Antimicrobial and insecticidal properties of isolated from seeds of the tropical forage legume *Clitoria ternatea* (L.)

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Introduction

Seeds use strategies to germinate and survive in soils that are densely inhabited by a wide range of microfauna and microflora. Various antimicrobial proteins such as chitinases, β-glucanases, thionins, ribosome-inactivating proteins and permatins have been detected in seeds. These are believed to play a role in plant defense because of their strong antimicrobial activity. This belief is supported by their ability to confer resistance (to pathogens) in transgenic plants containing genes that encode them.

Other plant-derived proteins have insecticidal properties that can, for example, protect seeds from attack by larvae of various bruchids and inhibit the growth and development of *Helicoverpa punctigera* (Wallengren) larvae.

We report here the isolation, purification, and characterization of a protein from *C. ternatea* seeds. This protein, designated 'finotin', has antifungal, antibacterial and insecticidal properties.

Materials and methods

Biological materials

The various test fungi and plant pathogenic bacterium were obtained from collections maintained at CIAT. The bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus* were obtained from the permanent mass-reared population maintained at CIAT.

Isolating and purifying the antifungal protein

Proteins were extracted from10 g seeds macerated in 100 mL of sterile distilled water for protein purification. The macerated suspension was filtered through several layers, of cheesecloth and centrifuged at 13 000 × *g* for 30 min. After several steps of cleaning, the supernatant was concentrated by lyophilization, then re-suspended in sterile distilled water at 10% of the original volume. The sample was resolved by preparative, granulated-bed IEF (Bio-Rad Laboratories), with a pH range of 3.5 to 9.5, according to the manufacturer's instructions and fractionated. Fractions with antifungal activity were analyzed.

Antifungal and antibacterial activity bioassay

Discs of thick filter paper (#7) containing 300-µL seed-extract filtrate were placed in petri dishes containing PDA. One sclerotium of *R. solani* was then placed in the center of the dish and incubated at 28 ºC. For the antibacterial activity assay, a 100-µL bacterial suspension in sterile deionized water with an optical density at 600 nm (OD_{600}) of 0.1 was evenly spread over nutrient agar (Difco, USA) in a plate. Microbial growth was assessed by measuring inhibition zones between the filter-paper disc and the visible microbial growth after 48 h of incubation for fungi and 24 h for bacteria.

Insect rearing and feeding tests

Tests were conducted with two species of bruchids that are key pests of stored beans around the world: the Mexican bean weevil or *Z. subfasciatus* (Boheman), and the bean weevil or *A. obtectus* (Say).

To test insecticidal effects of the protein on both bruchid species, 'artificial' seeds (with flour of a susceptible common bean variety) were prepared, using techniques devised by Shade et al. (1986. Environ. Entomol. 15:1286-1291).

Infestation procedures were as follows: for *Z. subfasciatus,* seeds for each protein concentration were infested with at least 8 pairs of bruchids per seed. After 5 days, the seeds were examined under a dissecting microscope and any eggs in excess of 5 to 6 per seed were destroyed with a needle. For *A. obtectus*, seeds for each protein concentration were infested with 5 to 6 neonate larvae per seed.

Result and Discussion

Antifungal activity

The crude extract from seeds of *C. ternatea* CIAT 20692 showed strong antifungal activity on the test fungus *R. solani.* This activity could be eliminated by treatment with Pronase E [Figure 1], indicating that the active compound is a protein. Seeds release this heat-stable, proteinaceous, antifungal compound after mechanical disruption of their seed coat or after germination (data not shown).

Figure 1. Antifungal activity of seed extracts from *Clitoria ternatea* CIAT 20692 is eliminated after treatment with Pronase E. One sclerotium of *Rhizoctonia solani* was placed in the center of each petri dish containing potato dextrose agar and incubated at 28ºC for 2 days. Seed extract filtrate (300 µL), either untreated (a) or treated with Pronase E (c) was applied to each of three filter paper discs placed per petri dish. In the control dish, the discs were given equal volumes of sterile distilled water (b). Fungal growth is whitish gray.

Identifying and purifying the antifungal protein

To identify the specific protein(s) responsible for the antifungal activity, we created a new protocol, which involves (1) resolving the seed extract by an IEF gel, (2) neutralizing the gel to eliminate the pH gradient, (3) lightly and uniformly coating the gel with warm PDA, (4) inoculating the gel/PDA composition with *R. solani* sclerotia, and (5) wrapping the inoculated gel/PDA with saran wrap to prevent loss of moisture and incubating it at 28 ºC for 2 days. This protocol, in fact, greatly facilitated our task.

R. solani was inhibited in the area where proteins with alkaline pI (isoelectric point) were found (Figure 2). The specific antifungal protein was identified by cutting out ultra-thin-layer polyacrylamide gel areas, corresponding to individual protein bands in a duplicate Coomassie-stained gel. Proteins were eluted from the sliced gels and tested for antifungal activity. The results of these tests show that a highly basic protein (numbered 1 in Figure 2) was responsible for the antifungal activity (Figure 3).

The peptide was well separated from the other proteins on IEF gels, making the purification procedure, using preparative, granulated-bed IEF, a relatively easy task. Five of the fractions showed activity with decreasing intensity, starting with the highly alkaline pI [Figure 4]. Both SDS-PAGE (Figure 5) and IEF gels (data not shown) showed that fraction 1 was pure and free of other proteins.

