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Insect Feeding Deterrents in Endophyte-Infected Tall Fescue†

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The presence of an endophytic fungus, *Acremonium coenophialum*, in tall fescue (*Festuca arundinacea*) deterred aphid feeding by *Rhopalosiphum padi* and *Schizaphis graminum*. Both species of aphid were unable to survive when confined to endophyte-infected tall fescue plants. Feeding deterrents and toxic factors to *R. padi* and *Oncopeltus fasciatus*, large milkweed bug, were primarily associated with a methanol extract obtained when endophyte-infected tall fescue seed was serially extracted with hexane, ethyl acetate, and methanol. The concentrations of pyrrolizidine alkaloids were determined to be 30 to 100 times greater in the methanol extract than in the hexane and ethyl acetate extracts.

An association between poor livestock performance and the presence of a fungal endophyte in tall fescue (*Festuca arundinacea* Schreb.) pastures has been corroborated by several lines of investigation (1, 9, 11). The endophyte was identified as *Epichloë typhina* (Pers.) Tul. (asexual state, *Sphacelia typhina* Sacc.) (1), but has been renamed *Acremonium coenophialum* Morgan-Jones et Gams (16). A strikingly similar association between an animal disorder and a fungal endophyte, *Acremonium loliae* Latch, Christensen et Samuels, is now known to occur in perennial ryegrass (*Lolium perenne* L.) (6, 15). Recent reports have demonstrated endophyte-free perennial ryegrass to be more susceptible to insect attack as compared with endophyte-infected cultivars (7, 19). With the development of new tall fescue cultivars (3, 17) that are relatively free of the endophyte and thus superior for livestock purposes, it is important to determine whether endophyte-free tall fescue is also more susceptible to insect damage.

To examine the possible relationships between endophyte presence and insect susceptibility a variety of insect feeding experiments were conducted representing choice and nonchoice situations on leaf blades, leaf sheaths, and seed extracts from endophyte-free and endophyte-infected plants.

MATERIALS AND METHODS

Insects. Four species of aphids (Homoptera:Aphididae) common in small grains in North America (18) were chosen: corn leaf aphid, *Rhopalosiphum maidis* (Fitch); oat bird cherry aphid, *Rhopalosiphum padi* (L.); English grain aphid, *Sitobion avenae* (Fab.); and greenbug, *Schizaphis graminum* (Rondani). Aphids were reared on barley (*Hordeum vulgare* L.) cultivar Barsoy and wheat (*Triticum aestivum* L.) cultivar Arthur in growth chambers at 22°C under alternating light and dark periods of 12 h each. For all aphid feeding tests, wingless aphids of all ages were shaken from the colony onto aluminum foil and then counted into small glass vials with polystyrene caps.

The large milkweed bug *Oncopeltus fasciatus* (Dallas) (Hemiptera:Lygaeidae), although not a pest on grasses, was chosen because it has been extensively used in research ranging from behavior to biochemistry. The insects were reared on sunflower seed by the method of Best (2). Five newly hatched nymphs less than 24 h old were placed in 18.3-ml clear plastic cups capped with a paper lid. These nymphs were held for 24 h without food or water. The test materials were delivered to the nymphs in the water, and the 24-h starvation period promoted a more rapid and uniform drinking response by the nymphs.

Plants. Portions of a tall fescue seed lot that was 100% infested with *A. coenophialum* at the time of seed harvest were stored under low- or high-temperature storage conditions for 11 months to result in seeds containing a viable or nonviable endophyte, respectively (21). Thus, plants grown from these seed sublots were similar to each other, except for endophyte content. Endophyte-free and endophyte-infected perennial ryegrass plants were selected from within a population of Nui perennial ryegrass, a cultivar from New Zealand. The presence or absence of endophyte in all plants used in the insect feeding experiments was confirmed by enzyme-linked immunosorbent assay (12, 13). All leaf blades and leaf sheaths tested were from seedlings that were at least 5 months old.

