Helminthosporium and Stagonospora Leafspot Resistance are Unrelated to Indole Alkaloid Content in Reed Canarygrass

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ABSTRACT

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Clones of *Phalaris arundinacea* representing a wide range of genetically controlled alkaloid concentrations were inoculated with *Stagonospora foliicola* or *Helminthosporium catenarium*. Resistance and susceptibility were found among both low-alkaloid and high-alkaloid clones. There was no apparent relation between disease severity and the type of alkaloids (gramine or tryptamine-carboline derivatives) in individual plants. Alkaloid concentrations in infected leaves did not differ significantly from the concentrations in noninoculated leaves. Radial growth rates of *S. foliicola*, *H. catenarium*, *H. avenae*, and *Botrytis cinerea* (the latter two fungi are not pathogenic on *P. arundinacea*) were measured on V-8 juice agar containing several alkaloids of reed canarygrass. Gramine, N-ω-methyltryptamine, N,N-dimethyltryptamine and 5-methoxy-N,N-dimethyltryptamine at 500 μg/ml caused little or no inhibition of growth. Hordenine slightly stimulated growth of *H. avenae* and *B. cinerea*. When inoculated leaf pieces were floated on cycloheximide solutions, the tissues became highly susceptible to penetration and colonization by all fungi, but the total alkaloid concentration of the tissues did not change. The results indicated that indole alkaloid concentration and alkaloid type in leaves are not related to resistance to *S. foliicola* or *H. catenarium*. Breeders can select lines combining low alkaloid levels with resistance to either, or both, of these pathogens.

Additional key words: resistance mechanisms, papillae, *Drechslera catenaria, Drechslera avenae,* palatability, forage quality.

We conducted this study to determine whether resistance to Helminthosporium and Stagonospora leafspots in reed canarygrass (*Phalaris arundinacea* L.) is related to the type or concentration of indole alkaloids in the leaves. This information was needed to determine whether possible alterations in disease resistance occur as a consequence of breeding for low-alkaloid cultivars.

Plant breeders are selecting low-alkaloid genotypes of reed canarygrass in efforts to improve animal performance on this species (6, 7). Palatability and intake of reed canarygrass forage are negatively correlated with alkaloid concentration (8, 10, 11, 12, 17). Sheep and cattle grazed on high alkaloid clones develop diarrhea and have reduced weight gains (12). Total alkaloid concentration is controlled by genotype (1) and environment (1, 10, 13). Simple inheritance determines the type of compound that is produced. Plants with dominant genes T and M synthesize tryptamine-carboline and methoxytryptamine-carboline derivatives, respectively; double-recessive plants produce gramine as the principal indole alkaloid (14).

Clones differ widely in susceptibility to tawny blotch, caused by *Stagonospora foliicola* (Bres.) Bubak, and to Helminthosporium leafspot, which is caused by *H. catenarium* Drechs. (24). An epidermal resistance mechanism involving enzyme-mediated papilla formation (16, 18, 20) stops many of the penetration attempts by these pathogens (19). The resistance mechanism totally prevents penetration by fungi nonpathogenic to reed canarygrass, including *H. avenae* Eidam and *Botrytis cinerea* Pers. ex Fr. (19). Treatment of leaves with cycloheximide solutions inhibits epidermal resistance and permits pathogens and nonpathogens to penetrate and colonize leaves readily (19, and Vance and Sherwood, *unpublished*). Removal of cycloheximide restores epidermal resistance but does not stop internal mycelial growth.

Hyphae of pathogens that penetrate normal, noncycloheximide-treated leaf tissue apparently are confronted with an internal resistance mechanism that differs from epidermal resistance. Host cell colonization and lesion expansion progress slowly (22, 23, 24). Papillae are not seen. No induced antifungal compounds have been detected (19, 21).

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Our attention was directed to indole alkaloids as possible resistance compounds by reports that tryptamine inhibited growth of *Sclerotinia trifoliorum* (9), and that many substituted indole compounds, including gramine, inhibited *Cladosporium cucumerinum* (4). To assess the role of alkaloids in reed canarygrass diseases, we studied (i) the relation of disease severity to genetically controlled alkaloid concentration and type in leaves, (ii) the effect of alkaloids on fungal growth rate, and (iii) the effect of cycloheximide on alkaloid concentration.

