Fungal endophytes of wild barley and their effects on *Diuraphis noxia* population development

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Abstract

Laboratory experiments were conducted to compare the expression of *Diuruphis noxia* (Mordvilko) (Homoptera: Aphididae) resistance in four plant introduction (PI) lines of wild barley *(Hordeum)* infected with different species or strains of endophytic fungi (tribe Balansieae, family Clavicipitaceae, *Neotyphodium* gen. nov. [formerly *Acremonium]).* Aphid densities were significantly lower on endophyte-infected plants of PI *3* 14696 *(H. bogdanii* Wilensky) and PI *440420 (H. brevisubulatum* subsp. *violaceum* (Boissier & Hohenacker)), compared with densities on endophyte-free plants of both PI lines in population growth experiments. This endophyte- associated resistance was the result of antibiosis effects or starvation. In other experiments, endophyte-free plants of PI 269406 and PI *440413 (H. bogdunii)* were not superior to endophyte-infected conspecifics as host plants of *D. noxia.* Our results demonstrate the influence of host plant species/genotype and endophyte species/strain on expression of aphid resistance, provide an explanation of the high levels of *D. noxia* resistance in PI 314696 and PI 440420 previously reported in the literature, and underscore the potential importance of endophytic fungi in conferring insect resistance in wild barley.

Introduction

Variation in plant quality for insect herbivores is well recognized (Fritz & Simms, 1992). This variation results from several factors such as plant chemistry and morphology based defense mechanisms, the environment in which the plant grows, dispersion among individual plants, and aspects of insect behavior, dispersion, and abundance (Denno & McClure, 1983). Microbial associates of plants also influence plant suitability for insect herbivores. For example, a defensive symbiosis between clavicipitaceous endophytic fungi *(Neotyphodium* Glen, Bacon & Haulin gen. nov. [formerly *Acremonium]*; Glen et al., 1996) and graminoid host plants is responsible for enhanced plant resistance to insect and mammalian herbivores (Joost &

Quisenberry, 1993; Bacon & White, 1994). Livestock toxicities resulting from grazing on endophyte-infected tall fescue *(Festuca arundinacea* Schreb.) and perennial ryegrass *(Lolium perenne* L.) provided the original impetus for research on this relationship (Bacon et al., 1977; Hoveland, 1993). Since the first reports linking *Neotyphodium (Acremonium)* endophytes in grasses to enhanced insect resistance appeared in the literature in the early 1980s, researchers have contributed a large body of information toward improving our understanding of endophyte-mediated resistance in grasses to phytophagous insects (Breen, 1994; Clement et al., 1994; Popay & Rowan, 1994; Rowan & Latch, 1994). Mammalian toxicoses and insect resistance are the result of the endophytic fungus or the grass-fungus interaction producing specific metabolites such as alkaloids (Porter, 1994). Both insect deterrence and toxicity are

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involved with the production of endophyte-associated alkaloids (Dahlman et al., 1991).

Most of the information on the anti-insect properties of endophyte-infected grasses comes from work on infected tall fescue and perennial ryegrass (Breen, 1994; Clement et al., 1994; Popay & Rowan, 1994; Rowan & Latch, 1994). A few reports document Neotyphodium-conferred insect resistance in other *Festuca* species and in *Poa ampla* Merr. (Clement et al., 1994; Funk et al., 1994). To our knowledge, there is only one report of Neotyphodium-enhanced resistance to an insect involving a host grass other than *Festuca, Lolium,* and *Pou* species, and it is a preliminary report of aphid resistance in Neotyphodium-infected wild barley (Clement et al., 1994).

In view of the potential application of using different species and strains of *Neotyphodium* fungi to create new endophyte-grass associations for improved resistance to pests (Clay, 1994; Siegel & Bush, 1994), it is important to learn more about the diversity and distribution of these fungi in grasses other than tall fescue and ryegrass and their association, if any, with enhanced plant resistance to insects. The need for information on insect-endophyte interactions involving wild cereal grasses is addressed in this paper.

This research was conducted after Wilson et al., (199 1) discovered *Neotyphodium-like* endophytes in Plant Introductions (PI) of perennial *Hordeum* that had varying levels of antibiosis resistance to Russian wheat aphid, *Diuruphis noxiu* (Mordvilko) (Homoptera: Aphididae), when compared with 'Steptoe' (Clement & Lester, 1990) and 'Winterwalt' cultivated barley, *Hordeum vulgare* L. (Kindler & Springer, 1991). Data is presented here on the rate of population growth and survival of *D. noxiu* on endophyte-infected (EI) and endophyte-free (EF) plants of wild barley accessions screened by Clement & Lester (1990) and Kindler & Springer (1991), thereby testing the hypothesis that endophyte infection is responsible for *D. noxiu*-resistance observed in these studies.

