The ribosome-inactivating protein restrictocin deters insect feeding on *Aspergillus restrictus*

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The fungus-feeding beetle, *Carpophilus freemani*, consumed equal quantities of young mycelia, fewer phialides bearing mature spores and much fewer phialides bearing developing spores of *Aspergillus restrictus* compared to those of *Aspergillus nidulans* when tested in diet choice assays. The degree to which specific fungal structures were consumed was inversely related to the localization of high levels of restrictocin, a ribosome-inactivating protein, to those structures. Pure restrictocin added to the insect diet at 1000 p.p.m. killed 38.5% of *C. freemani* larvae and 62.5% of *Spodoptera frugiperda* larvae in 48 h, but did not affect *C. freemani* adults or *Helicoverpa zea* larvae over the same interval. In diet choice assays, 1000 p.p.m. of restrictocin deterred feeding by adult *C. freemani* and *Sitophilus zeamais* compared to control diets. Thus, restrictocin production and localization may have a natural defensive role against insect feeding at times critical to spore formation by *A. restrictus*, and may have potential as an insect control agent.

Keywords: antifeedant, anti-insectan, pesticide, fungal defence

INTRODUCTION

Some species of fungi are more likely to be found associated with foodstuffs when certain insects are present. This has been shown to be the case with the sap beetles (Coleoptera: Nitidulidae) (Dowd, 1995), the granary weevil (*Sitophilus granarius*) (Agrawal *et al.*, 1957), and mites (Astigmata: Acaridae) (Armitage & George, 1986) on stored products. These insects inoculate clean grain with a variety of fungal spores present in their faeces and on the outsides of their bodies (Griffiths *et al.*, 1959; Agrawal *et al.*, 1957) and then feed upon the fungi that develop (Griffiths *et al.*, 1959). Thus insects can simultaneously be important vectors for the spread of fungal infestation and fungal antagonists that restrict fungal growth through feeding.

Some fungi possess defences against insect predators that are analogous to the defences of higher plants (Wicklow, 1988). Secondary metabolites produced by the fungi can be toxic to insects, or deter insect feeding (Wright *et al.*, 1982; Wicklow, 1988; Dowd, 1992a). While insects have adapted to feeding upon species of highly toxic fungi (Dowd 1992a, b), anti-insectan toxins can still contribute heavily to fungal defence (Wicklow *et al.*, 1988). The sclerotia of *Aspergillus flavus*, for example, have been

shown to contain substantial quantities of aflatoxin, as well as several aflavinine derivatives that deter insect predation (Wicklow *et al.*, 1988).

Plant and fungal resistance to insects is often associated with low-molecular-mass chemicals. Proteins, however, are increasingly being found to contribute to insect resistance (Gatehouse *et al.*, 1993). Ribosome-inactivating proteins (RIPs), such as ricin and saporin, are produced by a variety of higher plants (Stripe & Barbieri, 1986). Both ricin and saporin were found to be toxic to selected beetle larvae, but not to caterpillar larvae (Gatehouse *et al.*, 1990). It has also been suggested that the loss of RIPs in opaque-2 lines of corn has caused increased susceptibility to insect feeding (Bass *et al.*, 1992).

Fungi also produce RIPs (Conde *et al.*, 1978; Jimenez & Vazquez, 1985) such as restrictocin, which is produced by *Aspergillus restrictus*. This fungus is often closely associated with beetle infestations of stored grain products (Agrawal *et al.*, 1957). The production of restrictocin by *A. restrictus* is localized to the conidiophores and phialides during conidium formation (Brandhorst & Kenealy, 1992). It has been suggested that the protein toxin localization could deter insects from feeding until conidia are fully formed, after which time fungus-feeding insects could externally or internally carry spores to new resources for the fungus (Brandhorst & Kenealy, 1992).

In this study we examined the feeding of *Carpophilus*

Abbreviation: RIP, ribosome-inactivating protein.

freemani (an insect that preferentially feeds upon fungi; Tate & Ogawa, 1975) upon *Aspergillus nidulans* and *A. restrictus* cultures of different developmental stages. We also examined the effect of pure restrictocin upon the health and feeding habits of various insects that feed on agricultural crops.

METHODS

Insects. *Helicoverpa zea* (corn earworms) and *Spodoptera frugiperda* (fall armyworms) were reared on pinto bean diet (Dowd, 1988); *Carpophilus freemani* were reared on pinto bean diet (Dowd & Weber, 1991); *Sitophilus zeamais* (maize weevils) were reared on cracked corn (Dowd, 1994); all as previously described. Rearing conditions were $27 \pm 1^\circ\text{C}$, with a 14:10 light:dark cycle, at $50 \pm 10\%$ relative humidity for the *Sit. zeamais* and $40 \pm 10\%$ relative humidity for the other insect species.

