Feeding and oviposition by Argentine stem weevil on *Epichloë uncinata*-infected, loline-containing *Festulolium*

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Abstract Responses of adult Argentine stem weevil to meadow fescue-perennial ryegrass hybrids, known as *Festulolium*, infected with the endophyte *Epichloë uncinata* were investigated and compared with endophyte-free Grasslands Wana cocksfoot and Grasslands Samson perennial ryegrass, and with Samson infected with wild type and AR37 strains of *Epichloë festucae* var. *lolii*. *Epichloë uncinata* infection in the *Festulolium* seed-line reduced oviposition but not feeding compared with an endophyte-free seedline of the same plant genotype in Petri dish leaf-comb and potted plant assays. Feeding on *Festulolium* was similar to that on endophyte-free Samson but higher than on Samson infected with wild type and AR37 endophytes. In these experiments, the numbers of eggs laid on *E. uncinata*-infected plant material was similar to that on Samson perennial ryegrass infected with wild type and AR37 endophytes, but lower than on endophyte-free Samson.

Keywords forage grasses, *Listronotus bonariensis*, host plant resistance, fungal endophyte.

INTRODUCTION

Because of their natural role in biological protection of the grass hosts, *Epichloë* endophytes are widely recognised as beneficial mycosymbionts in natural grasslands and, especially, in pastoral and turf systems. There is considerable interest internationally in the development of forage and turf grasses infected with *Epichloë* endophytes. Understanding the role of different alkaloids in protecting plants against various herbivorous pests is critical to development of endophyte-containing grasses for commercial use.

Cropmark Seeds Ltd has been developing forage grasses based on meadow fescue (*Festuca pratensis* Hudson) and its loline-producing endophyte *Epichloë uncinata* (W. Gams, Petrini & D. Schmidt) Leuchtm. & Schardl. because of potential agronomic advantages, not least pest resistance and tolerance. Earlier research at Cropmark Seeds (Patchett 2007; Patchett et al. 2008) and elsewhere (Jensen et al. 2009; Popay & Lane 2000; Popay et al. 2009) indicated some level of resistance to Argentine stem weevil (ASW) (*Listronotus bonariensis* (Kuschel)) is afforded to meadow fescue and to meadow fescue-perennial ryegrass (*Lolium perenne* L.) hybrids (*Festulolium*) when plants are infected with *E. uncinata*. Further, data are becoming available from field situations to suggest that *E. uncinata* infected *Festulolium* supports lower ASW populations (Barker 2013). This paper describes two experiments that sought to clarify the influence of *Festulolium* infection by

E. uncinata on feeding and oviposition preferences of adult ASW under both choice and non-choice situations.

MATERIALS AND METHODS

Experiment 1 – Leaf-comb assays of ASW feeding

Plant material was established from seed sown December 2014 into Osmocote-amended pine bark potting medium in root trainers and maintained with regular watering in a greenhouse at Darfield, Canterbury. This material comprised Barrier *Festulolium* (line NW421, Barrier 1 selection) infected with *E. uncinata* strain U2; Barrier *Festulolium* endophyte free (Nil); Grasslands Samson perennial ryegrass infected with *Epichloë festucae* var. *lolii* strain AR37; Samson perennial ryegrass infected with *E. festucae* var. *lolii* strain Wild Type (WT), and Samson perennial ryegrass endophyte free (Nil). Grasslands Wana cocksfoot (*Dactylis glomerata* L.) (endophyte free) was propagated from rooted tillers collected from pasture and included in the assays as a standard because of its known favourability to feeding ASW (Barker 1989).

On 13 February 2015, feeding in choice and non-choice situations was determined using the leaf-comb assay method described by Kain et al. (1982a, b) and Barker et al. (1984). In brief, ASW were offered four 3 cm leaf segments in 9 cm diameter Petri dishes provided with a moistened filter paper floor. The leaf segments were fastened at one end to the moistened floor of the Petri dish with 10 mm wide masking tape. For the non-choice assay, the four leaf segments in each replicate Petri dish comprised two segments from each of two different plants, offered to 5 weevils. For the choice assay, material in each Petri dish comprised 2 leaf segments from each of the two plant treatments being compared, offered to 5 weevils. In both choice and non-choice assays, there was a minimum of 15 replicates of each treatment.