MATERIALS AND METHODS

Alkaloids and disease severity in leaves.—We used 25 clones known to differ widely in alkaloid concentration (8, 11, 17) and in leafspot reaction (24). The principal alkaloid type (gramine or tryptamine-carboline derivatives) produced by each clone also was known (8, 11, 17). In the present study no distinction was made between tryptamine- and methoxytryptamine-containing clones.

Ramets were grown in a peat moss:vermiculite mixture (1:1, v/v) in 15-cm diameter pots. Established plants were trimmed to 6 cm and fertilized with 2 g of 14-14-14 fertilizer. After the plants had regrown for 5 wk, we inoculated them with conidial/mycelial suspensions of *S. foliicola* or *H. catenarium* as described elsewhere (24). We tested *S. foliicola* on 25 clones in test 1 and on 22 of the 25 clones in test 2. We tested *H. catenarium* on the same 22 clones used in test 2. There were three treatments in each test: (i) noninoculated plants harvested on the day of inoculation, (ii) noninoculated plants harvested 14 to 17 days after inoculation, and (iii) inoculated plants harvested 14 to 17 days after inoculation. There were three replicate pots of each clone per treatment per fungus. Treatments 1 and 2 served as the control. After inoculation, the plants in treatments 2 and 3 were incubated in moisture chambers for 3 days, then arranged on a glasshouse bench in a randomized complete block design. We rated leaf disease severity of inoculated plants at harvest on a disease severity scale of 1 to 9 with 1 = 0%, 3 = 1%, 5 = 5%, 7 = 20%, 9 = 30% (or more) necrotic tissue (24).

We harvested and froze the leaf blades from each replicate pot separately. From each sample a 5-g (fresh weight) subsample was taken for alkaloid extraction and the remainder was used to determine the moisture content of the sample. Alkaloids were extracted by the method of Simons and Marten (17). Total base concentration was estimated by titration with toluene sulfonic acid (3), and an equivalent weight of 200 was used to calculate the alkaloid concentration as a percentage of dry weight (8).

Fungal growth rate on alkaloid-supplemented media.—We measured the growth rate of four fungi on V-8 juice agar (V-8A, see ref. 15) containing five of the nine known (5, 10) reed canarygrass alkaloids. Gramine, N-ω-methyltryptamine, N,N-dimethyltryptamine, and 5-methoxy-N,N-dimethyltryptamine (Aldrich Chemical Co., Milwaukee, WI 53233) were dissolved in chloroform (40 mg/ml) and added to molten V-8A. Hordenine-hemisulfate (Sigma Chemical Co., St. Louis, MO 63178) was dissolved in hot water, filter sterilized, and added to V-8A. All compounds were tested at 500 μg/ml. Gramine and hordenine were also tested at 50 μg/ml. Appropriate amounts of chloroform or water were added to alkaloid-free V-8A controls.

We also added mixtures of crude bases from reed canarygrass leaves to V-8A. Separate extracts were prepared from gramine and tryptamine clones by the method of Simons and Marten (17). The bases obtained in chloroform solution were further purified by partitioning into 0.1 N HCl, raising the pH with NH_4OH to 9.2, and partitioning into chloroform. Concentrations of alkaloids in the extracts were estimated by titration and use of an equivalent weight of 200. Extracts were added to V-8A at 500 μg crude base/ml.

The media were poured into 9-cm diameter petri dishes and inoculated with 5-mm-diameter plugs from cultures of *H. catenarium, S. foliicola, B. cinerea,* and *H. avenae* Eidam. Radial growth rate at 24 C was measured after the hyphae had reached a constant growth rate. The rate on alkaloid-amended media was expressed as a percentage of that on control media. We measured nine replicate cultures of the gramine and hordenine treatments and four replicate cultures of the other treatments.

Alkaloids in cycloheximide-treated tissue.—We tested the effect of cycloheximide on alkaloid concentration by using an excised leaf technique. Leaves from three glasshouse-grown clones were cut into 1-cm-long pieces and floated on water or on cycloheximide solutions (25 μg/ml) in 15-cm diameter petri dishes. The pieces were sprayed with water or with a water suspension of *H. avenae* conidia (2×10^5 conidia/ml). Dishes were kept on a laboratory table. Samples (5 g) taken 24 and 72 hr after the leaves were excised, were frozen and analyzed for alkaloid. The experiment was run twice on each of the three clones. Because the clones gave similar results the data were analyzed as six replications of a single experiment.