Tall fescue seed extracts. Kentucky 31 seed, 73% infested or 0% infested, hereafter called endophyte infected or endophyte free, respectively, were ground to pass a 1-mm screen. Thirty grams of each seed lot was extracted in a Soxhlet apparatus with a 600-ml total volume for at least 25 cycles with hexane, ethyl acetate, and methanol. Between each solvent extraction the seed residue dried overnight in the Soxhlet thimble in the laboratory. Solvent from each extraction was reduced in volume below 10 ml in vacuo at 40°C and then brought to 10 ml with the respective solvent.

Extracts were analyzed for levels of *N*-acetyl and *N*-formyl loline, pyrrolizidine alkaloids that have been shown to be consistently associated with endophyte infection in tall fescue (4). The extract preparations were tested for feeding deterrents and for antibiosis in choice and nonchoice situations.

For experiments with aphids, vegetative stems of endophyte-free tall fescue were collected, the outer leaf sheaths were removed, and the basal few millimeters of the stems were coated with paraffin. Stems were dipped to a depth of 3 to 4 cm in the extract solution to be tested or the appropriate solvent control. Because hexane proved to be an aphid repellent, hexane extracts were tested by bringing them to dryness and suspending in ethyl acetate. The dipped

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TABLE 1. Ratio of aphids preferring endophyte-free leaf sheaths over endophyte-infected leaf sheaths of tall fescue and perennial ryegrass[a]

Aphid species	Tall fescue	Perennial ryegrass
Rhopalosiphum padi	5.28[b]	0.95[c]
Schizaphis graminum	4.80[b]	1.07[c]
Rhopalosiphum maidis	1.30[d]	2.38[b]
Sitobion avenae	0.84[c]	0.88[c]

[a] Mean ratio of four test plates after 18 h. Significant preferences were calculated by the chi square test.
[b] $P < 0.001$.
[c] Not significant.
[d] $P < 0.05$.

portion of the stems were dried with a jet of warm dry air, and the paraffin-coated end and the distal nondipped region of the stems were excised.

Preference tests. Eight to 10 leaf blades, leaf sheaths, or vegetative stems were placed in the bottom of a glass petri plate (diameter, 15 cm) on water-moistened Whatman no. 1 filter paper. On each test plate, plant samples representing endophyte-infected material were alternated with samples representing endophyte-free material. Plant samples tested were approximately 3 cm in length. Fifty to 100 aphids were scattered onto the plant material and filter paper in each test plate. A thin sheet of Parafilm was used to seal the top and the bottom plates together to prevent aphid escape. Plates were placed in the growth chamber at h 8 of the 12-h light period. Counts of aphids probing or feeding (or both) on each plant sample were made after 18 h.

Nonchoice tests. Aphids were confined to individual endophyte-free or endophyte-infected tall fescue plants in sealed Plexiglas cylinders. Approximately 100 aphids were placed on each of four endophyte-free plants and four endophyte-infected plants. Counts of live aphids were made at 3-day intervals.

Nonchoice experiments were also conducted that confined aphids to detached vegetative tall fescue stems in petri plates. The treatments included endophyte-infected, endophyte-free, and endophyte-free stems dipped in 1/20 dilutions of the methanol extracts. After 18, 24, 42, and 66 h, the numbers of aphids dead, feeding, and wandering were counted and compared with the appropriate controls.

Experiments testing responses of the large milkweed bug, *O. fasciatus*, to tall fescue seed extracts were initiated by placing 300 μl of water or aqueous solution on a cotton pad (10-mm diameter, 5 mm thick) that had been cut from a dental roll. A cotton pad of this size has a holding capacity of approximately 300 μl. Test materials dissolved in organic solvents were first applied to the pad in the 300-μl volume, the solvent was permitted to evaporate before placement of the pad into the plastic cup with the nymphs, and the pad was then wetted with 300 μl of water. Aqueous solutions were applied directly to the pad in the cup. A single sunflower seed was placed in each cup as a food source. The paper-capped cups were held in closed plastic containers on moist paper toweling. Each third day the pad was replenished with 200 μl of water, and the sunflower seed was replaced. Daily counts of live nymphs were made for 12 consecutive days. The body of each nymph that died during the experiment was saved. At the end of 12 days, all nymphs still alive were frozen, and the collective dry weight of the nymphs from each treatment was determined.