ASW feeding scars were counted and scored (0-10 scale) after 24 and 96 h at ambient temperature (10-12°C minimum; 18-22°C maximum). For the feeding scores, 0 represented no feeding, and 10 the maximum feeding for the bioassay, which approximated 20 mm^2 leaf area consumed.

Experiment 2 - Whole plant assays of ASW feeding and oviposition

Plants of Barrier *Festulolium* infected with *E. uncinata* strain U2; Barrier *Festulolium* endophyte free (Nil); Samson perennial ryegrass infected with *E. festucae* var. *lolii* AR37; and Samson perennial ryegrass infected with *E. festucae* var. *lolii* WT were established in root trainers from seed sown December 2014 as described above. On 10 February 2015, plants were potted in pairs into Osmocote-amended pine bark medium in No. 5 black polyethylene polybags. For the non-choice assay, replicates comprised two different plants of the same treatment, while for the choice assay the paired plants were different treatments.

On 13 February 2015, cages were erected around each plant pair, comprising a slightly tapered cylinder of bisphenol A-free clear polycarbonate plastic, pressed 20 mm into the potting medium and extending 130 mm height. Ten ASW adults were added and each cage caped with mesh fabric secured with a plastic ring. In both choice and non-choice assays, there was a minimum of 10 replicates of each treatment, arranged in a randomised block design in a greenhouse.

After exposure to ASW for 96 h, counts were made of tillers per plant, ovipositions per plant, eggs per plant, eggs per oviposition, and feeding scars per plant. The positions of eggs on the plants were noted.

Source of insects

ASW used in experiments were collected during February 2015 by suction sampling an irrigated 3-year-old Arrow perennial ryegrass dairy pasture at Woodlands farm, Darfield. Dissection of a sample of weevils (n=54) indicated a male:female sex ratio 1.1:1.0, and females (n=26) were confirmed as reproductive with 2.3 ± 0.8 SE mature eggs in the calyces of each individual.

Determination of endophyte status and loline analyses

Incidences of *E. uncinata* and *E. festucae* var. *lolii* infections in *Festulolium* and perennial ryegrass seed-lines used in the experiments were determined for samples of 100 seeds by immunoblot (Hill et al. 2002) using the commercially available Agrinostics

(GA-USA) 'Phytoscreen Seed Endophyte Detection Kit', and staining seed squash preparations in aniline blue and examination under a compound brightfield microscope at 200-400× magnification. Because endophyte infection of sown seed was not 100%, *Epichloë* infection status in individual plants was determined using tiller immunoblots (modification of Gwinn et al. 1991) for two tillers per plant, cut with a scalpel through the meristem region at their base. Any replicate comprising a plant not conforming to the expected endophyte status was discarded.

Foliage harvested to ground level from five representative plants of Barrier U2 used in Experiments 1 and 2, confirmed by immunoblot as *E. uncinata*-infected, was submitted for determination of loline concentration. Using a method modified from Blankenship et al. (2001), freeze dried plant tissue (250 mg was ground to pass through a 1 mm screen and extracted in 5 ml of dichloromethane: ethanol (95:5) solvent containing 6 mg phenylmorpholine/100 ml as an internal standard, along with 250 µl saturated sodium bicarbonate. Samples were shaken at room temperature for 1 h at 200 rpm on an orbital shaker and left to settle for 10 min before being filtered into 2 ml GC vials. Samples were analysed using a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector. Hydrogen was used as the carrier gas through an Rtx-624 column. For the four most abundant loline compounds, retention times were as follows: n-methyl-loline (12.8 minutes), n-acetyl-norloline (17.4), n-formyl-loline (18.2), n-acetyl loline (18.8).

Statistical analyses

Data on numbers of feeding scars, visual scores of feeding, numbers of eggs per plant, numbers of eggs per oviposition site, and tillers per plant were subject to analysis of variance (ANOVA), with sum of squares formulae accommodating slight variation in sample sizes. Square root transformation was performed on numbers of feeding scars to assure homogeneity in variances. These statistical analyses were performed using S-Plus version 4. Data on visual scores of feeding are best treated as ordinal data and accordingly were also analysed by the non-parametric Kruskal-Wallis test in Microsoft Excel to confirm results from ANOVA.