Chemicals. The momordin, saporin, and ultra-low-temperature-gelling agarose were obtained from Sigma. The sorbic acid was obtained from US Biochemicals. The restrictocin was obtained as previously described (Brandhorst *et al.*, 1994). All other chemicals were of reagent grade.

Fungal cultures. *Aspergillus restrictus* (strain NRRL 2869) and *A. nidulans* (strain WI: *argB*, *metH2*, *biA1*) (Cullen *et al.*, 1987a, b) were maintained and grown as previously described (Brandhorst *et al.*, 1994). Agar plate fungal cultures were held at 22°C for 8 h during week days, and at 4°C the rest of the time in order to carefully observe development so that assays could be set up with the fungal cultures of the desired maturity (and thus restrictocin content). Fungi were grown both directly upon the agar surfaces of the culture plates and upon sterile filters (MSI, nitrocellulose, $0.4\ \mu\text{m}$ pore size) overlaying the agar.

Choice assays. Cultures used in choice assays with *C. freemani* adults included those in which all growth was mycelial, those in which conidiophores were in the early stages of conidiation, and those in which conidia were profuse and fully mature. Choice assays with adult *C. freemani* were set up in arenas consisting of 3.5 cm diameter Petri dishes. Squares (colonized by fungi) measuring $1 \times 1\text{ cm}$ were cut from the agar and placed on opposite sides of the plates. In the case of fungal material that had been grown on filter paper, $1 \times 1\text{ cm}$ squares were cut out and placed upon a 3 cm diameter piece of moistened filter paper in the bottom of the Petri dish. Five 1-week-old *C. freemani* adults were placed in the dishes and allowed to feed for 24 h on the fungal material covering the squares. Eight arenas of each treatment were used in each series of assays and each series was replicated for verification. Consumption was rated on an integer scale of 0 to 10, with 0 corresponding to no detectable feeding, and 10 to all fungal material consumed. At the end of the 24 h period, the remaining fungal material was frozen for 24 h and then fixed in ethanol. The fixed material was examined microscopically to identify the remaining fungal structures.

Estimation of restrictocin content of differentiated structures. The mean number of conidiophores present on one agar plate culture of *A. restrictus* was estimated by microscopic examination of intact culture surfaces and homogenates of plate-grown cell material. The mean number of phialides per conidiophore head was likewise estimated by microscopic examination. The mean length and diameter of the phialides, taken from both literature values (Frey *et al.*, 1979) and microscopic measurements by the authors, were used to calculate the approximate volume and weight of the average phialide (assuming a cylindrical shape). The concentration of restrictocin upon the phialides could be estimated given the

quantity of restrictocin present on agar plate cultures of *A. restrictus* (Brandhorst & Kenealy, 1992).

Feeding activity and mortality assays. Pure restrictocin, momordin and saporin were incorporated in cold-gelled powdered corn at 1000 p.p.m. by a method described previously (Dowd, 1994). Briefly, the incorporation involved adding an acetone solution of sorbic acid (as a mould inhibitor) to the powdered corn (the concentration in the final product would be 1000 p.p.m.), evaporating the acetone, adding 2 parts water, and rehydrating at 4°C overnight. A RIP was added in an additional part of water and the mixture was chilled at 4°C for 2 h. Ultra-low-temperature-gelling agarose was added to a concentration of 5% (w/v) and allowed to gel for at least 1 h before setting up in assays. For assays with adult *C. freemani*, approximately 0.6 g of material was used and five adult insects were included in each treatment. A feeding rating based on a 1–4 scale (Dowd, 1990) was used in feeding activity assays. For assays with *Spo. frugiperda*, 1.25 g of material was placed in dishes along with 20 newly hatched larvae per treatment. Larvae were examined for mortality after 48 h. Assays with *C. freemani* larvae were run in a similar fashion to those for *Spo. frugiperda*, except that second-instar (approx. 0.5 mg) larvae were used. For assays with *H. zea*, 20 newly hatched larvae per treatment were individually caged in the wells of a 24-well tissue-culture plate with approximately 0.1 g of material. Plates were sealed as described previously (Dowd, 1988).

