RESISTANCE TO ARGENTINE STEM WEEVIL IN PERENNIAL RYEGRASS INFECTED WITH ENDOPHYTES PRODUCING DIFFERENT ALKALOIDS

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ABSTRACT

Four pot trials were carried out to test the ability of endophytes which produce different alkaloids, to reduce Argentine stem weevil adult feeding, oviposition and larval damage on Nui or Ruanui perennial ryegrass. Nui ryegrass with the wildtype endophytes which produce peramine, ergovaline and lolitrem B, and two endophytes which produce peramine, but not ergovaline or lolitrem B, had lower adult feeding and oviposition than endophyte-free (E-) plants or plants infected (E+) with an endophyte (AR37) which produces none of these alkaloids. An endophyte which produces ergovaline, but not peramine or lolitrem B, had a small effect on adult feeding and may reduce oviposition. The wildtype and AR37 endophytes, and one of the three endophytes producing peramine, but not lolitrem B or ergovaline, reduced larval tiller damage. Of the peramine-producing endophytes resistance was lowest where mean peramine concentration was lowest and highest where mean peramine concentration was highest. A significant relationship between peramine and adult feeding was found.

Keywords: *Acremonium* sp., peramine, ergovaline, lolitrem B, insect resistance

INTRODUCTION

Alkaloids produced in endophyte (*Acremonium* sp.) associations with perennial ryegrass (*Lolium perenne*) are responsible for toxicity to grazing animals and adverse effects on some graminivorous insects, such as Argentine stem weevil (ASW) (*Listronotus bonariensis*), a major pasture pest in New Zealand. Research into the effects of endophytes on ASW has focused on peramine, a pyrrolopyrazine alkaloid, ergovaline, an ergot alkaloid, and lolitrem B, a member of the indole diterpene group of compounds. From laboratory feeding studies where alkaloids were incorporated into artificial diet we know that both peramine (Rowan and Gaynor 1986) and ergovaline (Popay *et al.* 1990) are highly deterrent to ASW adults. To a lesser extent, peramine is also a deterrent to larvae (Dymock *et al.* 1989a) whereas ergovaline has no effect on this stage (A.J. Popay, unpublished). Lolitrem B, however, has no effect on ASW adults but is toxic to larvae (Prestidge and Gallagher 1985; Dymock *et al.* 1989b). Of these three alkaloids, ergovaline and lolitrem B are mammalian toxins (Gallagher *et al.* 1984; Fletcher 1993).

In New Zealand all endophytes in perennial ryegrass produce peramine, lolitrem B and ergovaline. In parts of Europe, however, endophytes have been found in perennial ryegrass and tall fescue (*Festuca arundinacea*) which produce various combinations, or which do not produce any, of these alkaloids. Inoculation of these endophytes into a single ryegrass cultivar has enabled studies to be made of the effect of these alkaloids *in planta* on Argentine stem weevil with the aim of evaluating the degree to which ASW resistance of these novel associations could be accounted for by the peramine, ergovaline and lolitrem B content. This paper reports the results of some of these studies.

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METHODS

Four pot trials were conducted at the Ruakura Agricultural Research Centre in 1994 (Trials 1 and 2) and 1995 (Trial 3 and 4). The different endophyte strains used in these trials, their host grasses and alkaloid profiles are given in Table 1. At the completion of each trial all plants were checked for the presence of endophyte by staining leaf sheath tissue in aniline blue and microscopic examination.

TABLE 1: The occurrence of peramine, ergovaline and lolitrem B in host grasses of different endophytes tested for their resistance to Argentine stem weevil.

Trial	Host ryegrass	Endophyte	Alkaloid presence or absence		
			Peramine	Ergovaline	Lolitrem B
Trial 1 ¹	Nui	AR555	+	+	+
		AR553	-	+	-
		AR501	+	-	-
		AR37	-	-	-
Trial 2 ²	Parent	AR40	-	-	-
	Nui	AR553	-	+	-
Trial 3 ³	Nui	Wildtype	+	+	+
		AR1	+	-	-
		AR501	+	-	-
		AR37	-	-	-
Trial 4 ³	Ruanui	AR22	+	-	-

¹Trial included two Nui endophyte-free controls, one was the same clone as the host of AR555, the other was from seed.