Materials and methods

Aphids. Aphids were obtained from a colony maintained on *D. noxiu* susceptible 'Steptoe' barley in a growth chamber at 20 ± 1 °C with a photoperiod of L14:D10 (Clement & Lester, 1990). This colony was initiated with aphids collected in a *Hordeum* spp. germplasm nursery near Pullman, Washington, in summer 1988. Experiments were conducted in a separate growth chamber maintained under similar conditions of temperature and photoperiod.

Plant material. Four accessions of perennial *Hordeum* from the Asian areas of origin of *D. noxia* were evaluated: one accession of *H. brevisubulutum* subsp. *violuceum* (Boissier & Hohenacker) Tzvelev (PI 440420, origin Kazakhstan) and three accessions of *H. bogdunii* Wilensky (PI 269406, Afghanistan; PI 3 14696, Kazakhstan; PI 4404 13, former USSR).

In June 1991, 20 ramets from two 1 1-month-old EI plants, one each of PI 440413 and PI 440420, were transplanted separately into 15 cm pots containing potting soil. After 2 months of growth in a greenhouse (15–30 °C; L 14–1 6:D8–10; weekly fertilizer applications [20–20–20; NPK]), half of the plants of each PI line were split to provide 10 ramets (one per plant) for planting in 15 cm pots with soil (55% peat moss, 35% pumice, 10% sand). These clones were clipped to 8 cm above the soil line and treated with a soil drench solution (to run off, approximately 30 ml per pot) of a systemic fungicide (propiconizole; CIBA, Greensboro, N.C.) at 3 mg a.i./ml. Treated clones were not watered for 2 days. After an 8 week greenhouse-maintenance period in potting soil, microscopic examination of leaf sheath samples (Wilson et al., 199 1) revealed the presence of endophyte hyphae in treated clones. Control of endophyte occurred after these clones were placed in a sand medium and treated a second time with propiconizole (same procedure and rate). The propiconizole treatments killed approximately half of the clones, and surviving plants experienced growth distortions and reduced height and herbage growth. It was not until January 1992 that surviving plants had outgrown the phytotoxic effects of the fungicide treatments.

From the above procedure, we selected single EI (untreated) and EF (treated) plants of PI 4404 13 and PI 440420 and maintained them as sources of experimental material. Thus, for each PI line experimental ramets in the EI and EF states were genetically equivalent. By contrast, single EI and EF (untreated) plants of PI 269406 and PI 3 14696 provided experimental material for experiments involving these two lines. These two plants were started from seed in March 1990. The eight 'parent plants' were maintained in a standard potting soil in a greenhouse (15-30 °C; L1O-1 6:D8–14) with frequent watering and bi-weekly fertilizer applications. They were subdivided and repotted several times before experiments were conducted in summer 1994.

Isolation and identijcation of endophytes. In March 1994, 5 weeks before the first experiment was initiated, the endophyte status of each parent plant was confirmed by isolating endophytes on potato dextrose agar (PDA) from surface-sterilized basal stem sections as described by Clement et al. (I 996). The status (EI or EF) of test ramets was confirmed 7-10 days after each experiment by isolating stem sections on PDA and examining for mycelia after 3 weeks.

Scanning electron micrographs were prepared from plugs of mycelia from cultures (one per EI parent plant) and fixed in 2.5% glutaraldehyde/2.0% paraformaldehyde (buffered in 0.1 M Pipes buffer), rinsed in 0.1M phosphate buffer, postfixed in osmium tetroxide, buffer-rinsed, dehydrated in an ethanol series, critical point dried, and sputter coated with gold. The cultures were characterized by measuring the lengths of 38 conidia per culture using an Hitachi S-570 scanning electron microscope. Conidial dimensions were used previously by Christensen & Latch (199 1) to help differentiate different endophyte isolates (and possibly different species) in tall fescue. Herein, the endophytes isolated from wild barley are called '*Neotyphodium*-like isolates' because their taxonomic identity has not been established.

