Naturally-Occurring and Synthetic Loline Alkaloid Derivatives: Insect Feeding Behavior Modification and Toxicity[1,2]

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ABSTRACT Methanol extracts of tall fescue plants (*Festuca arundinacea* Schreb.) infected with an endophytic fungus (*Acremonium coenophialum* Morgan-Jones and Gams) contain loline alkaloids which deter feeding and are toxic to insects. This study was conducted to determine the effect of several naturally-occurring and semi-synthetic loline alkaloid derivatives on insects. The influence of these compounds on the feeding behavior and weight of fall armyworm larvae, *Spodoptera frugiperda* Smith, and European corn borer larvae, *Ostrinia nubilalis* Hübner, was evaluated using two-choice, diet-incorporated feeding behavior modification bioassays. Toxicity of these compounds to greenbugs, *Schizaphis graminum* Rondani, was evaluated and compared with the toxicity of the insecticide nicotine sulfate. Fall armyworm larvae were more susceptible, in terms of feeding behavior modification and reduced weight gain, to specific loline derivatives than European corn borer larvae. N-acetyl loline appeared to show toxic effects, in terms of reduced larval weight in the absence of feeding behavior modification, toward both fall armyworm and European corn borer larvae. Several of the naturally-occurring loline alkaloids, namely N-formyl loline, N-acetyl loline and N-methyl loline, had LC_{50} values against apterous greenbug adults similar to nicotine sulfate.

KEY WORDS Loline alkaloids, toxicity, feeding deterrence, fall armyworm, *Spodoptera frugiperda*, European corn borer, *Ostrinia nubilalis*, greenbug, *Schizaphis graminum*.

The plant kingdom uses chemical substances as defenses against insects (Jacobson 1981). A recent report which outlined one such plant-insect interrelationship (Johnson *et al.* 1985) indicated that tall fescue plants (*Festuca arundinacea* Schreb.) infected with an endophytic fungus (*Acremonium coenophialum* Morgan-Jones and Gams) produced compounds which deter the feeding of aphids, *Rhopalosiphum padi* (L.), *Schizaphis graminum* (Rondani) and milkweed bugs, *Oncopeltus fasciatus* Dallas. In an effort to identify the compounds which were active in deterring feeding, these authors prepared extracts of tissue from endophyte-infected plants. Because the extracts contained a mixture of alkaloid types, active compounds were not specifically identified.

Using extracts from seeds of endophyte-infected plants, Yates *et al.* (1989) showed that N-formyl loline and related alkaloids were potent toxins to milkweed

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bugs. Further fractionation of the extracts revealed the presence of several derivatives of loline, namely N-methyl loline and N-acetyl loline, as well as N-formyl loline (Yates *et al.* 1990). The role of individual loline derivatives in insect resistance is a topic deserving further study. As a first step towards this goal, we investigated the effect of 3 naturally-occurring and 16 synthetic loline derivatives on the feeding behavior of fall armyworm, *Spodoptera frugiperda* Smith, larvae and European corn borer, *Ostrinia nubilalis* Hübner, larvae. We also measured the toxicity of these compounds against greenbugs, *Schizaphis graminum* Rondani.

Materials and Methods

Loline Derivative Preparation. N-acyl loline derivatives and N-methyl loline were prepared from pure loline (Fig. 1) as previously described (Petroski *et al.* 1990).

Two Choice, Diet Incorporated Feeding Behavior Modification Bioassay. Loline and loline derivatives were dissolved in solvent (methanol or methylene chloride depending upon solubility) and applied to powdered cellulose. After drying, the cellulose powder-loline derivative mixtures were placed into test tubes (7.5 × 1.0 cm), and the tub filled to the 4-cm mark with liquid artificial fall armyworm diet medium (Bioserv #F9179). Test tube contents were thoroughly mixed, and the diet medium was drawn into a plastic soda straw (0.5 cm diameter) to a height of 10 cm. After solidification, the diet medium was pushed out of the straw and cut into 0.5 cm sections. Final loline derivative concentration in the diet was 2000 ppm. Two sections containing the compound to be tested were placed opposite one another, in a 5-cm plastic petri dish. In the same dish, also placed opposite one another were two sections of diet that were prepared the same way, using the same solvent but containing no loline derivatives. Each diet section was equidistant from the one next to it and approximately 0.5 cm from the edge of the petri dish.