Papilla formation, penetration, and colonization were assessed in inoculated pieces each time of sampling. The leaf pieces were stained in cotton blue-lactophenol and examined microscopically (19).

RESULTS

Alkaloids and Helminthosporium leafspot.—Clones differed significantly ($P < 0.01$) in Helminthosporium leafspot ratings and in alkaloid concentrations (Fig. 1-A). Disease ratings ranged from 2.7 to 6.3, and alkaloid concentrations ranged from 0.040 to 0.460%. There was no significant difference in mean alkaloid concentration among treatments; viz., (i) noninoculated leaves harvested at the time of inoculation, (ii) noninoculated leaves harvested at the time of disease rating, and (iii) inoculated leaves harvested at the time of disease rating (Table 1). There was no significant correlation between alkaloid concentration at the time of inoculation and disease severity 17 days later ($r = -0.291$) or between alkaloid concentration and disease severity at the time of rating ($r = -0.196$).

Resistant and susceptible plants occurred among both the low- and high-alkaloid clones (Fig. 1-A). Alkaloid type (gramine or tryptamine-carboline derivatives) showed no relation to disease rating or alkaloid concentration.

Alkaloids and Stagonospora leafspot.—Differences among clones for tawny blotch rating and alkaloid

concentration were significant (Fig. 1-B, 1-C). Differences in alkaloid concentration among treatments 1, 2, and 3 were not significant (Table 1).

Resistant and susceptible clones occurred among both low- and high-alkaloid clones. However, in test 1 several of the most susceptible clones had higher than average alkaloid concentrations and six of the eight most resistant clones had lower than average alkaloid concentrations. Accordingly, in test 1 the correlation between disease intensity and alkaloid concentration was positive and significant (Fig. 1-B). In test 2 the correlation was not as strong (Fig. 1-C).

Alkaloid type was not related to disease rating or alkaloid concentration (Fig. 1-B, 1-C).

Fungal growth rate on alkaloid-supplemented media.—Growth rates of the four fungi on V-8A containing alkaloids are given in Table 2. In most combinations of fungi and alkaloids tested, the alkaloid had little or no effect upon growth rate. Hordenine-hemisulfate at 500 μg/ml slightly stimulated *H. avenae* and *B. cinerea* and slightly inhibited *S. foliicola*. N-ω-methyltryptamine inhibited the four species about 11 to 23%. N,N-dimethyltryptamine reduced growth of three fungi about 28 to 40% but did not inhibit *S. foliicola*. The crude base extracts from plants inhibited *H. catenarium* about 22 to 46%. The plant extracts stimulated *H. avenae*.

Alkaloids in cycloheximide-treated tissue.—There were no significant ($P < 0.05$) effects of cycloheximide treatment, inoculation, or time of sampling on alkaloid concentration in leaf pieces (Table 3). The differences among replications (clones and runs) were significant ($P < 0.01$). Inoculated leaf pieces floated on water showed papilla formation, but no penetration, at 24 and 72 hr. Pieces on cycloheximide showed penetration by 24 hr and colonization of the mesophyll by 72 hr.

DISCUSSION

The occurrence of low- and high-alkaloid clones within both disease-resistant and susceptible clones (Fig. 1) indicated that alkaloid concentration is inherited independently of disease resistance. Alkaloid type also appeared to be independent of resistance. Earlier studies showed that resistance to *S. foliicola* was independent of resistance to *H. catenarium* (24). We conclude that it is feasible to select lines combining low alkaloid level with superior leafspot resistance. Selection must be directed toward each character separately, because selection for one will not automatically change the other.

We observed a weak positive correlation between susceptibility to *S. foliicola* and high alkaloid concentration (Fig. 1-B). This may reflect the bias given the data by a few plants at the extremes of this relatively small sample of clones. The relation might disappear if additional clones were tested. The results indicate that neither epidermal resistance nor internal resistance require high levels of alkaloids.

Alkaloid concentration did not change in response to inoculation and infection (Table 1). This indicates that the pathways of alkaloid biosynthesis or degradation (2) are not markedly activated or inhibited by infection. We believe that the role of alkaloids in animal performance would not be strongly influenced by plant infection.