Population growth experiments. Replicate tillers from EI and EF parent plants of each PI line were rooted in potting soil in plastic Supercells (Ray Leech Conetainers, Canby, Oreg.) (3.8 by 20.6 cm) placed in holding racks positioned over metal trays filled with water. These were clipped to a uniform height of approximately 10 cm and allowed to grow for 3-4 weeks under greenhouse conditions (15–30 °C; L10–16:D8–14). The plants were then moved to a growth chamber where each was infested with 25 adult apterous aphids. Aphids were transferred with a camel's-hair brush to the base of each plant. Clear plastic tubes (3.6 by 30 cm), capped with nylon organdy screen, were tightly inserted into Supercells to confine the aphids.

Four separate experiments were conducted, each with an equal number of EI and EF ramets (22-28 per experiment) of PI 269406 (experiment 1), PI 440413 (experiment 2), PI 314696 (experiment 3), or PI 440420 (experiment 4). Ramets in supercells were arranged in a completely randomized design. The number of aphids on each ramet was recorded every 2 days over 8 (experiments l-3) or 10 (experiment 4) day periods. On the last census, adult and immature aphids were counted separately on each plant.

Statistical analyses. Data on dimensions of endophyte conidia and aphid count data from the last census in population growth experiments were analyzed by analysis of variance. Means were separated using Tukey's mean separation test. The overall results of the population growth experiments were analyzed with SAS-GLM repeated-measures analysis of variance (SAS Institute, 1987). All data were transformed by $\log_{10} (x + 1)$ to meet the normality and homogeneity of variance assumptions of analysis of variance with untransformed means reported here.

Results

Endophyte isolation. After experiments, endophyte cultures developed only from plant samples of test ramets derived from EI parent plants. On the basis of mean conidial lengths (Table 1) ($F = 121.19$; df = 3, 148; P = 0.0001) the endophytes from four *Hordeum* lines fell into three groups: PI 269406 with long conidia, PI 4404 13 with conidia intermediate in length, and PI 3 14696 and PI 440420 with short conidia. In another study (TePaske et al., 1993), grass samples from endophyte-infected PI 3 14696 and PI 440420 contained loline (N-Formylloline) and ergot (ergovaline) alkaloids whereas samples from infected plants of PI 269406 and PI 4404 13 contained no measureable levels of these alkaloids (Table 1). Based on conidial lengths and alkaloid profiles, the endophytes in PI 3 14696 (*H. bogdunii)* and PI *440420 (H. brevisubulatum* subsp. *violaceum)* may fall into one taxonomic grouping.

Population growth experiments. Aphid populations increased on EI and EF ramets of PI 269406 and PI 440413 (Figure 1). The repeated measures analysis revealed no significant differences in aphid counts among EI and EF ramets of these two PI lines (P > 0.37, P > 0.09). In both experiments, the effect of time was significant (P < 0.0001) because aphid populations increased over time on both plant types (Table 2; experiments 1 and 2). By contrast, aphid populations increased only on EF ramets of PI 440420. In the case of PI 3 14696, aphid numbers initially declined on both plant types before they stabilized and increased on EF ramets (Figure 1). The repeated- measures analysis showed significant differences in aphid mortality among EF and EI plants of PI 3 14696 and PI 440420 (P < 0.0001) and over time (P< 0.0001, P < 0.0082) on these two plant types of both PI lines. Also, there were

Table *1*. Length of conidia of *Neotyphodium* fungi in **vitro** and production of alkaloids in *vivo* of infected wild barley

Host	Length of conidia (μm)[1]		Alkaloid Production'	
	Range	Mean $\pm$ SE	N-Formylloline	Ergovaline
H. bogdanii				
PI 269406	**4.4-6.5**	5.5±0.1a	▪	▪
PI 440413	**3.3-5.7**	4.4±0. 1b	▪	
PI 3 14696	3.1–4.1	3.5±0.0c	+	+
H. brevisubulatum				
spp. violaceum				
Pl 440420	**3.0-5.0**	3.6±0.0c	+	+

[1]n=38; values with the same letter within a column are not significantly different at P>0.05 (Tukey's mean separation test).
[2]+, metabolite present in host grass; −, absent in host grass. Information from TePaske et al. (1993).
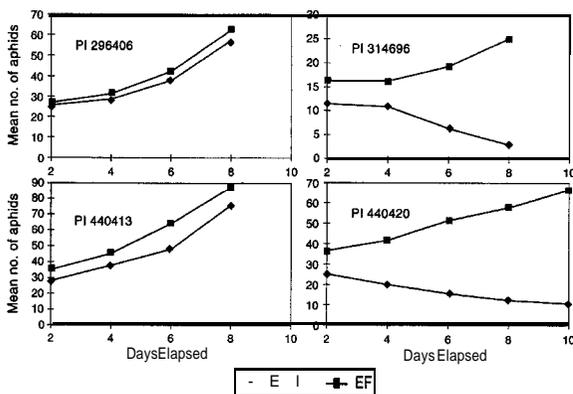