Twenty newly-hatched fall armyworm or European corn borer larvae were placed into each petri dish. Petri dishes containing insects were placed in darkness at 27°C for 16 to 20 h; following this the dishes and contents were frozen. The number of larvae present on each diet type as well as nonresponding larvae in each dish were recorded. The feeding behavior modification bioassay was a test patterned after that used to study phagostimulants for European corn borers (Bartelt et al. 1990). Newly hatched larvae typically wander about the petri dish for several hours, appearing to "taste" the diet materials they encounter. Within 16-20 h, they normally settle down to feed on an acceptable piece of diet and remain there; the corn borer larvae also produce a silk "nest" at this feeding site. In preliminary observations, the larvae did not leave these feeding sites when the petri plate was placed in the freezer. Freezing facilitated counting all of the larvae at each diet section but did not change the larval distribution. The number of nonresponding larvae in the tests was low (generally $< 10\%$) because at least the control diet was readily accepted by the larvae of both species. The final distribution of larvae was interpreted as a measure of feeding behavior modification. If the larvae were equally distributed between the two types of diet, then there was no evidence for feeding behavior modification, but if the larvae were found predominantly at the control diet, then the test compound was concluded to modify feeding behavior.

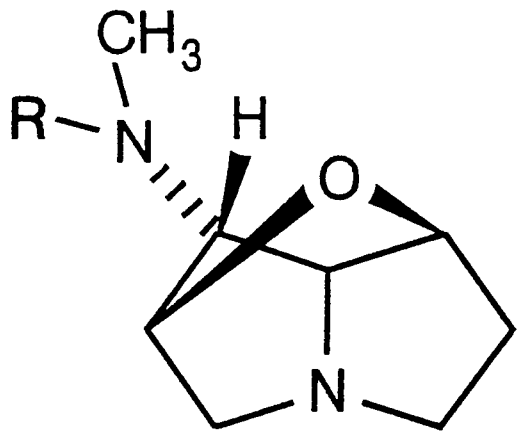

Fig. 1. Structure of the parent compound. "R" = H for loline, for all other N-Acyl derivatives, R is explained in the compound name.

Statistical analysis was by Chi-square tests. The null hypothesis was a 50:50 distribution of larvae between the test and control diets. A significant Chi-square statistic ($P < 0.05$) was evidence for a difference from the control. Using five replications gave greater power to this Chi-square test and also allowed the consistency of the ratio, treatment: control, to be evaluated among replications, again with Chi-square tests. These tests were non-significant ($P > 0.05$) in almost all cases, indicating the data were "well-behaved" and that the Chi-square test for detecting differences between treatments and controls was appropriate. The data were summarized as "larvae distribution ratios," defined as the total number of larvae at the treatment divided by the total at the control diet.

No Choice Toxicity Bioassay. Toxicity tests involved incorporating samples into diet in the same manner as shown above. Fifty (50) mg of sample was incorporated into 2.5 g of liquid diet and then drawn into 5 soda straws to the 21 cm mark in each. After solidification, the straws were cut into 3 cm sections and placed one per cup, (cups were clear plastic with a 1 oz. volume). Newly hatched fall armyworm or European corn borer larvae (15 per trial, one larva per cup) were placed in cups containing treated diets as well as controls containing diet only and diet plus solvent (replicated 5 times). Insects were allowed to feed for 8-9 days at which time mortality and weights of surviving insects were recorded. Significant differences within larval weight ratios were determined by ANOVA.

Greenbug Toxicity Studies. Clones of field-collected greenbug were maintained in culture on seedlings of barley (*Hordeum vulgare* cv Hazen) in a growth chamber under 16 h photoperiods at 23°C and 85% RH.

