40	86^{b}	24.0^{c}	0.32^{b}	14.3
	5	16.7^{c}	40	64^{c}	25.6^{c}	0.40^{ab}	35.7

[1]Aerated-steam treatments were tested on seeds of *Lolium perenne* cv. Ellett PI accession. [2]Endophyte-viability percentages (%) followed by different letter(s) were significantly different at (p = 0.001), according to Fisher's protected LSD tests following arcsin transformations.[3]Plant response values followed by different letters were significantly different at (p = 0.0001) for plant height and (p=0.05) for dry weight, according to Fisher's protected LSD tests. Delay in seed germination indicates the number of days that it took for all viable seeds to germinate following the first 12 days of observation after aerated-steam seed treatments.[4]Percent mortality of maturing plants following successful seed germination

Seed germination began to decrease significantly to 90% for aerated-steam treatments of 58° for 5 min and was reduced to only 64% when treated with aerated steam at 60° for 5 min. Seed germination was also delayed for about 40 days by aerated-steam treatments of 58° for 10 min and more rigorous treatments. Seedling heights were significantly reduced by increasing exposure of aerated-steam applied to seeds at 58° for 5 min and at longer exposures and greater temperature. Dry weights of seedlings were reduced only by aerated-steam treatments for 58° for 10 min and 60° for 3 min. Mortality of maturing plants that survived to the 5 week seedling stage after

Table 4: Effects of supercritical fluid CO_2-extraction of seeds on viability of *Neotyphodium lolii* and plant responses of *Lolium perenne* cv. Ellett

Seed treatment[1]		Endophyte[3]		Plant responses[4]	
ATMs[2]	Min	Viability (%)	Growth	Seed germination (%)	Mortality (%)
Control		100[a]	A	100[a]	0
100	10	98[a]	A	98[a]	0
	15	98[a]	M	98[a]	0
	20	96[a]	M	98[a]	0
200	10	94[a]	M	96[a]	0
	15	74[b]	M	96[ab]	0
	20	62[bc]	L	94[ab]	0
300	10	48[c]	L	94[ab]	0
	15	34[cd]	L	92[bc]	0
	20	10[d]	T	88[c]	0
400	10	0[e]	–	86[c]	0
	20	0[e]	–	84[c]	0

[1]Supercritical fluid CO_2-extractions for each treatment were tested using 50 seeds of *Lolium perenne* cv. Ellett PI accession 462339 derived from the USDA-ARS, National Small Grains Collection (NSGC). [2]Seeds were extracted with SFE-CO_2 at 100 to 400 atmospheres (ATMs) of pressure at 21°C for the indicated treatment times (min). [3]Growth of endophytic hyphae within seedling leaf sheaths were scored according to the following scale: A = Abundant, more than 6 hyphae found in intercellular spaces between leaf sheath cells; M = Moderate, 4-6 hyphae found in leaf sheath intercellular spaces; L = 1-3 hyphae found in intercellular spaces; T = No more than one hypha found in an intercellular space of leaf sheaths. Endophyte-viability percentages (%) followed by different letters were significantly different according to Fisher's protected LSD tests ($p = 0.0001$). [4]Percent mortality of maturing plants following successful seed germination. Seed germination percentages (%) followed by different letter(s) were significantly different according to Fisher's protected LSD tests ($p = 0.0001$)

germination was observed only with aerated-steam treatments of seeds at 60°. Mortality was greater in seeds exposed to aerated-steam for 5 min than for 3 min at this temperature.

Supercritical fluid CO_2-seed extraction tests: The effects of SFC-CO_2-seed extraction treatments on *Neotyphodium*-endophyte viability in *L. perenne* and on plant responses were highly significant ($p<0.0001$). Extractions of perennial ryegrass seeds using supercritical fluid CO_2 at high pressures caused differential effects on the viability of the *N. lolii* endophyte compared to effects on seed viability and germination. Endophyte viability was not significantly reduced until extraction pressures of at least 200 atmospheres were performed for a minimum of 15 min (Table 4). Under these extraction conditions, viability of *N. lolii* was reduced by 26% and only moderate numbers of hyphal strands were observed in leaf sheafs following treatment. Higher pressures and duration of extractions continued to decrease endophyte viability at an accelerated rate up to 400 atmospheres where no endophyte viability remained after treatment at this pressure for 10 and 20 min.