None of the five alkaloids or two plant extracts tested

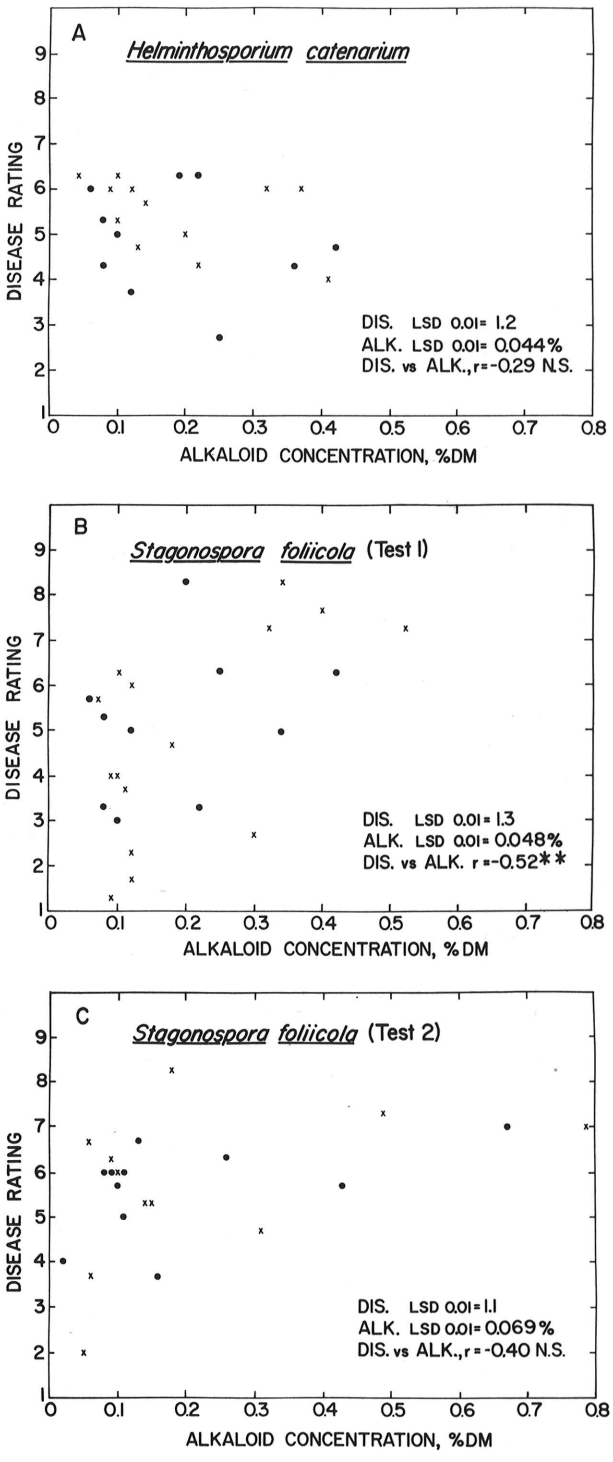

Fig. 1-(A to C). Relation of disease severity to total alkaloid concentration in leaves of 25 reed canarygrass clones. Alkaloid concentration was measured at the time of inoculation, and disease rating (1 = none, 9 = 30% of leaf area necrotic) was made 17 days later. **A)** Test with *Helminthosporium catenarium*. **B)** First test with *Stagonospora foliicola*. **C)** Second test with *S. foliicola*. Symbols: • = clones containing predominantly gramine, × = clones containing predominantly tryptamine-carboline derivatives.

greatly affected the growth rate of the four test fungi at 500 μg/ml in vitro (Table 2). The concentration tested was equivalent to about 0.20% alkaloid on a dry weight basis. This estimate was calculated on the assumption, substantiated by our experience, that reed canarygrass leaves grown in our glasshouse contain about 25% dry matter. The levels of alkaloids in our leaves ranged from 0.020 to 0.801%, with an average of 0.195% (Table 1). On a tissue basis, alkaloids in some of the resistant clones were present at much lower concentrations than the

TABLE 1. Alkaloid concentrations in noninoculated leaves of 22 reed canarygrass clones and in leaves of the same clones infected by *Helminthosporium catenarium* or *Stagonospora foliicola* in the glasshouse

Treatment	Alkaloid concentration (%, dry wt basis)			
	H. catenarium expt.		*S. foliicola* expt.[a]	
	Range (%)	Mean[b] (%)	Range (%)	Mean[b] (%)
Noninoculated leaves sampled on the day of inoculation	0.045-0.417	0.189	0.020-0.801	0.209
Noninoculated leaves sampled 17 days after inoculation	0.040-0.432	0.185	0.028-0.729	0.207
Infected leaves sampled 17 days after inoculation	0.043-0.462	0.187	0.017-0.673	0.186

[a]Two tests gave similar results. Only test 2 is reported here.
[b]Differences between means in a column are not significant, $P = 0.05$.