Ten adult apterous greenbugs were transferred to 8-day-old barley plants (one-leaf fully expanded) which were then covered with a 7 × 40 cm vented, clear-plastic tubular cage. After greenbugs were on the plants for 24 hours, the plants were sprayed with loline solutions. Immature greenbugs born during the 24 h were not removed before spraying. Loline derivatives were dissolved in 95% ethanol,

which was quantitatively added to application solutions which contained a final concentration of 0.05% (v:v) ethanol and 0.1% (v:v) polyoxyethylene sorbitan monooleate (a surfactant). Solutions containing 0, 400, or 4000 μg/ml nicotine sulfate, loline, or loline derivatives were applied to plants using a thin-layer chromatographic atomizer, and the cages were replaced. Each cage was inspected for wandering greenbug adults 2 hours after spray application. The number of live apterous adult greenbugs, as well as the number of dead adult greenbugs, was determined 18 h after spray application. Each concentration of each loline derivative tested was replicated 3 times. Greenbug-infested plants sprayed with solutions containing the same concentration of ethanol and polyoxyethylene sorbitan monooleate served as controls.

The amount of solution sprayed on each plant was previously measured on uninfested plants. Plants were clipped below the soil level and weighed. The clipped plants were placed back in the pot, held in an upright position with soil. After spraying with the atomizer, plants were immediately weighed again. The difference in weights before and after spraying was approximately 46 mg.

Initial screening of loline derivatives identified 5 compounds with insecticide activity against the greenbug equal to or greater than nicotine sulfate. Several additional experiments were conducted to determine the LC_{50} of these compounds. Between 6 and 14 concentrations of each compound, replicated on 3 separate plants, were evaluated. Probit regression analysis (POLO-PC, LeOra Software, 1987) using maximum likelihood estimates of the intercept, slope, and threshold response rate for the assay data was performed. The 95% confidence limits for LC_{50} were calculated for these tests by probit methods.

Results

It is clear from the data presented in Table 1 that some tested compounds had significant effects upon the feeding behavior of fall armyworm and European corn borer larvae. The number of fall armyworm larvae feeding on diet treated with 2000 ppm loline derivatives divided by the number of larvae feeding on untreated diet (larvae distribution ratios) were significantly reduced by a greater number of compounds tested and to a greater extent than the larvae distribution ratios of the European corn borers (Table 1). This species response differential was also evident in weights of larvae. Fall armyworms tended to show slower weight gain than European corn borers. Compounds that caused significant effects on both species in both parameters studied included N-lauroyl loline, N-palmitoyl loline, and N-cinnamoyl loline.

In the two choice antifeedant bioassay, the number of nonresponding insects was low. The number of nonresponding European corn borer larvae averaged less than 10% per treatment with a range of 0-19% (data not shown). For fall armyworm larvae, nonresponse averaged 11% per sample with a range of 0-47% nonresponse. The compounds N-propionyl and N-butyryl loline showed unusually high nonresponse (47% and 43%, respectively). Because the responding larvae showed no preference between treated and control diets, this phenomenon was not further investigated.

In the no-choice toxicity test, European corn borers had no mortality in either samples or controls after 9 days (Table 1). Larvae in the 7 control groups had a mean weight of 19.5 mg (range of 13.5-24.8 mg). In the fall armyworm, larvae in the 7 control groups had a mean weight of 84.6 mg (range of 70.3-103.8 mg). Mean

Table 1. Results of loline bioassays against fall armyworms and European corn borers (2000 ppm).

Compound name	Armyworms		Corn borers	
	2-choice test	No-choice test	2-choice test	No-choice test
	Larvae distribution ratio†	Larval weight ratio‡ (%)	Larvae distribution ratio†	Larval weight ratio‡ (%)
Loline	.70	92	.60	150
N-formyl loline	.24***§	40***	.73	89
N-acetyl loline	.71	71**	.86	60**
N-methyl loline	.65	95	.65*	147
N-propionyl loline	.96	89	.94	61
N-butryl loline	.84	70**	.59*	76
N-isobutryl loline	.39***	100	.85	75
N-cyclopropanoyl loline	.56**	89	1.12	76
N-hexanoyl loline	.42***	98	.92	104
N-octanoyl loline	.27***	77*	1.00	71*
N-nonanoyl loline	.12***	58***	1.11	48**
N-decanoyl loline	.11***	49***	1.25	46***
N-lauroyl loline	.12***	38***	.61*	67*
N-myristoyl loline	.11***	48***	.81	81
N-palmitoyl loline	.07***	6***	.58*	42***
N-benzoyl loline	.36***	48***	.56*	85
N-cinnamoyl loline	.29***	62***	.45***	58**
N-hexylbenzoyl loline	.10***	57***	.94	42***
N-dimethylacroyl loline	.62*	78*	.71	83

† Feeding ratio = # of larvae on treated diet/# of larvae on control diet.