Feeding puncture assays. For assays with *Sit. zeamais* adults, cold-gelled corn powder was prepared as just described. Initial studies in choice-assays as described for *C. freemani* adults indicated relatively limited feeding over a 1 week period, and difficulty in counting the numbers of feeding punctures due to the friable nature of the material. A different diet was used that was of a smoother consistency so that feeding punctures could be counted. This diet was analogous to the pinto bean diet except that 240 g frozen sweet corn was substituted for the frozen pinto beans. A 5 g quantity of the diet was freeze dried (to 1.25 g dry weight) and then rehydrated with 3.75 ml deionized water containing 5 mg restrictocin (final concentration 1000 p.p.m.). An analogous control diet without restrictocin was also prepared. Each block was sectioned into eight pieces, and pairs of these pieces were set up in an arena test with 10 adult *Sit. zeamais*. The number of feeding punctures in each block was counted after 2 and 4 d.

Digestion assays. The susceptibility of restrictocin to digestion by *H. zea* and *Spo. frugiperda* was determined by comparing degradation over a discrete time interval with different dilutions of gut homogenates. Midguts from three different last-instar caterpillars of each species were dissected individually in ice-cold 0.1 M glycine buffer, pH 9.6. Each individual midgut was homogenized in 1 ml of the same buffer using a ground-glass tissue grinder. The homogenate was centrifuged at 10000 g for 10 min at 4°C , and the supernatant (excluding any floating lipid layer) was used as an enzyme source. Protein determinations were done with the Bio-Rad packaged assay using bovine serum albumin as a standard. Protein content of homogenates used in assays was adjusted to 1 mg ml^{-1} ($\pm 10\%$) by dilution with buffer. Incubations consisted of 25 μl diluted homogenate and 25 μl 1 mg ml^{-1} solution of restrictocin in buffer. Enzyme-free restrictocin was also tested over the interval. All solutions were incubated at $27 \pm 1^\circ\text{C}$ for 1 h, then frozen at -20°C until examined electrophoretically.

Native polyacrylamide gel electrophoresis was performed as described previously (Dowd, 1994) with minor modifications. Because of the basic nature and small size of restrictocin, the gel was positioned with the sample slots nearest the anode, and

electrophoresis was carried out for only 30 min. The gel was removed, fixed in trichloroacetic acid/sulfosalicylic acid for 2 h, rinsed rapidly three times with deionized water, stained overnight with Pharmacia PhastGel Blue R (Coomassie R350) (prepared according to directions included with the material) and destained with repeated changes of 30% (v/v) methanol.

RESULTS

Choice assays

The presentation of insects with fungal cultures at specific developmental stages required careful observation of the cultures. This necessitated the overnight storage of plate cultures when there were no observers present. The cold storage of the plates did not dramatically alter the morphology of the fungal cultures and development proceeded according to the time of incubation at growth temperatures.

The *C. freemani* adults consumed the mycelia of both *A. nidulans* and *A. restrictus* with no noticeable discrimination (Table 1). Feeding by *C. freemani* adults on *A. restrictus* decreased somewhat when conidiophores were being formed (data not shown). When cultures entered the early stages of conidium formation, *C. freemani* adults fed extensively upon the phialides and the immature conidia of *A. nidulans*, as indicated by the marked absence of these structures after feeding. In comparison, these structures remained almost entirely unconsumed in cultures of *A. restrictus*. Overall, there was a marked decrease in feeding on *A. restrictus* compared to *A. nidulans* when conidia were starting to develop (Table 1). Although not shown, this was independent of the type of assay (filter paper vs agar), results not being significantly different. Shortly after the maturation of the conidia of *A. restrictus*, the adult *C. freemani* resumed feeding upon the phialides and fed upon the mature conidia as well; however, this level of feeding was still significantly lower than for *A. nidulans* (Table 1).

Table 1. Effects of fungal development on the feeding of *C. freemani* adults on *A. nidulans* and *A. restrictus* cultures

The feeding scores are based on a 0–10 scale, with 0 indicating no feeding. Values are means ($\pm$ standard errors) based on eight arenas used in each series of evaluations. Values for *A. restrictus* followed by an asterisk are significantly different from those of *A. nidulans* ($P < 0.05$) by paired *t*-test analysis.