RESULTS

Experiment 1 - Leaf-comb assays of ASW feeding

There were significant differences in intensity of feeding by adult ASW between the grass genotype/endophyte combinations included in the experiment (Table 1). For Samson perennial ryegrass infected with AR37 and particularly with WT endophyte, a large proportion of feeding scars was minute 'test bites', indicating that an interest in feeding by ASW adults was offset by a deterrent in the plant material. Such effects were not apparent with Barrier U2 *Festulolium* where the nature of the feeding scars was similar to that on Barrier Nil, Samson Nil and on the standard, Wana cocksfoot. ANOVA of visual scores of feeding (0-10 scale) (supported by the Kruskal-Wallis test; $H = 660.8$, df 14, $P < 0.0001$) indicated adult ASW fed at similar levels on Barrier U2 and Barrier Nil (Table 1), suggesting no effect of *E. uncinata* infection. Feeding on Barrier U2 and Barrier Nil was similar to that when ASW were offered Samson Nil, but less than when weevils were offered Wana cocksfoot. Feeding was lowest on Samson AR37 and Samson WT perennial ryegrasses. In general, feeding on Barrier Nil and Barrier U2 was not influenced by whether the leaf material was presented in a choice or a non-choice situation.

Experiment 2 - Whole plant assays of ASW feeding and oviposition

The size of plants did not differ significantly between treatments, with a mean of 6.3 tillers/plant ($P=0.634$). ANOVA of visual scores (supported by Kruskal-Wallis test; $H=521.5$, df 24, $P<0.001$) indicated the extent of feeding by ASW varied between treatments (Table 2). Feeding on plants of Barrier U2 was similar to that on Barrier Nil and Samson Nil, but statistically higher than on Samson AR37 and Samson WT.

ANOVA indicated the number of eggs deposited by ASW varied between treatments (Table 2). Numbers of eggs deposited on Barrier U2 were less than on Barrier Nil and Samson Nil,

Table 1 Feeding intensity (numbers of feeding scars or visual feeding score) of ASW on leaf material from various festuloliums, perennial ryegrasses and cocksfoot, varying in endophyte status, in choice and non-choice situations for 96 h.

	Numbers of feeding scars (back-transformed means)		
Plant material	Barrier Nil	Barrier U2	G. Samson Nil
Barrier Nil	17.7[1]	17.0	18.2
Barrier U2	16.3	15.9[1]	16.2
G. Samson AR37	11.8	11.6	10.4
G. Samson WT	5.5	6.0	9.5
G. Samson Nil	16.5	17.0	16.1[1]
G. Wana cocksfoot	17.1	18.4	17.3
	LSD (P=0.05)=2.12; P <0.0001		
	Visual score of feeding (0-10 scale)		
Barrier Nil	6.5[1]	6.0	6.9
Barrier U2	6.1	6.3[1]	6.5
G. Samson AR37	4.4	4.8	4.1
G. Samson WT	3.5	3.7	4.0
G. Samson Nil	6.9	6.5	7.2[1]
G. Wana cocksfoot	8.5	8.7	8.9
	LSD (P=0.05)=1.40; P <0.0001		

[1]Non-choice situations. All other cases shown in the table represent choice situations.

but similar to numbers deposited on Samson AR37 and Samson WT. No differences were detected between treatments in the position of oviposition sites as in the great majority of cases eggs were placed by female weevils in the lower one-third of the tiller. The number of eggs per oviposition site varied significantly (P=0.029) only between Barrier U2 (mean 1.28, range 1-2, n=14) and Barrier Nil (mean 2.03, range 1-5, n=62).

Total loline concentrations in the above-ground tissues of the Barrier U2 material used in Experiments 1 and 2 averaged 5599 (± 471 SE) µg/g, and comprised 64% N-formyl loline (NFL), 13% N-acetyl loline (NAL), 12% N-acetyl norloline (NANL) and 11% N-methyl loline (NML).

DISCUSSION

The experiments clearly demonstrated that *E. uncinata* U2 infection in Barrier did not reduce feeding, but suppressed egg laying by ASW relative to endophyte-free Barrier (and endophyte-free Samson perennial ryegrass). Furthermore, in the case of oviposition, the level of protection afforded to this plant material was similar to that of Samson AR37 and Samson WT. The absence of a reduction in ASW feeding in *E. uncinata* infection in *Festulolium* in the present work is consistent with the results obtained by Jensen et al. (2009) with *E. uncinata* infected meadow fescue plants and artificial diets containing different loline concentrations. The reduction of feeding and oviposition of ASW on Samson AR37 recorded in the present work was contrary to earlier studies by Popay & Wyatt (1995) and A.J. Popay (AgResearch, personal communication), which showed an absence of deterrency to adult ASW afforded by host plants infected with AR37.