TABLE 2. Counts of live aphids confined to individual plants of endophyte-free (−) and endophyte-infected (+) tall fescue

Aphid species	Endophytes	Live aphids on day after introduction[a]:				
		3	6	9	12	15
R. padi	−	19	20	51	68	161
	+	1	0	0	0	0
S. graminum	−	120	166	333[b]		
	+	0	0	0		

[a] Approximately 100 aphids were placed on each plant on day 0. Data represent means of four plants.
[b] Severe necrosis of plants due to toxins in the saliva of *S. graminum*.

RESULTS

R. padi and *S. graminum* preferred endophyte-free tall fescue leaf sheaths for feeding by greater than a 4-to-1 margin over endophyte-infected leaf sheaths (Table 1). In contrast, *R. padi* and *S. graminum* showed no apparent preference for endophyte-free leaf sheaths of perennial ryegrass over the endophyte-infected sheaths (Table 1). *R. maidis* did appear to prefer endophyte-free leaf sheaths of perennial ryegrass, but the magnitude of this preference was less than that of *R. padi* and *S. graminum* for endophyte-free tall fescue. *Sitobion avenae* showed no apparent preference for either endophyte-free or endophyte-infected leaf sheaths of tall fescue or perennial ryegrass (Table 1). The ratios of *R. padi* and *S. graminum* preferring endophyte-free leaf blades of tall fescue over leaf blades from endophyte-infected plants were 1.77 and 1.74, respectively (significant at $P < 0.001$). Neither *R. padi* nor *S. graminum* was able to survive when confined to endophyte-infected tall fescue plants (Table 2).

Because the feeding preferences of *R. padi* appeared to be the most affected by endophyte infection in tall fescue, this aphid was used to assay the tall fescue seed extracts for feeding deterrents. The majority of the aphid feeding deterrent activity appeared to be concentrated in the methanol extract prepared from endophyte-infected seed. The aphids demonstrated a preference for stems dipped in methanol solvent over stems dipped in the methanol extract even when it was diluted 100- to 200-fold in methanol (Table 3). In

TABLE 3. Number of *R. padiaphids* feeding on stems dipped in various dilutions of extracts prepared from endophyte-infected (+) or endophyte-free (−) tall fescue seed in preference tests with stems dipped in appropriate control solvent

Extract	Endophytes	*N*-Acetyl and *N*-formyl loline[a] (μg/ml)	No. of aphids with dilution of extract[b]:				
			1/5	1/10	1/50	1/100	1/200
Hexane	+	150		15			
Control solvent				22			
Ethyl acetate	+	454	7	15			
Control solvent			27	18			
Methanol	+	15,578		1	1	4	16
Control solvent				20	23	29	30
Hexane	−	0		28			
Control solvent				25			
Ethyl acetate	−	0	4	9			
Control solvent			34	22			
Methanol	−	0		13	22	28	
Control solvent				28	18	21	

[a] Concentration in undiluted extract.
[b] Data represent means of eight stem segments after 18 h.

contrast, the methanol extract from endophyte-free seed was not inhibitory to feeding at 1/50 and 1/100 dilutions, but was inhibitory at a 1/10 dilution (Table 3). Ethyl acetate extracts from endophyte-infected seed were no more inhibitory to aphid feeding than were the ethyl acetate extracts from endophyte-free seed (Table 3). Hexane extract prepared from endophyte-infected seed and then suspended in ethyl acetate appeared to be only slightly more inhibitory to aphid feeding than that prepared from endophyte-free seed (Table 3). Furthermore, in a preference experiment between stems dipped in ethyl acetate resuspensions of the hexane extracts at 1/10 dilutions, the ratio of feeding preference on endophyte free over endophyte infected was only 1.05, and thus not significant.