Growth stage of culture	Feeding rating	
	<i>A. nidulans</i>	<i>A. restrictus</i>
Mycelia	10.0 $\pm$ 0.0	10.0 $\pm$ 0.0
Immature conidia (on agar)	9.9 $\pm$ 0.1	0.9 $\pm$ 0.0*
Immature conidia (on filter overlay)	9.5 $\pm$ 0.3	0.8 $\pm$ 0.2*
Mature conidia	9.9 $\pm$ 0.1	6.0 $\pm$ 0.1*

Table 2. Effects of restrictocin on feeding activity of *C. freemani* adults

Feeding activities are based on a 1–4 scale (Dowd, 1990) using cold gel incorporation. A score of 1 would indicate no feeding. Eight arenas were used in each series of evaluations. The results are means $\pm$ standard errors.

Restrictocin in diet (p.p.m.)	Feeding activity
0	3.2 $\pm$ 0.3
500	3.1 $\pm$ 0.3
1000	2.5 $\pm$ 0.4
2500	1.6 $\pm$ 0.3

When presented with a choice of a diet containing restrictocin or a diet without additives, *C. freemani* adults were not significantly deterred from feeding on the restrictocin-containing diet until higher concentrations of restrictocin (1000 and 2500 p.p.m.) were used (Table 2).

Estimation of restrictocin content of differentiated structures

The concentration of restrictocin present upon the phialides of the conidiophores was estimated in order to compare the concentrations of restrictocin used in the artificial diets of the choice and mortality assays to the concentration present in *A. restrictus*. Cell-associated restrictocin reaches a peak concentration of 18 μ g per agar plate culture (Brandhorst & Kenealy, 1992). At this time, there are an average of 1×10^7 conidiophores per plate and approximately 60 phialides per conidiophore. The volume of a phialide was estimated to be 21 μ m³. The product of these last three figures yields 12.6 mm³ of phialides per agar plate culture. If we assume that all the restrictocin is secreted by the phialides, the concentration of restrictocin upon the phialides may be approximated: 18 μ g restrictocin/12.6 mm³ phialides = 1400 p.p.m.

Mortality assays

Restrictocin (1000 p.p.m.) was used to test mortality of adult and larval forms of *C. freemani*. The mortality rates for *C. freemani* adults and *H. zea* larvae were not significantly affected by restrictocin at 1000 p.p.m. (Table 3). The mortality of *Spo. frugiperda* and *C. freemani* larvae feeding on the restrictocin-containing material was significant at 48 h. No mortality of *Spo. frugiperda* larvae fed on gelled corn powder containing the RIPs momordin or saporin was noted after 48 h (Table 3).

Feeding puncture assays

Restrictocin at 1000 p.p.m. significantly reduced feeding by *Sit. zeamais* adults after 2 d and 4 d. The number of feeding punctures in the restrictocin-containing diet was

Table 3. Effects of different RIPs at 1000 p.p.m. on the mortality of different insect species

Values are based on both live and dead insects found. Values followed by an asterisk are significantly different from the control ($P < 0.05$) by chi-square analysis. (N) indicates the number of insects included in each experiment.

RIP	Insect species	Percentage mortality (48 h)			
		Control	(N)	+ RIP	(N)
Restrictocin	<i>C. freemani</i> adult	0	(20)	0	(20)
	<i>C. freemani</i> larvae	0	(16)	38.5*	(13)
	<i>H. zea</i> larvae	0	(20)	5.2	(19)
	<i>Spo. frugiperda</i> larvae	0	(14)	62.5*	(16)
Momordin	<i>Spo. frugiperda</i> larvae	4.8	(21)	0	(19)
Saporin	<i>Spo. frugiperda</i> larvae	4.8	(21)	0	(18)

Table 4. Effects of restrictocin on feeding by adult *Sit. zeamais*

Values are means ($\pm$ standard errors) based on eight arenas used in each series of evaluations. Values followed by an asterisk are significantly different from the control ($P < 0.05$) by paired *t*-test analysis.

Days of exposure	Feeding punctures	
	Control diet	1000 p.p.m. restrictocin
2	8.9 $\pm$ 1.5	2.6 $\pm$ 0.7*
4	23.5 $\pm$ 3.9	8.2 $\pm$ 2.0*

reduced approximately threefold compared to the control diet (Table 4).

Digestion assays

Digestion of restrictocin by *H. zea* midgut homogenates could be discerned on native polyacrylamide gels at a homogenate dilution of 1:10, while digestion by the *Spo. frugiperda* midgut homogenate was only discernible with undiluted homogenate. *H. zea* and *Spo. frugiperda* appeared to digest the restrictocin differently. An obvious secondary band was noted with the *H. zea* homogenate, while no secondary bands were noted with the *Spo. frugiperda* homogenates. In these latter digestions, a smear of partially degraded protein was observed instead.