²For each endophyte there were endophyte-free clones.

³Endophyte-free controls were seed of the same host cultivar used in each trial.

Trials 1 and 2

In Trial 1 four perennial ryegrass plants cv. Grasslands Nui containing the endophytes AR37, AR553, and AR501 were each divided into four to give a total of 12 plants containing each endophyte. Five clonal Nui ryegrass plants containing the AR555 endophyte and five E- plants were similarly divided to give 12 plants of each. Endophyte-free Nui ryegrass plants grown from seed were also divided to provide a second E- control. The plants, with between four and 20 tillers each, were grown in standard commercial potting mix in 8 cm polystyrene cups with drainage holes and maintained throughout the trial, with regular watering and trimming as necessary, in the glasshouse.

On February 9, 1 month after dividing plants, six plants, representing the four different endophytes and two E- controls, were placed adjacent to one another, in two rows of three pots, in a polystyrene tray. There were 12 replicate groups of such plants with pots arranged randomly. Six adult ASW, collected from the field the previous evening, were released onto each plant and wire frames with fine nylon net covers were placed over each of the 12 trays.

In Trial 2, conducted in the glasshouse at the same time as Trial 1, two sets of 12 clonal endophyte-free and 12 endophyte-infected ryegrass plants (see Table 1 for host plant) containing either endophyte AR40 or AR553 were obtained by division as above. For each endophyte, 12 replicates of a positive and negative clonal pair were placed together in liver pails. Five adult ASW were released onto each plant and liver pails were covered with a fine nylon net.

One week later covers were removed from both trials. Each tiller on each plant was scored for adult feeding on a scale of 0 - 5 (0 = no feeding; 5 = numerous feeding scars over entire leaf blade) and checked *in situ* for presence of eggs. The number of eggs on each tiller was recorded and, because of the erratic nature of the oviposition, eggs were removed from plants in Trial 1 on which oviposition was exceptionally high, to leave approximately 20/plant. These eggs, and others obtained from field collected weevils, were sterilised and placed together on artificial diet in petri dishes at 20°C. One month later mixed age larvae were released at a rate of 2/plant, onto all plants in Trial 1 on which no oviposition had been recorded, in order to test for larval resistance.

On March 25 all tillers were cut at the base and plant material was stored at 4°C while each tiller was examined by dissection for larval damage. Larval damage was scored as light (small entrance/exit hole only, no mining of the tiller), moderate (tiller partially mined) or severe (tiller completely mined). The number of live and dead tillers on each plant was also recorded and the green leaf area was measured.

Trials 3 and 4

Trial 3, conducted in a shadehouse in 1995, was similar to Trial 1. Nui ryegrass infected with four different endophytes (Table 1) and endophyte-free Nui were planted into 12 cm dia. pots. Ten replicate pots of E- Nui ryegrass plants and those infected with the wildtype endophyte were obtained from seed and replicate plants with the three other endophytes were obtained by division of existing plants. Five pots, representing one of each treatment, were placed together in polystyrene trays as described for Trial 1. Six field collected adult ASW were released onto each pot and trays were covered as before.

In Trial 4, set up at the same time as Trial 3, ten replicate plants of E- Ruanui perennial ryegrass (obtained from seed) and Ruanui with endophyte AR22 (obtained by plant division) in 12 cm dia. pots, were placed individually into liverpails. Ten adult ASW were released onto each pot and liverpails were covered.

Covers were removed from both trials 1 week later and adult feeding and oviposition was recorded as described above on 20 tillers per plant. Sixteen days later 20 tillers were removed from each plant and checked for larval damage as in Trials 1 and 2. After a further 2 weeks plants were cut at the base and all tillers from each plant were freeze-dried and finely ground. They were stored below 0°C before HPLC analysis of peramine content.