The percentages of aphids dead, wandering, and feeding after introduction to petri plates containing tall fescue stem segments dipped in a 1/20 dilution of the methanol extract from endophyte-infected tall fescue seed were comparable to those of aphids confined to endophyte-infected stem segments. In both cases, a majority of *R. padi* aphids were wandering at 18 h after introduction, and mortality had reached 100% by 66 h after introduction (Table 4). The percent aphid feeding remained high and mortality remained low when aphids were confined to endophyte-free stems or to endophyte-free stems dipped in a 1/20 dilution of the methanol extract from endophyte-free tall fescue seed (Table 4). An additional nonchoice experiment (data not shown) indicated that after 1 and 6 h the number of aphids feeding on endophyte-infected stems was consistent with the number feeding on endophyte-free stems. After 18 h, however, the number feeding on the endophyte-infected stems was sharply reduced.

It is clear from the data in Table 5 that one or more components toxic to *Oncopeltus fasciatus* nymphs are associated with endophyte-infected tall fescue seed and that the major portion of this toxic factor can be removed with a methanol extraction. No toxicity was associated with the ethyl acetate fraction (Table 5), but results from preliminary tests with slightly older nymphs suggest a low level of toxicity in this fraction. No activity was observed in the hexane fraction. There also appeared to be some factor associated with the methanol extract of the endophyte-free seed, because *O. fasciatus* nymphs in this treatment had a small total dry weight even though the percent mortality was relatively low.

When the methanol fraction from the endophyte-infected tall fescue seed was diluted with methanol and tested for *O. fasciatus* response, it became apparent that the response was concentration dependent and that as little as 0.39 μl per 300 μl (a 1/769 dilution) still produced significant mortality and somewhat less mean dry weight per nymph (Table 6). A similar study with the ethyl acetate fraction showed *O. fasciatus* response to only the highest level tested (50 μl per 300 μl).

TABLE 4. Nonchoice tests with aphids confined to petri plates containing stem segments dipped in methanol extracts from either endophyte-infected or endophyte-free tall fescue seed[a]

Tall fescue stems	% Dead, wandering, feeding at h after introduction			
	18	24	42	66
Dipped[b]				
Infected extract	10, 70, 20	65, 25, 10	90, 5, 5	100, 0, 0
Free extract	<5, <5, 95	ND[d]	5, 5, 90	ND
Nondipped[c]				
Infected	10, 55, 35	30, 50, 20	75, 20, 5	100, 0, 0
Free	<5, <5, 95	<5, <5, 95	10, 10, 80	20, 5, 75

[a] Approximately 200 *R. padi* aphids were added to each plate. Data are presented as the percent dead, percent wandering, and percent feeding, respectively, and are the means of two test plates. Each plate contained six stem segments.

[b] Aphids confined to test plates containing endophyte-free stems dipped either in a 1/20 dilution of the methanol extract from endophyte-infected tall fescue seed or in the same from endophyte-free seed.

[c] Aphids confined to test plates containing either nondipped endophyte-infected stems or nondipped endophyte-free stems.

[d] ND, Not determined.

TABLE 5. Percent mortality of *O. fasciatus* nymphs in response to 50-μl extracts from endophyte-free and endophyte-infected tall fescue seed[a]

Solvent	Tall fescue seed	% Mortality on day:						Mean dry wt (μg) per nymph after 12 days
		2	4	6	8	10	12	
Methanol	Infected	73	87	100	100	100	100	53
	Free	7	7	7	20	20	20	179
	Solvent	0	0	0	7	13	20	566
Ethyl acetate	Infected	7	7	7	7	7	7	473
	Free	0	0	0	0	7	13	326
	Solvent	0	0	7	7	20	30	464
Hexane	Infected	7	7	13	13	13	13	560
	Free	7	7	7	7	13	13	580
	Solvent	7	7	7	13	13	13	360
Water		0	0	7	7	7	13	840

[a] Data are computed from three replications of five nymphs per cup.

