PRELIMINARY RESULTS ON THE BIOCHEMICAL RELATIONSHIP BETWEEN ADULT ARGENTINE STEM WEEVIL AND TWO ENDOPHYTES IN RYEGRASS

D.L. Gaynor*, D.D. Rowan**, G.C.M. Latch***, S. Pilkington***

*Entomology Division**, *Applied Biochemistry Division***, *Plant Diseases Division****
DSIR, Palmerston North

SUMMARY

Bioassays on cut leaves and leaf extracts have revealed that an extractable feeding deterrent present in *Acremonium* endophyte-infected perennial ryegrass (*Lolium perenne*) reduced adult Argentine stem weevil (*Listronotus bonariensis*) feeding preference for infected plants. This effect was not found with ryegrass containing a second, as yet unnamed, endophyte nor with extracts of cultured *Acremonium* endophyte. Separation of plant extracts has shown that the active substance is hydrophilic which does not correspond with the reported lipophilic properties of the lolitrems associated with ryegrass staggers.

INTRODUCTION

The larvae of Argentine stem weevil feed within ryegrass tillers causing tiller death while the adults chew the leaves but generally cause little damage. Kain *et al* (1982b) have identified stem weevil resistant perennial ryegrasses on which adult weevils produce fewer eggs and larvae. Stem weevil resistance has since been linked to the presence of endophytic fungal infection within the resistant plants (Prestidge *et al* 1982; Gaynor and Hunt 1983). Endophyte infection has also been linked to the presence of tremorgenic lolitrems (Gallagher *et al* 1981) associated with ryegrass staggers, a serious stock disorder (Fletcher and Harvey 1981). Two endophytic fungi have been isolated from New Zealand ryegrass (Latch 1983). The more common fungus is an *Acremonium* species while the other, generally referred to as a C-type endophyte, has not yet been identified.

The purpose of this study was to test whether the association between stem weevil resistance and endophyte infection held for both types of endophyte and to investigate the biochemical nature of the resistance mechanism.

METHODS

Bioassay with intact leaves

The bioassay of Kain *et al* (1982a), was modified to test the adult weevil feeding preference for 'Grasslands Nui' perennial ryegrass infected with either *Acremonium* endophyte only or with both C-type and *Acremonium* endophytes. 'Grasslands Ruanui' perennial ryegrass and 'Grasslands Ariki' hybrid ryegrass infected with only the C-type endophyte were also tested. Endophyte-free clones were produced by benomyl fungicide (Benlate 0.1 g ai/litre potting mix) treatment using the method of Latch and Christensen (1982). The plants were repotted several months prior to testing and checked microscopically for infection.

A 3 cm section of the youngest fully developed leaf was cut at the ligule from a tiller of an endophyte-infected plant and compared with an identical section of leaf cut from an endophyte-free clonal replicate. Both leaves were placed on moist filter paper in a petri dish containing one field-collected adult weevil. Twenty-four weevils were used per test and maintained at 16°C and 16:8 h photoperiod for 48 h. Each leaf was then

*Present address: Massey University, Palmerston North

scored for feeding damage on a 0-3 scale ('0' having no feeding holes, '1' having only 1 feeding hole, '2' having 2-3 feeding holes, '3' having >3 feeding holes). The feeding scores were analysed using the Wilcoxon signed-rank test (Siegel 1956) and are summarised as the percent weevils showing a feeding preference.

Plant extraction procedure

The bottom 8 cm of foliage from a test plant was blended twice in 95% ethanol. The total filtered extract was evaporated to dryness under vacuum and partitioned according to Fig. 1. Dialysis was against 10% ethanol.

Bioassay with plant extracts

To determine adult weevil preferences for different plant extracts two 1 cm diameter agar discs containing 4% agar, 4% cellulose powder and 5% sucrose were presented to one adult weevil in a petri dish. One disc contained a known weight of extract from endophyte-infected ryegrass adsorbed onto the cellulose powder, while the other disc contained the same weight of extract from endophyte-free clonal replicates of the infected plants. The final concentration of the extract in the agar was 2.5% which corresponded to concentrations of extractable material in the plant. Twenty-five to sixty weevils were used in each test and maintained at 16 °C with a 16:8 h photoperiod for 72 h. The amount of feeding on each disc was scored on a 0-3 scale ('0' having no sign of feeding, '1' having 1-2 small areas of the disc broken-up by feeding, '2' having 4-10 areas broken up, '3' having large areas of the disc shredded). The feeding scores were analysed using the Wilcoxon signed-rank test (Siegel 1956) and are summarised as the percent weevils showing a feeding preference.

Bioassay with cultured endophyte

In order to test whether fungus alone produced compounds which affect weevil feeding behaviour, mycelia from a liquid potato-dextrose culture of *Acremonium* endophyte were extracted in ethanol as above. An ethanol extract from an endophyte-free plant was adsorbed onto cellulose powder and to this was added the hyphal extract to give bioassay concentrations corresponding to 0.7 and 7 mg whole endophyte/g agar.