Analysis

An analysis of variance was carried out on adult feeding scores, number of eggs, number of tillers with larval damage and peramine concentration, with log transformation where this was appropriate, in Trials 1 and 3. In Trials 2 and 4 data were compared using student's *t* - test. Linear regression analyses were used to investigate the relationship between green leaf area and larval damage in Trial 1, egg numbers and larval damage in Trial 3, and peramine and ASW damage (adult feeding, oviposition and larval damage) in Trials 3 and 4. For the latter regressions, E- and endophytes not producing peramine were excluded from the analysis. Proportions of damaged tillers which were severely damaged were analysed using a generalised linear model.

RESULTS

Trial 1

ASW adult feeding, oviposition and larval damage and green leaf area were recorded on a per plant and a per tiller basis. Differences found between treatments were the same for each parameter and so only the latter data are presented. Adult feeding was lower ($P < 0.001$) on ryegrass containing the endophytes AR555, AR501 and AR553, which produce peramine and/or ergovaline, than on E- plants or plants containing the endophyte AR37, which does not produce these alkaloids (Table 2). There were no significant differences in egg numbers, but three endophytes, AR37, AR555 and AR501, had less larval damage ($P < 0.05$) compared to the E- controls. AR37 had the lowest ($P < 0.05$) proportion of severely damaged tillers (29%). For all other E+ plants and the E- controls the greater proportion of damaged tillers were severely damaged. Green leaf area/tiller was highest on plants which had the lowest

level of larval damage (AR37) and lowest on endophyte-free plants and plants with the endophyte AR555. Green leaf area on plants with the AR501 or AR553 endophytes was less ($P<0.05$) than on the AR37 plants and more ($P<0.05$) than on the E- and AR555 plants. There was a significant relationship between green leaf area/tiller and the number of tillers damaged/10 tillers (slope = -47; $P<0.001$). When adjusted for the number of damaged tillers, green leaf area was greater ($P<0.01$) for plants with the AR37 endophyte than for plants with the AR553 and AR501 endophytes and the E-plants obtained from seed (Table 2). The plants containing the AR555 endophyte and its E- clones had a lower green leaf area ($P<0.05$) for a given number of damaged tillers than all others.

TABLE 2: Green leaf area and adult Argentine stem weevil adult feeding and oviposition, number of tillers with larval damage and proportion of those which were severely damaged on ryegrass infected with different endophytes in Trial 1.

Endophyte	Adult feeding score/tiller	No. of eggs/10 tillers	Tillers with larval damage No./10 tillers	Proportion with severe damage ($\pm$ SE)	Green leaf area/tiller (mm^2)	Green leaf adjusted for damaged tillers
AR555	2.5	3.4	2.4	0.84 (0.08)	416	400
AR553	2.4	1.7	2.6	0.72 (0.09)	561	557
AR501	1.9	1.9	1.8	0.61 (0.09)	564	521
AR37	3.7	3.5	0.9	0.29 (0.13)	876	788
Nil	3.8	1.8	4.4	0.74 (0.06)	418	493
Nil ¹	4.1	3.1	4.4	0.76 (0.07)	328	405
LSR ²		1.75	1.71			
SED	0.22				90	82.7

¹ Plants in this endophyte-free control were the same genotype as plants with AR555 endophyte

² Least significant ratio

Trial 2

Adult feeding score/tiller was lower ($P<0.05$) on ryegrass with the endophyte AR40 than on its E- clonal pair (means 3.5 and 3.1, SEM 0.17). There were no differences in the number of eggs/10 tillers (means 4.0 for E+ and 4.2 for E-) but the number of tillers with larval damage/10 tillers was less ($P<0.05$) on the E+ than on the E- (means 0.1 and 2.7, SEM 1.59).

There was no difference in the feeding score/tiller between plants with AR553 endophyte and their E- pairs but feeding score/plant was less ($P<0.05$) on the E+ plants (means 23.8 and 32.9, SEM 3.09). The mean number of eggs/tiller for the E+ and E- plants respectively was 3.2 and 7.1 but this difference was not significant due to a high variability between plants. Larval damage was lower ($P<0.01$) on E+ than on the E- plants (means 0.9 and 4.1 damaged tillers/10 tillers, SEM 0.86).