DISCUSSION

The presence of endophytes deterred aphid feeding in tall fescue and perennial ryegrass. However, this deterrence was dependent on the species of aphid and host plant. *R. padi* and *S. graminum* were deterred by endophyte-infected tall fescue, but not by endophyte-infected perennial ryegrass. In contrast, *R. maidis* appeared to be deterred by endophyte-infected perennial ryegrass to a greater extent than by endophyte-infected tall fescue (Table 1). *S. avenae*

TABLE 6. Percent mortality of *O. fasciatus* nymphs in response to dilutions of a methanol extract of endophyte-infected tall fescue seed[a]

Extract concn (μl/300 μl)	% Mortality on day:						Mean dry wt (μg) per nymph after 12 days
	2	4	6	8	10	12	
50	48	74	88	98	100	100	110
25	52	66	96	100	100	100	85
12.50	48	74	86	98	98	100	80
6.25	32	76	88	96	98	98	90
3.12	24	54	78	90	94	98	83
1.56	8	26	44	56	64	66	85
0.78	4	26	48	60	64	70	105
0.39	2	8	24	32	38	48	330
0.20	0	4	12	12	12	14	620
0.10	0	0	2	6	16	24	705
Methanol blank	2	2	6	12	16	24	420
Water blank	2	2	4	4	14	18	812

[a] Data are computed from 10 replications of five nymphs per cup.

was unaffected by endophyte infection of tall fescue and perennial ryegrass in our preference test (Table 1).

This lack of uniformity among species of aphids has been corroborated in a recent study by Latch et al. (G. C. M. Latch, M. J. Christensen, and D. L. Gaynor, N. Z. J. Agr. Res., in press). They also found *R. padi* to be deterred by endophyte presence in tall fescue and not in perennial ryegrass. In addition, they reported that two species of aphids, *Metopholophium dirhodum* (Walker) and *Sitobion fragariae* (Walker), showed no preference for either endophyte-free or endophyte-infected plants of tall fescue or perennial ryegrass.

R. padi and *S. graminum* were unable to survive on endophyte-infected tall fescue plants. The large increases in numbers of aphids on endophyte-free tall fescue plants under growth chamber conditions (Table 2) may signify an increased susceptibility to insects by endophyte-free tall fescue in the field. The high numbers of *S. graminum* on endophyte-free plants only 9 days after being introduced could be especially significant because of the excessive injury of plant cells by toxins in the insects' saliva (18). Another possible result of large increases in aphid numbers on endophyte-free tall fescue in the field could be a concomitant increase of tall fescue as a reservoir of barley yellow dwarf virus (5, 8). However, increased susceptibility to insects by endophyte-free tall fescue in the field has not been observed (20), but might be as more pastures low in endophyte content are established.

In tests with tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera:Sphingidae), and tobacco budworm, *Heliothis virescens* (Fab.) (Lepidoptera:Noctuidae) (data not shown), we found no consistent response when larvae were fed artificial diets containing either ground plant material or plant extracts from endophyte-infected tall fescue as compared with similar preparations from endophyte-free tall fescue. Greenhouse experiments with another lepidopterous species, *Spodoptera eridania* (Cramer), southern armyworm, also failed to show susceptibility to toxic factors that may be present in endophyte-infected tall fescue plants. In contrast, high levels of endophyte in perennial ryegrass cultivars have been associated with resistance to sod webworm, *Crambus* spp. (7).

Feeding deterrents and toxic factors to *R. padi* and *O. fasciatus* were primarily associated with the methanol extract prepared from endophyte-infected tall fescue seed (Tables 3 and 5). This extract was determined to contain 30- to 100-fold higher concentrations of pyrrolizidine alkaloids (*N*-acetyl and *N*-formyl loline) than the hexane and ethyl acetate extracts (Table 3). *N*-Acetyl and *N*-formyl loline may reach concentrations as high as 0.5% per unit dry weight in stems, leaves, and seeds of endophyte-infected tall fescue plants (14) and have not been detected in endophyte-free tall fescue plants or in pure cultures of *Acremonium coenophialum*. Pyrrolizidine alkaloids are known to be involved in some livestock poisonings (10). Studies involving further fractionation of the methanol extract from endophyte-infected tall fescue seed and testing of purified loline alkaloids for insect feeding deterrence and antibiosis are being conducted.

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