Antifungal and antibacterial activity

Finotin was active against several important plant pathogens: *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. and *Xanthomonas axonopodis* pv. *phaseoli* (Smith) Dye from common beans; *Lasiodiplodia theobromae* (Pat.) Gr. & Maub. and *C. gloeosporioides* (Penz.) Sacc. from *Stylosanthes* spp.; *Bipolaris oryzae* and *Pyricularia grisea* Cav. from rice; and *R. solani* from *Brachiaria* spp.

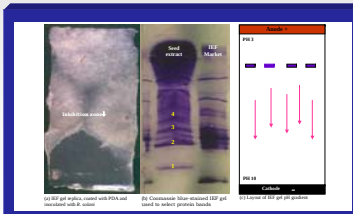

Figure 2. Extracts from *Clitoria ternatea* seeds resolved by isoelectric focusing (IEF) gel, (a) neutralized in a buffer, coated with potato dextrose agar (PDA), and inoculated with one *Rhizoctonia solani* sclerotium. The growth inhibition zone indicated that the protein(s) responsible for antifungal activity were in the alkaline part of the gel. A triplicate IEF gel was superimposed on an identical Coomassie blue 1 to 4 (b) were cut out to identify the antifungal protein; (c) IEF gel pH gradient layout.

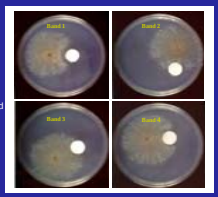

Figure 3. Identifying the specific antifungal protein band (finotin). The protein band with the most alkaline pI (isoelectric point) numbered 1 in Figure 2b inhibited growth of the test fungus *Rhizoctonia solani* around the disc.

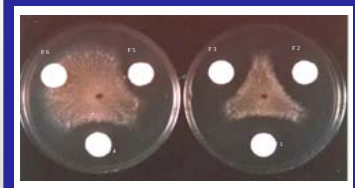

Figure 4. Purification of an antifungal protein from *Clitoria ternatea* seeds, using preparative, granulated bed isoelectric focusing. Five fractions, scooped from the gel (starting from the alkaline part of the gel as fraction 1 or F1), demonstrated antifungal activities, with F5 having the least inhibitory activity against growth of *Rhizoctonia solani*.

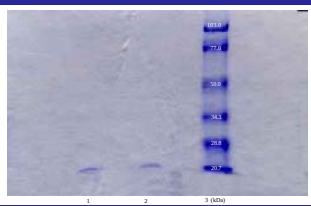

Figure 5. SDS-PAGE analysis of purified protein fraction from *Clitoria ternatea* seeds. Lanes 1 and 2, fraction 1 from two separate purifications; lane 3, SDS-PAGE molecular weight marker (low range).

Insecticidal activity

Mortality of the bruchids *Zabrotes subfasciatus* (Boheman) and *Acanthoscelidius obtectus* (Say) was very low (less than 3%) on artificial seeds. The artificial seeds were enriched with increasing concentrations of the test protein (finotin), leading to increasing levels of mortality. Maximum levels (100% larval mortality) were reached at the dosage of 5% for *Z. subfasciatus* and 1% for *A. obtectus*. Probit analysis (Table 1) showed that the protein is highly toxic to both bruchid species at LC_{50} values, which can be considered as low (less than 2%). The LC_{50} value for *A. obtectus* (0.36%) was about four times less than that for *Z. subfasciatus* (1.21%), meaning that the protein is more toxic for *A. obtectus*. The protein is highly toxic to first-instar larvae of both bruchid species; dissection of infested seeds revealed that up to 75% of larvae did not reach second-instar stage.

Responses to different concentrations in terms of days to adult emergence are shown in Table 2. Developmental times of those few insects that survived the various concentrations were prolonged. Response correlated with dosage: the higher the dosage the longer the developmental time. This is further proof of the protein's toxicity to both bruchid species.

Table 1. Toxicological responses of bean bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus* to a purified protein (finotin) isolated from *Clitoria ternatea* seeds.

	Bruchids	
Parameter	*Z. subfasciatus*	*A.obtectus*
No. tested	147	155
LC_{50} (95% FL)[a]	1.21 (0.99 -1.47)	0.36 (0.28-0.43)
LC_{95} (95% FL)[a]	2.88 (2.17-5.21)	0.77 (0.61-1.28)
Slope ± SEM	4.3 ± 0.88	4.9 ± 0.96
X^2	2.78	0.84

[a] FL, fiducial limits

Table 2. Effects of increasing concentrations of a purified protein (finotin) isolated from *Clitoria ternatea* seeds on the biology (days to adult emergence) of the bean bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus*.

Protein concentration*	**Days to adult emergence****	
(% w/w)	*Z. subfasciatus*	*A. obtectus*
0.0	43.1e	34.4c
0.0625	45.0e	33.8c
0.125	51.5d	35.2c
0.25	55.6c	49.4b
0.5	57.3c	63.4a
1.0	72.7b	NE
2.0	80.0a	NE
5.0	NE	NE

*Background flour was prepared from seeds of common bean cv. ICA Pijao.

** Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD. ANOVA on data testing for differences among dosages (protein concentrations): for *Z. subfasciatus*, F =106.9, df = 6.21, P< 0.001; and for A. obtectus, F = 184.9 df = 4.16, P < 0.0001. NE means no adult emergence, that is, 100% larval mortality.