Trial 3

Nui ryegrass with the wildtype or AR501 endophytes had lower adult ASW feeding ($P<0.001$) than ryegrass without endophyte or with the endophytes AR1 or AR37 (Table 3). Egg numbers were lowest ($P<0.001$) on plants with AR1, AR501 and wildtype endophytes compared with E- or AR37 plants but the least ($P<0.001$) number of tillers with larval damage occurred on plants infected with AR37 and the wildtype endophytes. The number of tillers with larval damage was no fewer on plants infected with the AR1 or AR501 endophytes than it was on E- plants. Only 8% of the damaged tillers on plants with AR501 were severely damaged compared with 36% and 51% on plants with AR1 and E- plants respectively. Plants with the wildtype endophyte also had a very low proportion of severely damaged tillers.

TABLE 3: Adult Argentine stem weevil adult feeding and oviposition, number of tillers with larval damage and proportion of those which were severely damaged on ryegrass infected with different endophytes in Trials 3 and 4, and mean concentration of peramine in plants.

Endophyte	Adult FS/tiller	No. eggs/20 tillers	Tillers with larval damage No./20 tillers	Propn. with severe damage ($\pm$ SE)	Peramine conc (μ g/g)
Trial 3¹					
Wildtype	0.51	7.3	3.6	0.06 (0.06)	40.2
AR1	1.70	9.6	11.1	0.36 (0.05)	15.4
AR501	0.93	11.4	10.4	0.08 (0.03)	20.1
AR37	1.79	20.9	1.6	0.28 (0.11)	0
Nil	1.45	21.6	12.2	0.51 (0.05)	0
LSR		1.66	1.49		
SED	0.214				4.38 ³
Trial 4²					
AR22	2.16	8.3	5.2	0.04 (0.03)	34.5
Nil	3.12	17.5	10.3	0.36 (0.05)	0
SEM	0.22	4.2	1.64		

¹Host plant was Grasslands Nui

²Host plant was Grasslands Ruanui

³SED for all plants containing peramine (including AR22)

Over all treatments there was a relationship between the number of eggs laid and number of damaged tillers (ln damaged tillers/20 tillers adjusted for ln eggs/20 tillers, slope 0.325 ± 0.101 , $P < 0.01$). There were fewer damaged tillers for a given number of eggs in plants with the AR37 and wildtype endophytes compared with that on endophyte-free plants and plants with the AR1 and AR501 endophytes (Fig. 1).

FIGURE 1: Relationship between number of damaged tillers and number of eggs for different endophytes in Nui and Ruanui perennial ryegrass.

Trial 4

In Ruanui the AR22 endophyte significantly reduced adult feeding ($P < 0.01$) and larval damage ($P < 0.05$) compared with the E- controls but the difference in oviposition was not significant (Table 3). There were fewer tillers damaged per eggs laid in plants with AR22 than in E- plants (Fig. 1).

Peramine concentration in plants with the wildtype and AR22 endophytes was higher ($P < 0.001$) than in plants with the AR501 or AR1 endophytes (Table 1). There was a significant negative relationship between peramine concentration and adult feeding score for all peramine-producing plants pooled (slope -0.016 ± 0.0075 , $P < 0.05$) but there was no relationship between peramine levels and oviposition or larval damage.

DISCUSSION

Because of the mammalian toxicity caused by the presence of ergovaline and lolitrem B in endophyte-infected plants, it is of great importance to assess the ability of endophyte-infected ryegrass to resist ASW in the absence of these two alkaloids. Previous field studies have shown that ryegrass infected with an endophyte which does not produce lolitrem B is as resistant to ASW attack as the wildtype endophyte which produces this alkaloid (Fletcher *et al.* 1991; Popay *et al.* in prep.). The aim of the trials reported here was to evaluate the importance of ergovaline in endophyte-mediated resistance to ASW and the capacity of endophytes which produce peramine, but not ergovaline or lolitrem B, to prevent ASW damage to ryegrass.

Although adult ASW are highly sensitive to ergovaline (Popay *et al.* 1990), concentrations of this alkaloid are low in leaf blades where adult ASW are feeding compared with the leaf sheath, stems and pseudostems (Beleskey *et al.* 1989; Tapper 1993). Nevertheless, the endophyte AR553 (ergovaline, no peramine or lolitrem B) was found to cause some reduction in adult feeding, although this was not always significant. Unlike ergovaline, peramine is translocated freely in the plant (Tapper 1993) and a significant relationship was found between peramine concentration and adult feeding score for all endophytes which produced peramine in the trials reported here.