‡ Larval weight ratio = mean larval weight for treated diet/mean larval weight for control diet (x 100).

§ *, **, ***, represent significant differences from the controls at the 95%, 99%, 99.9% confidence levels using chi-square analysis for two-choice tests and ANOVA for larval weight data.

mortality in the controls was 10% after 8 days (range of 0-20%). N-formyl loline has the highest mortality of the samples tested (33%). Chi-square analysis indicated this was very close to being active at the 95% level. No other sample had greater than 15% mortality (data not shown).

Results of screening various concentrations of loline derivatives for toxicity against greenbugs are shown in Table 2. Nicotine sulfate, a contact insecticide, was also included in this test. Two hours after spray application, no differences in greenbug behavior (wandering) were apparent (data not shown). Results of greenbug counts taken 18 h after spraying indicated that several compounds showed toxicity equal to or greater than nicotine sulfate against adult greenbugs at concentrations of 4000 μg per ml of spray solution (Table 2). These compounds included N-formyl loline, N-acetyl loline, N-methyl loline, N-propionyl loline, and N-myristoyl loline. These compounds were further tested to determine LC_{50}. Concentrations of several loline derivatives needed to reach LC_{50} compared favorably with nicotine sulfate (Table 3). The slopes of the regression lines for all of the loline derivatives

Table 2. Average number of greenbugs alive on plants after spray treatment with nicotine sulfate, loline, or loline derivatives tested at 0, 400, and 4000 μg/ml.

Compound	Number of alive greenbugs*		
	0	400 μg/ml	4000 μg/ml
Nicotine sulfate	8.3 ± 0.9	3.3 ± 0.7	0.3 ± 0.3
Loline	6.6 ± 1.2	7.7 ± 1.2	3.3 ± 0.8
N-formyl loline	6.7 ± 1.8	7.7 ± 0.9	0
N-acetyl loline	8.3 ± 1.2	2.5 ± 0.5	0
N-methyl loline	7.7 ± 1.3	3.7 ± 0.9	0.3 ± 0.3
N-propionyl loline	6.7 ± 0.3	5.0	0.3 ± 0.3
N-butyryl loline	7.0 ± 0.6	8.0 ± 0.6	3.0 ± 0.6
N-isobutryl loline	7.3 ± 1.2	7.0 ± 1.2	4.7 ± 1.3
N-cyclopropanoyl loline	7.0 ± 1.5	6.0 ± 0.6	2.3 ± 0.3
N-hexanoyl loline	7.7 ± 0.9	6.7 ± 1.9	7.0 ± 0.6
N-octanoyl loline	7.7 ± 1.2	5.0 ± 1.5	6.0 ± 0.6
N-nonanoyl loline	5.0 ± 2.1	6.0 ± 0.6	6.3 ± 1.8
N-decanoyl loline	6.0 ± 1.5	7.3 ± 0.9	1.7 ± 1.2
N-lauroyl loline	7.7 ± 0.3	5.7 ± 0.9	2.3 ± 1.5
N-myristoyl loline	8.7 ± 1.2	6.3 ± 0.7	0.3 ± 0.3
N-palmitoyl loline	6.0 ± 1.0	5.7 ± 0.7	3.0 ± 0.6
N-benzoyl loline	8.0 ± 1.0	6.7 ± 1.3	5.7 ± 0.9
H-hexylbenzoyl loline	6.7 ± 1.8	7.0 ± 1.5	5.7 ± 0.3
N-dimethylacroyl loline	5.3 ± 0.9	8.0 ± 0.6	1.3 ± 0.7
N-nicotanoyl loline	7.0 ± 1.5	9.3 ± 0.7	6.3 ± 1.8

* Values represent mean ± SE.