Figure I. Population trends of *D. noxia* on experimental ramets of *Hordeum bogdanii* (PI 269406, PI 3 14696, PI 4404 13) and *Hordeum brevisubulatum* subsp. *violaceum* (PI 440420) with (EI) and without (EF) fungal endophyte.

significant time X endophyte interactions (P< 0.0001), indicating that the effect of time on aphid mortality on EF and EI ramets was different (Table 2; experiments 3 and 4).

On the last census, adult and immature densities on EI and EF ramets of PI 269406 and PI 440413 were similar (P > 0.05). Therefore, it appears that aphid reproduction and developmental rates were unaffected by endophyte in these lines. By contrast, fewer immatures (P < 0.05) and adults were found on EI ramets of PI 314696 and PI 440420 than on EF conspecifics of each line. Low adult aphid populations on EI ramets of PI 3 14696 and PI 440420 did not permit

statistical comparisons of counts between EI and EF plants (Table 3). These findings and Figure 1 show that EF ramets of PI 3 14696 and PI 440420 are far superior to EI conspecifics as host plants of *D. noxia.* This endophyte-associated resistance was the result of antibiosis effects or starvation resulting in low aphid reproduction and abundance.

Discussion

The four PI lines in this study were screened by Clement & Lester (1990) for resistance to *D. noxia,* whereas Kindler & Springer (1991) screened three lines (PI 3 14696, PI 440413, PI 440420). Although these lines were resistant to *D. noxia* in both studies, PI 440420 supported the fewest aphids of all accessions screened. In addition, this line was 'immune to leaf curling' (Kindler & Springer, 199 1). Overall, PI 3 14696 ranked second and PI 269406 and PI 440413 third and fourth (Clement & Lester, 1990).

Incidence of endophyte infection was 98% in the seed lot that produced plants of PI 440420 (Wilson et al., 1991) for Clement & Lester's (1990) and Kindler & Springer's (199 1) studies. Thus, these researchers probably screened EI plants. This information, coupled with our results, support the hypothesis that endophyte infection was responsible for the very high level of aphid resistance in PI 440420 observed by these researchers. Although endophyte infection of PI 314696 confers resistance to *D. noxia* (Figure 1), the role played

Table 2. Results of repeated-measures analysis of variance on mean numbers of *D. noxia* on endophyte-infected (EI) and endophyte-free (EF) wild barley plants

Source	df	Sum of squares	Mean squares	*F*	*P*
Experiment 1. PI269406 (EI vs. EF)					
Effect of treatment					
Treatment	1	0.04	0.04	0.85	>0.37
Error	26	1.265	0.05	—	
Effect of time					
Time	3	2.04	0.68	108.08	<0.0001
Time x treatment	3	0.003	0.001	0.17	>0.91
Error	78	0.49	0.01	—	
Experiment 2. PI 440413 (EI vs. EF)					
Effect of treatment					
Treatment	⟩ 1	0.20	0.20	3.21	>0.09
Error	20	1.22	0.06	—	
Effect of Time					
Time	3	2.04	0.68	97.01	<0.0001
Time x treatment	3	0.01	0.004	0.63	>0.60
Error	60	0.42	0.01		
Experiment 3. PI 314696 (EI vs. EF)					
Effect of treatment					
Treatment	1	3.72	3.72	39.95	<0.0001
Error	22	2.05	0.09	—	
Effect of time					
Time	3	0.57	0.19	11.39	<0.0001
Time x treatment	3	2.07	0.69	41.26	<0.0001
Error	66	1.10	0.02	—	
Experiment 4. PI 440420 (EI vs. EF)					
Effect of treatment					
Treatment	1	10.85	10.85	43.24	<0.0001
Error	26	6.50	0.25	—	—
Effect of time					
Time	4	0.21	0.07	3.63	<0.0082
Time x treatment	4	2.61	0.65	35.55	<0.0001
Error	104	1.91	0.02	—	—

Table 3. Mean numbers of adult and immature *D. noxia* on endophyte-infected (EI) and endophyte-free (EF) ramets of wild barley on last censuses in population growth tests