The effects of SFE-CO_2 seed extraction on plant responses were not as severe and much more gradual with increasingly rigorous seed-extraction conditions. Seed germination was not significantly reduced in seed extractions of <300 atmospheres, but seed germination was reduced when seeds were exposed to extractions of at least 300 atmospheres for 15 min. Under the most rigorous extraction conditions of 400 atmospheres for 20 min, seed germination was reduced by only 16% (Table 4). Seedlings derived from extracted seed did not exhibit any indications of retarded growth (stunting), chlorosis, or any other adverse growth effects from the extraction process. Also, none of the plants, derived from germinated seeds that survived to maturity, showed any apparent mortality associated with the seed-extraction treatments. Thus, seed viability was only minimally affected by SFE-CO_2 extractions under the conditions tested in this study. Vegetative growth in seedlings appeared normal and healthy for all SFE-CO_2 extraction treatments.

DISCUSSION

The abundant needs and requirements of scientists and agronomic producers to develop endophyte-free grass cultivars from *Neotyphodium* endophyte-infected varieties, for various tests and applications, has prompted new investigations focused on determining the best methods and approaches to achieve this without causing appreciable mortality in parent plants, significant reductions in seed viability, or adverse growth effects in progeny plants. The evaluation of fungicides from different chemical classes, previously found to have some success in controlling *Neotyphodium*-endophytes via seed-treatments of various *Festuca* and *Lolium* species (Saiga *et al*., 2003), were evaluated here with *Neotyphodium*-endophytes in seedlings of similar grasses and a *Hordeum* species from the NSGC. The *Neotyphodium*-endophyte in seedlings of *H. brevisubulatum* subsp. *violaceum* (PI accession 440420), collected in Kazakhstan in 1978 (Wilson, 2007), was not sensitive to benomyl, prochloraz, or fenarimol at the 3-5 ppt range and only marginally sensitive to imazalil. However, this endophyte was highly sensitive to propiconazole and was totally eliminated from leaf sheaths

and other vegetative parts of seedlings. These results differed from those of Saiga *et al.* (2003) who showed that benomyl soakings of ryegrass seedlings harboring *N. lolii* and tall fescue seedlings harboring *N. coenophialum* were very effective in eliminating these endophytes from young plants. The differential sensitivity of various *Neotyphodium*-endophytes to fungicides suggest that there are different metabolic mechanisms present in different *Neotyphodium* species and even strains, that have different levels of effectiveness in detoxifying these materials. Many fungicides have been shown to be ineffective in controlling these fungi when applied to plants (Dernoeden *et al.*, 1990; Latch and Christensen, 1988). Thus, seed treatments with fungicides generally are considered more effective than applications to live plants for controlling *Neotyphodium*-endophytes (Williams *et al.*, 1984; Bilotti *et al.*, 1989; Maddaloni *et al.*, 1989; Leyronas *et al.* 2006).

Similar fungicide-drench tests for activity against *N. lolii* in *L. perenne* cv. Ellett (PI accession 462339), collected in New Zealand in 1981 (Wilson *et al.*, 1991), also indicated that this ryegrass endophyte was not sensitive to benomyl within the plant. The cultivar Ellett has a long history of commercial selection in New Zealand. It was selected for its high level of vigor and resistance to the Argentine stem weevil, *Listronotus bonariensis* (Kuschel) and high rate of endophyte infection (99%) which helped reduce grazing pressure in a 60-year-old Auckland pasture of the Mangere District. Many other endophyte-free cultivars have succumbed to *L. bonariensis* attack during field selection in New Zealand (Barker *et al.*, 1985). Similar results were observed with *N. coenophialum* in *F. arundinacea* cv. Tribune which had little sensitivity to benomyl drenches. However, the fungicides imazalil, prochloraz and propiconazole significantly reduced viability of *N. coenophialum* up to 32% in this fescue at concentrations of 2-3 ppt, compared with controls, although there were minor reductions in plant height and dry weight suggesting some phytotoxicity to these fungicides. These results indicate that there are some exceptions to fungicidal activity against certain *Neotyphodium* species in specific hosts and that fungicide activity must be assessed on a case-by-case basis relative to any endophyte-host combination. Thus, it cannot be assumed that just because a certain fungicide has not had activity against a given *Neotyphodium*-endophyte in a certain cultivar, that the fungicide could not have activity against the same endophyte species in a different plant cultivar or host species. The corollary case must also be stated: that a fungicide found to be effective against a specific endophyte species in certain host species or cultivars, does not necessarily have the same activity or effectiveness against the same endophyte species in a different host species or cultivar.