A reduction in oviposition is the primary defence mechanism against ASW in grasses infected with the wildtype endophyte and it is therefore important to retain this attribute. Unfortunately egg numbers were highly variable in Trials 1 and 2 in 1994 and no endophytes significantly lowered egg numbers although both the AR553 (ergovaline, no peramine or lolitrem B) and the AR501 (peramine, no ergovaline or lolitrem B) endophytes had relatively low oviposition. In Trial 3, the wildtype and AR501 endophyte had the lowest number of eggs and both had fewer eggs than endophyte-free plants and another peramine-only (ie. no ergovaline or lolitrem B) endophyte AR1. The ergovaline-only (ie. no peramine or lolitrem B) endophyte was not included in this trial. Further work is needed to determine if the effects of ergovaline are at least additive, if not synergistic, with peramine in reducing oviposition. No direct relationship was found between peramine concentration and oviposition in the plants.

A reduction in larval damage occurred on plants infected with the ergovaline-only endophyte in Trial 2, despite evidence from artificial diet studies that ergovaline does not affect larvae, but this difference may have been due to the low oviposition on infected plants or to the presence of another factor which affects larvae. In Trial 1 the same plants did not have significantly fewer damaged tillers compared with E- plants and, furthermore, 74% of the damaged tillers were severely damaged indicating that there was no deterrence to larvae. In Trial 1 the peramine-only endophyte AR501 reduced the number of tillers damaged whereas in Trial 3, where the relative roles of oviposition and larval resistance in endophyte-mediated defence against ASW were more closely examined, both peramine-only endophytes had as many tillers damaged for a given number of eggs as did the endophyte-free controls. Although the latter result suggests that peramine levels were not sufficient to reduce larval damage, the low severity of damage recorded for the AR501 endophyte compared with that for the AR1 endophyte and endophyte-free plants implies otherwise. In the AR501 endophyte-

infected plants peramine levels may have been sufficient to deter larvae from completely mining tillers and cause them to transfer frequently to new tillers, hence the high number of tillers damaged. Severity of damage is likely to increase as larvae mature, however. In Trial 1 tillers were not sampled until a month after egg laying and by this time tillers in plants with the AR501 endophyte were as severely damaged as in the endophyte-free controls. Both the AR22 endophyte in Ruanui (Trial 4) and the wildtype endophyte in Nui (Trial 3) provided some resistance to larvae. Mean peramine levels were higher for the latter endophytes than for the AR501 and AR1 endophytes, which may be an important factor in determining larval resistance, although no relationship was found between peramine levels and larval damage over all peramine-producing plants. We cannot rule out the possibility of the presence of another factor in some of these endophytes which directly affects larvae. An endophyte (AR555) in Trial 1, similar to the wildtype endophyte in Trial 3, did not show high levels of resistance to ASW, but plants containing this endophyte were poor as evidenced by the low green leaf area recorded for both the clonal E+ and E- plants.

Most surprising from the results presented here, was the very low levels of larval damage recorded for the endophytes AR40 and AR37 which do not produce any of the known alkaloids. The AR40 endophyte caused a small but significant reduction in adult feeding in its original host plant but no such reduction was caused by AR37 in Nui ryegrass and neither endophyte reduced oviposition. These results provide evidence of another compound which mainly affects ASW larvae. Production of this compound by other endophytes (or by host grasses infected with particular endophytes) may explain the larval resistance observed in other plant/endophyte combinations in these trials. A similar endophyte to the AR40 endophyte included here has also been shown to reduce feeding by adult black beetle (Ball *et al.* 1994).

In the trials reported here, the wildtype endophyte has provided a stronger defence against ASW than endophytes which produce peramine only. Ergovaline appears to have a role in enhancing endophyte-mediated resistance to ASW but further work is required to elucidate this. Mean peramine concentrations were high where resistance to ASW was strong, but a relationship between peramine and ASW was only established for adult feeding. These trials have also revealed the existence of another, as yet unidentified, factor which is a potent deterrent and/or toxin to ASW larvae.

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