DISCUSSION

C. freemani adults fed upon colonies of *A. nidulans* and *A. restrictus*. The phialides of the conidiophores and the emerging, immature conidia of *A. restrictus* were not eaten during a particular time frame. A dramatic buildup of restrictocin is seen upon the surfaces of these structures during this same time frame (Brandhorst & Kenealy,

1992). This suggests an involvement of the restrictocin in the deterrence of insect feeding. Fungi are an attractive food source for insects, particularly the fruiting bodies, which are rich in nitrogen and energy (Southwood, 1973), so it would be an advantage for the fungus to develop mechanisms to protect these structures.

Adult *C. freemani* were not killed by restrictocin at 1000 p.p.m., but they exhibited an aversion to restrictocin in their diet, as did *Sit. zeamais*. Larvae of *C. freemani* and *Spo. frugiperda*, but not *H. zea*, were killed by the toxin. The ability of gut proteinases to hydrolyse ricin and saporin appeared to explain the lack of susceptibility of caterpillar larvae to these toxins (Gatehouse *et al.*, 1990). The difference in effect of restrictocin on *Spo. frugiperda* vs *H. zea* may be at least partly explained by different rates of digestion. At equivalent protein concentrations under the conditions used, the *H. zea* midgut homogenates were able to digest restrictocin approximately 10 times faster than those of *Spo. frugiperda*.

Restrictocin may act in concert with one or several of the other compounds present in the fungi to effectively deter feeding by adult *C. freemani*. The results of our experiments with pure restrictocin indicated that restrictocin by itself, when present at concentrations of 1000 p.p.m., deterred feeding by adult *C. freemani*. The natural concentration of restrictocin coating fungal phialides may be in excess of 1000 p.p.m., and was estimated at 1400 p.p.m. by calculating the volume and number of phialides per plate at maximum restrictocin production (Brandhorst & Kenealy, 1992).

Restrictocin may protect *A. restrictus* conidiophores from insect feeding by one or more mechanisms. Accumulation of a lethal or sublethal dose of the toxin by fungivorous insects could check extensive feeding upon toxin-coated conidiophores. Alternatively, restrictocin toxicity may be targeted to specific cells. Poisoning cells of the gut lining might cause sufficient gastric distress to inhibit further feeding, while a specificity to taste receptor cells could hamper their normal functioning sufficiently to produce

an antifeedant response. Finally, the restrictocin molecule could act as an insect repellent. While *Bacillus thuringiensis* crystal protein, for instance, acts as an anti-feedant for some insects (Gould *et al.*, 1991), the lethal portions of the toxin have been found to be distinct from the portions that cause the anti-feedant/repellent response (Rajendran *et al.*, 1994).

In tests of opaque-2 and normal corn lines, the adults of *Sitophilus oryzae* were significantly more likely to be found with the opaque-2 lines (Gupta *et al.*, 1970). The absence of the maize RIPs in the opaque-2 lines is now thought to be responsible for this increased susceptibility (Bass *et al.*, 1992). It is possible that *S. oryzae* was able to sense the presence of the maize RIP, as we found for *Sit. zeamais* and restrictocin in the present study.

Once conidia have matured, restrictocin levels on the conidiophore heads diminish substantially (Brandhorst & Kenealy, 1992) and the feeding deterrence effect wanes as well. It is probable that this is advantageous to the reproductive efficiency of the fungus, as fungivorous insects are an important vector for the dissemination of conidia. Investigators have noted that the majority of spores in the guts of Acaridae (Griffiths *et al.*, 1959), *Sitophilus granarius* (Agrawal *et al.*, 1957) and *Carpophilus* spp. (Jewell, 1956; Moller & Devay, 1968) remain undigested and pass through the gut maintaining full viability.

Prior tests with the plant-derived RIPs ricin and saporin indicated activity against beetle larvae, but not caterpillar larvae (Gatehouse *et al.*, 1990). We also found that the *Spo. frugiperda* larvae were not affected after 48 h by the plant-derived saporin and momordin. However, both beetle (*C. freemani*) larvae and some species of caterpillar larvae (*Spo. frugiperda* but not *H. zea*) were affected by restrictocin at 1000 p.p.m. The differing spectrum of insect toxicity of restrictocin compared to the plant-derived RIPs that have been tested suggests some fundamental differences in pharmacological activity. Fungal RIPs, such as restrictocin, may have ecologically relevant activity and value in insect control through genetic engineering that cannot be predicted based on comparisons with plant-derived RIPs.

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Disclaimer: names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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