The data presented here has demonstrated the feasibility of controlling *Neotyphodium*-endophytes in seeds of various grass hosts using aerated steam. Endophyte viability of *N. lolii* was reduced up to 37% without appreciable reductions in seed viability or seedling growth. However, 83% reduction in endophyte viability was achieved using aerated steam only at 60°C for 5 min, but significant reduction in seed germination resulted from this treatment. The more rigorous treatments also delayed seed germination. Although the *N. lolii* endophyte in *L. perenne* cultivar Ellett was not completely controlled with aerated-steam treatments of seed, the treatment provided a means for effectively producing a large proportion of seedlings that are endophyte-free. Subsequent vegetative propagation and seed production from these endophyte-free progeny provided large numbers of endophyte-free plants of this cultivar. Once endophyte-free lines of a cultivar were generated and confirmed, all subsequent progeny produced from seed increases within the germplasm facilities remained uninfected. Thus, both endophyte-infected and endophyte-free lines of each cultivar may be maintained indefinitely and stored separately in germplasm storage facilities (Wilson, 1996, 2007).

The use of aerated-steam seed treatments for control of *Neotyphodium*-endophytes in grasses has not been reported previously, but this control method has been used at various levels of success with different seed-borne pathogens in other plant hosts. Titone *et al.* (2003) compared fungicide seed treatments to aerated-steam treatments (64 or 72°C for 5 min and 67 or 75°C for 2 min) for the control of Bakanae disease of rice, caused by *Gibberella fujikuroi* (Sawada) Ito in Ito and K. Kimura. All treatments yielded good control of the pathogen with aerated steam and carbendazim treatments, providing an average disease reduction of 98%. Efficacy was demonstrated for aerated-steam thermal treatments and chemical treatments with carbendazim, mancozeb, iprodione + propiconazole and carboxin + thiram, whereas fludioxonil did not control *G. fujikuroi*. A month after treatment, germinability was not reduced, but 3 and 6 months later a progressive decrease in germinability was observed following treatment at 72°C for 5 min. Carboxin + thiram caused a higher percentage of abnormal seedlings compared to the control. In the field, the treatment at 72°C for 5 min reduced the number of plants compared to the control. Treatment of infected lentil seed with aerated steam at 45-75°C for 30 min reduced inoculum levels of *Ascochyta lentis* Vassiljevsky in seeds, but did

not totally control this seed-borne pathogen (Kaiser and Hannan, 1987). Forsberg *et al.* (2005) found the efficacy of aerated steam treatments for the decontamination of cereal grains from seed-borne diseases, such as seed-borne *Fusarium* sp. and *Tilletia caries* (DC.) Tul. and C. Tul. in wheat, *Drechslera teres* (Sacc.) Shoemaker, *D. graminea* (Rabenh.) Shoemaker and *Bipolaris sorokiniana* (Sacc.) Shoemaker in barley and for *D. avenae* (Eidam) Scharif and *Ustilago avenae* (Pers.) Rostr. in oats, was equivalent to chemical seed dressing. By contrast, aerated steam was not as effective as fungicides for the control of *Ustilago tritici* (Pers.) Rostr. (= *U. nuda*) in barley. They concluded that aerated steam treatments had the potential of being a competitive alternative to chemical seed dressings for the control of seed-borne diseases in cereal grasses.

The novel testing of SFE-CO_2 seed extraction to control seed-borne inoculum of *Neotyphodium*-endophytes is reported here for the first time. The decline in *N. lolii* viability within seeds of *L. perenne* cv. Ellett occurred inversely proportional to increasing atmospheres and duration of extraction, but without appreciable effects on seed viability or plant growth. Total control of seed-borne endophyte inoculum was obtained at 400 atmospheres for 10 or 20 min. The potential effectiveness of the SFE-CO_2 extraction method for controlling many other seed-borne pathogens warrants further investigation because of its operational safeness, selective or target-specific effects on seed-borne pathogens (achieved without appreciable damage to seeds) and the innocuous nature of this method. However, commercialization of this method will require the design and construction of large-scale extraction equipment to achieve the necessary large-volume throughput required for routine disinfestations and decontaminations of seeds in bulk. This decontamination method also offers promise for the potential control not only of internal seed-borne pathogens, but also external contaminants carried outside of the seed coat. Thus, some other related uses, such as the disinfesting of cereal grains used for seed planting or for food, are also within the range of possible applications. The latter application would be particularly useful in such cases as carnal bunt (*Tilletia cares*) infestations of wheat that preclude the purchase and importation of bunt-contaminated grain into certain countries having regulatory trade embargoes, designed to exclude nonnative (exotic) disease pests.

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