Table 3. Average LC_{50} values and confidence intervals for nicotine sulfate and selected loline derivatives for adult greenbugs.

Compound name	Concentration range tested (μg/ml)*	LC_{50} (μg/ml)	0.95 C.I.	Slope†
Nicotine sulfate	0-4000	199	45-459	1.2 ± 0.3
N-formyl loline	0-3300	437	237-615	3.1 ± 0.7
N-acetyl loline	0-500	343	190-532	2.3 ± 0.8
N-methyl loline	0-4000	361	81-613	1.6 ± 0.4
N-propionyl loline	0-4000	551	234-907	2.5 ± 0.5
N-myristoyl loline	0-4000	1683	1115-3109	3.9 ± 0.9

* Between 6 and 14 different concentrations within the ranges indicated, replicated 3 times, were evaluated for each compound.

† Values represent slope ± SE.

tested, with the exception of N-methyl loline, were similar. The slopes of the regression lines for nicotine sulfate and N-methyl loline were also similar.

Discussion

The presence of N-acyl loline derivatives in the diet modified fall armyworm and European corn borer feeding. However, the specific effective derivative and the magnitude of this effect was dependent upon the species of larvae tested. Fall armyworm larvae feeding ratios were significantly reduced by 14 of the 19 loline derivatives tested, while European corn borer feeding ratios were significantly reduced by only 6 of the 19 tested. These results suggest that different insect species respond differently to loline derivatives.

For many loline derivatives, significant decreases in fall armyworm larvae distribution ratios also coresponded to lower larval weights in the no-choice toxicity test. These results suggest that lack of weight gain with these derivatives may have resulted simply from decreased consumption of diet rather than toxicity.

Previous experimental results (Mikolajczak *et al.* 1989) showed that fall armyworm larvae with weights less than 5% of controls after 13 days would not survive to maturity. Based on this result, N-palmitoyl loline, which caused no mortality after 8 days with a mean weight equal to 6% of the controls, could be expected to cause fairly high mortality by the time insects reach maturity. In several loline derivative treatments, notably N-acetyl loline, fall armyworm and European corn borer larval weights did not attain the levels seen in larvae feeding on control diet, even though no significant feeding behavior modification effects were recorded. Results such as these could be expected if these compounds were acting as metabolic toxins. This hypothesis is supported by the work of Yates *et al.* (1989) who found that N-acyl loline derivatives were toxic to the milkweed bug.

To further investigate this toxicity against insects, loline derivatives were sprayed onto plants infested with greenbugs. The level of susceptibility of adult greenbugs to several specific loline derivatives, as measured by LC_{50}, was very close to the level seen for the potent insecticide nicotine sulfate. It has been postulated that loline derivatives, when present within plant tissue, act as feeding deterrents for greenbugs (Johnson *et al.* 1985). Consequently, it was important to rule out the possibility that mortality seen in response to loline derivative application to the plant surface was the result of feeding deterrence followed by starvation, and not toxicity. Data taken 2 h after spray application of loline derivatives indicated no differences in greenbug behavior across all treatments. These results would suggest that loline derivatives act as toxins and not feeding deterrents when sprayed on greenbugs. Of interest is the observation that the slopes of regression lines for nicotine sulfate and N-methyl loline were similar. Consequently it is tempting to postulate that these two compounds have the same mode of action. It appears that the slopes of the other loline derivatives are different from those of nicotine sulfate and N-methyl loline. Again, it is tempting to postulate that the mode of action of these compounds is different from the mode of action of nicotine sulfate and N-methyl loline.

Additional work, in terms of chemical and physical measurements of the loline derivatives as well as more precise topical applications of known quantities of toxicants, is needed before specific conclusions about modes of action can be answered.

In conclusion, it is of interest to note here that those compounds that show activity against insects (namely N-formyl, N-acetyl, N-methyl and N-propionyl loline) do not inhibit the germination and growth of alfalfa (Petroski *et al.* 1990). These results, taken together, suggest that these loline derivatives could be used as insecticides or in improving plant resistance to insects without causing harm to the plant. Further studies, with the goal of commercial development of loline derivatives for insect control agents, are warranted.

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