Larvae, resulting from eggs deposited in the sheath tissues, are the principal damaging stage of ASW. In grass species or cultivar evaluations, a first approximation of likely susceptibility to oviposition is provided by those grasses being unfavourable to adult feeding in simple leaf assays (Kain et al. 1982a,b; Barker 1989; Popay et al. 2005). That grasses favourable for adult feeding are favourable for oviposition does however not always apply, as some grasses, for example, are rejected as oviposition hosts despite being utilized

Table 2 Feeding intensity (visual score) and numbers of eggs deposited by ASW on whole potted plants of various festuloliums and perennial ryegrasses, varying in endophyte status, in choice and non-choice situations for 96 hours.

	Visual score of feeding (0-10 scale)				
Plant material	Barrier Nil	Barrier U2	G. Samson AR37	G. Samson WT	G. Samson Nil
Barrier Nil	5.5[1]	5.7	5.7	6.1	5.7
Barrier U2	5.6	5.8[1]	5.9	5.6	6.6
G. Samson AR37	3.5	3.8	3.5[1]	3.6	3.6
G. Samson WT	2.6	2.9	3.1	3.0[1]	2.6
G. Samson Nil	7.0	6.1	6.7	6.1	6.3[1]
	LSD (P=0.05)=1.62; P <0.001				
	Numbers of eggs per plant				
Barrier Nil	3.45[1]	3.37	4.02	3.56	3.41
Barrier U2	0.15	0.28[1]	0.28	0.32	0.19
G. Samson AR37	0.92	0.79	0.80[1]	0.86	0.77
G. Samson WT	0.46	0.60	0.57	0.65[1]	0.62
G. Samson Nil	3.16	3.05	4.04	3.89	3.36[1]
	LSD (P=0.05)=2.22; P <0.001				

[1]Non-choice situations. All other cases shown in the table represent choice situations.

for feeding by gravid female weevils (Firth et al. 1993; Barker & Firth 1994). The results of Jensen et al. (2009) and the present study suggest that *E. uncinata* infection in meadow fescue and *Festulolium* leads to a further case where the correlation between susceptibilities to feeding and oviposition breaks down – that is, the high susceptibility of *E. uncinata*-infected meadow fescue and *Festulolium* to feeding by ASW does not predict the low susceptibility to ASW oviposition.

The results of Jensen et al. (2009) and the present study are, however, contrary to those of Patchett (2007) and Patchett et al. (2008) where feeding by ASW on field grown plants was found to be reduced by *E. uncinata* infection in meadow fescues and declined with increasing loline concentrations in the plant leaf tissues. In a laboratory experiment, Patchett (2007) and Patchett et al. (2008) found lower feeding on *E. uncinata* infected meadow fescue when plants were offered in a choice situation with an endophyte-free meadow fescue, but this difference failed to reach statistical significance. The reasons for these conflicting results are unknown, but they raise the possibility that deterrence in *E. uncinata* infected *Festuca* and *Festulolium* is conditional on plant genotype, environmental factor(s) influencing loline concentration and other plant quality attributes, and/or the phenological phase and physiological state of ASW employed in experiments.

Popay & Latch (1993) reported that NFL and NAL at 110 μg/g concentration failed to deter ASW from feeding. The loline content of the meadow fescue plant material used by Jensen et al. (2009) is unknown, but their artificial diet work included concentrations of different loline alkaloids over the range from 0 to 10,500 μg/g dry weight yet too failed to provide conclusive evidence of feeding deterrence. In the present experiments, total loline concentrations in the above-ground tissues of Barrier U2 were ~5600 μg/g, at which there was no evidence of feeding deterrence. In contrast, Patchett (2007) and Patchett et al. (2008) observed consistent feeding deterrence in field grown meadow fescue at loline concentrations 400 μg/g and above. Despite these uncertainties, it is clear that *E. uncinata* infection affords in large measure protection of Barrier from egg laying by ASW. This, coupled with the adverse effects of loline alkaloids on the development and feeding of the larval stage (Popay et al. 2009), may offer considerable agronomic advantage to Barrier U2 as a forage grass.

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