TABLE 2. Growth rates of fungi on V-8 juice agar containing alkaloids

Alkaloid	Alkaloid concentration (μg/ml)	Growth rate ± standard deviation of means[a]			
		Stagonospora[b] *foliicola* (%)	*Helminthosporium*[b] *catenarium* (%)	*Helminthosporium avenae* (%)	*Botrytis cinerea* (%)
Gramine	50	96 ± 4	102 ± 4	100 ± 2	103 ± 2
	500	99 ± 9	68 ± 7	61 ± 4	100 ± 2
Hordenine-hemisulphate	50	101 ± 3	110 ± 7	116 ± 8	114 ± 5
	500	86 ± 6	102 ± 7	119 ± 3	118 ± 6
N-ω-methyltryptamine	500	77 ± 6	89 ± 3	80 ± 7	88 ± 3
N,N-dimethyltryptamine	500	102 ± 2	57 ± 2	72 ± 6	60 ± 1
5-methoxy-N,N-dimethyltryptamine	500	100 ± 5	82 ± 4	104 ± 5	90 ± 2
Alkaloids from 'gramine' plants[c]	500	100 ± 1	78 ± 3	136 ± 7	90 ± 1
Alkaloids from 'tryptamine' plants[d]	500	110 ± 5	54 ± 7	125 ± 4	89 ± 3

[a]Rate of growth was based on radial growth of a colony growing at a constant rate and was expressed as a percentage of the rate on V-8 juice agar. Averages of four to nine replications.
[b]Pathogenic on reed canarygrass.
[c]Alkaloid fractions extracted from reed canarygrass plants containing predominantly gramine.
[d]Alkaloid fractions extracted from plants containing predominantly tryptamine-related alkaloids.

TABLE 3. Alkaloid concentrations in excised reed canarygrass leaf pieces inoculated, or noninoculated, with *Helminthosporium avenae* and floated on water or on cycloheximide solution for 24 or 72 hr

Treatment	Alkaloid concentration ± standard deviation of means[a]			
	Water		Cycloheximide[b]	
	24 hr (%)	72 hr (%)	24 hr (%)	72 hr (%)
Noninoculated	0.157 ± 0.037	0.145 ± 0.042	0.133 ± 0.035	0.133 ± 0.038
Inoculated	0.173 ± 0.058	0.146 ± 0.042	0.147 ± 0.045	0.142 ± 0.042

[a]Percentage alkaloid expressed on a dry wt basis. Differences between means were not significant, $P = 0.01$.
[b]Cycloheximide concentration, 25 μg/ml.

concentrations in V-8A tolerated by these fungi. This showed that high concentrations of alkaloids are not necessary for resistance. There was no obvious difference in tolerance between the pathogens and the nonpathogens of reed canarygrass. In contrast to these results, tryptamine at 400 μg/ml reportedly stopped growth of *Sclerotinia trifoliorum* (9); gramine at 500 μg/ml completely inhibited *Cladosporium cucumerinum* but not three other fungi (4).

Recent studies have shown the presence of three β-carboline alkaloids in *P. arundinacea* (5). These compounds were not tested separately in the in vitro studies (Table 2). However, they are associated with the presence of tryptamines and would have been extracted by the technique used for extraction of total alkaloids.

Inoculation of leaf pieces with *H. avenae* induced enzyme-mediated epidermal resistance (18, 20). In this study we found no change in alkaloid concentration during the period when resistance was induced. Treatment with cycloheximide solution inhibited epidermal and internal resistance (19). There was no change in alkaloid concentration in response to cycloheximide treatment. These observations, together with the observations on alkaloid concentration and type in relation to leafspot reaction and the observations on hyphal growth on V-8A containing various alkaloids, all indicate that there is no direct relation between genetically controlled alkaloid content and resistance to *S. foliicola* or *H. catenarium*.

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