TABLE 1: Feeding preference of adult Argentine stem weevil when offered a choice of leaves cut from endophyte-free and endophyte-infected ryegrass.

Endophyte Type	Plant Type	No. Weevils/ Test	% Weevils Showing a Feeding Preference			
			Endophyte Free Leaf Preferred	Endophyte Infected Preferred	No Preference	No Feeding
<i>Acremonium</i>	Nui†	20	75***††	0	5	20
		20	70***	0	20	10
<i>Acremonium</i> and C-type	Nui	20	65*	10	5	20
		20	65*	15	15	5
C-type	Ruanui	40	20	42*	25	13
		28	18	43*	25	14
	Ariki	40	32	35 ns	30	3
		30	17	47*	26	10

† duplicate tests

†† Significant preferences were calculated on Wilcoxon signed-rank test (Siegel 1956)
ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

The choice test compared extracts of ryegrass without endophyte with extracts of the same plant to which cultured *Acremonium* had been added.

RESULTS AND DISCUSSION

The leaf feeding tests (Table 1) support the observations of Prestidge *et al* (1982), and Gaynor and Hunt (1983) that adult weevils prefer to feed on ryegrass free of *Acremonium* endophyte. Weevils did not show the same preferences when tested against ryegrass infected with the C-type endophyte but instead a possible preference for the infected material was indicated. When both endophytes were present preference for endophyte-free leaves remained.

The plant extract results (Table 2) confirmed the leaf feeding test data and demonstrated that feeding preference is affected by an extractable substance present in *Acremonium*-infected plants. They also showed that the C-type endophyte is associated with a reversal of this preference but the *Acremonium* influence is dominant when both endophytes are present.

TABLE 2: Feeding preference of adult Argentine stem weevil when offered a choice of agar discs containing ethanol extracts from endophyte-free and endophyte-infected ryegrass.

Endophyte Type	Plant Type	No. Weevils/ Test	% Weevils Showing a Feeding Preference			
			Endophyte Free Disc Preferred	Endophyte Infected Preferred	No Preference	No Feeding
<i>Acremonium</i>	Nui	25	68*	20	12	0
		57	47***	14	7	32
		60	47**	17	10	27
<i>Acremonium</i> and C-type	Nui	45	67***	16	13	4
		57	49***	21	5	25
		60	60***	15	5	20
C-type	Ariki	40	10	40**	17	33

See notes Table 1

Separation of the plant extract (Fig.1.) has suggested that the active compound has the following properties.

1. High activity is maintained after 200-fold purification.
2. It is dialysable and therefore of low molecular weight.
3. It is a highly polar hydrophilic compound and therefore differs from the lipophilic properties reported by Gallagher *et al* (1981) for the tremorgenic lolitrems which have been associated with ryegrass staggers.

The feeding preferences shown in the choice tests can be explained by either the presence of a feeding deterrent in the *Acremonium*-infected ryegrass or by higher levels of some feeding stimulant in the uninfected material. To clarify this a choice test was used to compare a 2.5% ethanol extract of endophyte-free Nui ryegrass with the same extract to which had been added 0.023% of a highly active fraction from an *Acremonium*-infected plant (Fig. 1. — chloroform:ethanol:water (10:30:1) fraction). Preference was shown ($p < 0.001$) for the discs that did not contain the added fraction, indicating that the added active fraction deterred the weevils from feeding. This clearly indicates that *Acremonium*-infected ryegrass contains a feeding deterrent active against the adult weevil.

Fig. 1: Scheme showing separation of endophyte-infected extract. (Significant reductions in weevil feeding in choice test are indicated as ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

Acronium grown in culture (Table 3) did not affect weevil preference at 0.7 and 7 mg/g concentrations. The concentration of endophyte in the plant has since been shown to range between 5 and 200 mg/g depending on conditions (Musgrave 1983). However, bioassays on ryegrass extracts at one-tenth the natural concentration of plant material did detect significant feeding deterrent activity, suggesting that activity should be detectable at the low level of 7 mg/g used in these tests. This suggests that the chemicals affecting feeding preference do not occur in pure fungal culture but arise by the plant-fungus interaction.

TABLE 3: Feeding preference of adult Argentine stem weevil when offered a choice of discs containing ethanol extracts from endophyte-free ryegrass and identical extracts to which was added cultured *Acronium* endophyte.

<i>Acronium</i> Concentration	No. Weevils/ Test	% Weevils Showing a Feeding Preference			
		Endophyte Free Disc Preferred	Endophyte Infected Preferred	No Preference	No Feeding
0.7 mg/g	42	24	33 ns	10	33
7.0 mg/g	42	36 ns	19	2	43

CONCLUSIONS

Acronium-infected ryegrass contains a feeding deterrent active against adult Argentine stem weevil. Initial results suggest that this compound is produced through the plant-fungal interaction and is not one of the lolitrems associated with ryegrass staggers. The deterrent does not appear to be present in ryegrass infected with only the C-type endophyte which emphasises the importance of careful identification of the endophyte during any breeding programme. Work on the *in vitro* endophyte activity and characterisation of the deterrent is continuing.

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