Host	Endophyte status	Mean (&SE) no. aphids'	
		Adults	Immatures
PI 269406	EI	15.64±2.42	41.79zt3.82
	EF	17.57±2.41	45.21±4.47
	F values (*df*)	0.32(1,26)ns	0.34(1,26)ns
PI440413	EI	22.27zt3.54	52.64±7.18
	EF	28.00±3.38	59.82zt5.49
	F values (*df*)	1.37(1,20)ns	0.63(1,20)ns
PI 3 14696	EI	0.08±0.09	3.58±0.49
	EF	6.50zt0.85	18.67±1.91
	F values (*df*)	—[2]	58.46(1,22)***
PI 440420	EI	0.64±0.50	10.2912.38
	EF	20.43± 1.72	46.21±3.06
	F values (*df*)	—[2]	86.08(1,26)***

'Asterisks indicate levels of significance (by ANOVA) (ns, $P>0.05$; ***, $P<0.001$). Comparisons made within clusters of two means.

[2] Low adult populatons on EI propagules precluded statistical comparison.

by the endophyte in conferring resistance in Clement & Lester's (1990) and Kindler & Springer's (1991) studies is less clear. They probably screened a mix of EI and EF plants of PI 3 14696 because their seed came from a source with an endophyte infection level of 62% (Wilson et al., 1991). Two mechanisms may contribute to the resistance of PI 314595 to *D.* noxia, namely, a plant genetic component and an endophyte component. Indeed, a strong plant genetic component is suggested by the data in Figure 1, which shows that EF ramets of PI 3 14696 supported fewer aphids (about 2.5 per plant) than did EF ramets of the other lines (57-88 aphids per plant) after 8-10 days. Although the data in Figure 1 suggest differences in susceptibility to *D. noxia* among the lines (independent of endophyte infection), we did not statistically analyze this aspect because the four experiments were conducted at different times. Clearly, resistance in PI 269406 and PI 4404 13 is not associated with the presence of endophyte (Figure 1).

Experimental ramets of PI 440413 and PI 440420 in the EI and EF states were genetically equivalent, mak-

ing it possible to test the hypothesis that endophyte infection alone is responsible for enhanced resistance to   in these lines. Because genetically equivalent EI and EF experimental ramets of PI 269406 and PI 3 I4696 were not available, the only alternative was to use progenies from single EI or EF parent plants. Notwithstanding this limitation, we believe the experiments with PI 269406 and PI 3 14696 tested the possible effects of endophytes on aphid performance because they showed that endophyte infection may (PI 3 14696) or may not (PI 269406) alter plant suitability to **D. noxia** (Figure 1). In all experiments, potential differences (soil, water, nutrient, light, temperature) between plants except for the presence or absence of endophytes were controlled.

Reports on grass-endophyte systems have shown that host grass genotype or species (tall fescue or perennial ryegrass) and the endophyte species or strain involved can affect the expression of insect resistance (Breen, 1994; Clement et al., 1994; Popay & Rowan, 1994). Our results extend this phenomenon to host grasses other than tall fescue and perennial ryegrass by showing that aphid resistance varies with the genotype or species of a crop relative (Figure 1) and the **Neotyphodium-like** species or strain involved (Table 1).

Although EI grass samples from each PI line were not analyzed for alkaloids in this study, it is possible that variation in alkaloid content mediated **D. noxia** responses to the lines. Support for this hypothesis comes from TePaske et al. (I 993), who isolated N-Formylloline from EI plants of PI 314696 and PI 440420, but not from EI PI 269406 and PI 440413. This loline alkaloid, along with N-acetylloline, has been implicated in the resistance of EI tall fescue to the aphid *Rhopalosiphum padi* L. (Eichenseer et al., 1991). Therefore, the presence (PI 314696, PI 440420) or absence (PI 269406, PI 440413) of this loline alkaloid may explain the variation in plant quality for **D. noxia** among the wild barley lines in this study. TePaske et al. (1993) also detected ergot alkaloids in EI samples from PI 314696 (ergovaline) and PI 440420 (ergovaline, ergosine, ergotamine) (Table 1), but it is not known if these metabolites mediate resistance to aphids (Dahlman et al., 1991).

The discovery of **Neotyphodium-like** endophytes in wild barley (Wilson et al., 1991) foretells the probable existance of an important endophyte resource in crop relatives. This study underscores the potential importance of these fungi in conferring natural plant resistance to insects.

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