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A N I N T E R N A T I O N A L J O U R N A L

LC_{50} of the Peptide Produced by the Entomopathogenic Fungus *Nomuraea rileyi* (Farlow) Samson Active Against Third Instar Larvae of *Anticarsia gemmatalis* (Lep.: Noctuidae)

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ABSTRACT

The entomopathogenic fungus Nomuraea rileyi *(Farlow) Samson produced a peptide active against* Anticarsia gemmatalis *3rd instar larvae. To produce this peptide,* N. rileyi *was cultivated aerobically in Saboraud, maltose, yeast-extract broth at 26 ± 1°C for 12 days, after which the medium was filtered and separated in a liquid/liquid extractor, concentrated and the peptide purified chromatographically. The crystals obtained were kept refrigerated until needed for LC_{50} analysis. The LC_{50} of this peptide against* A. gemmatalis *3rd instar larvae was determined in triplicate experiments using solutions containing 1.0, 0.2, 0.1, 0.01, 0.001 and 0.0001 mg/ml of*N. rileyi *peptide. The results of these experiments were used to calculate a linear equation in which Y = 6,81176 + 1,01382 * LOGx , giving a LC_{50} value of 0.0163 mg/ml.*

Key words: *Anticarsia gemmatalis*; *Nomuraea rileyi*, integrated control, secondary metabolite, toxins

INTRODUCTION

Many fungi produce secondary metabolites, which act on other organisms, sometimes causing inhibition of growth, disease and even death. Examples of such metabolites include the aflatoxins produced by some Aspergillus flavus strains (Diener and Davis, 1969), ochratoxin produced by *A. ochraceus* (Myokey et al., 1969; Kodaira, 1969) and the toxins and antibiotics produced by members of the genus *Penicillium*. Some entomopathogenic fungi produce metabolites, which can affect other microorganisms and insects (Onofre, et al., 1999), *e.g.* the fungus *Metarhizium anisopliae* produces an insecticidal cyclodepsipeptide called destruxin, which inhibits the growth of various bacterial strains (Kodaira, 1962; Kaijiang and Roberts, 1986; Dumas et al., 1995; Jegorov et al., 1995). Fungi such as *Beauveria bassiana, Paecilomyces*

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fumosoroseus and *Fusarium moniliforme* also produce cyclodepsipeptides, including be auvericin and the enniatin complex (Kucera and Sansinakova 1968; West and Buggs, 1968; Hamil et al., 1969; Richard et al., 1995; Logrieco et al., 1996). Studies have reported that the fungus *Nomuraea rileyi* produces metabolites active against insects (Ignoffo et al., 1976; Wasti and Hartmann, 1978; Kucera and Sansinakova 1968; Mohamed and Nelson, 1984; Ye et al., 1993), including some metabolites showing toxic activity against the larvae of *Heliothis zea*, *H. virescens* (Mohamed and Nelson, 1984) and *Bombyx mori* (YE et al., 1993).

Defoliating caterpillars are important pests of Brazilian soybeans and beans, and among these *Anticarsia gemmatalis* (the soybean caterpillar) being the most important (Costa, 1958; Redaelli, 1960; Bertels and Ferreira, 1973; Corseuil et al., 1974). This pest can be controlled using chemical insecticides and biological agents such as virus, bacteria and fungi (Ignoffo et al., 1976) and it has been shown that *N. rileyi* can be used for the biological control of *A. gemmatalis* when applied during the first stages of larval development (Ignoffo et al., 1976). The aim of the present work was to isolate and purify a peptide produced by *N. riley* and to study the effect of this peptide on insect mortality.

MATERIALS AND METHODS

Fungal Strain: *Nomuraea rileyi (Farlow) Samson* strain SA-86101 (Biological Control Division, Biotechnology Institute, University of Caxias do Sul, Grande do Sul, Brazil) was isolated from *Anticarsia gemmatalis* (*Lep.: Noctuidae*).

Media: Saboraud, maltose, yeast-extract (SMY) broth (4% maltose, 1% peptone and 0.5% yeast extract, pH 6.0) was autoclaved, cooled and inoculated as shown in Fig. 1.

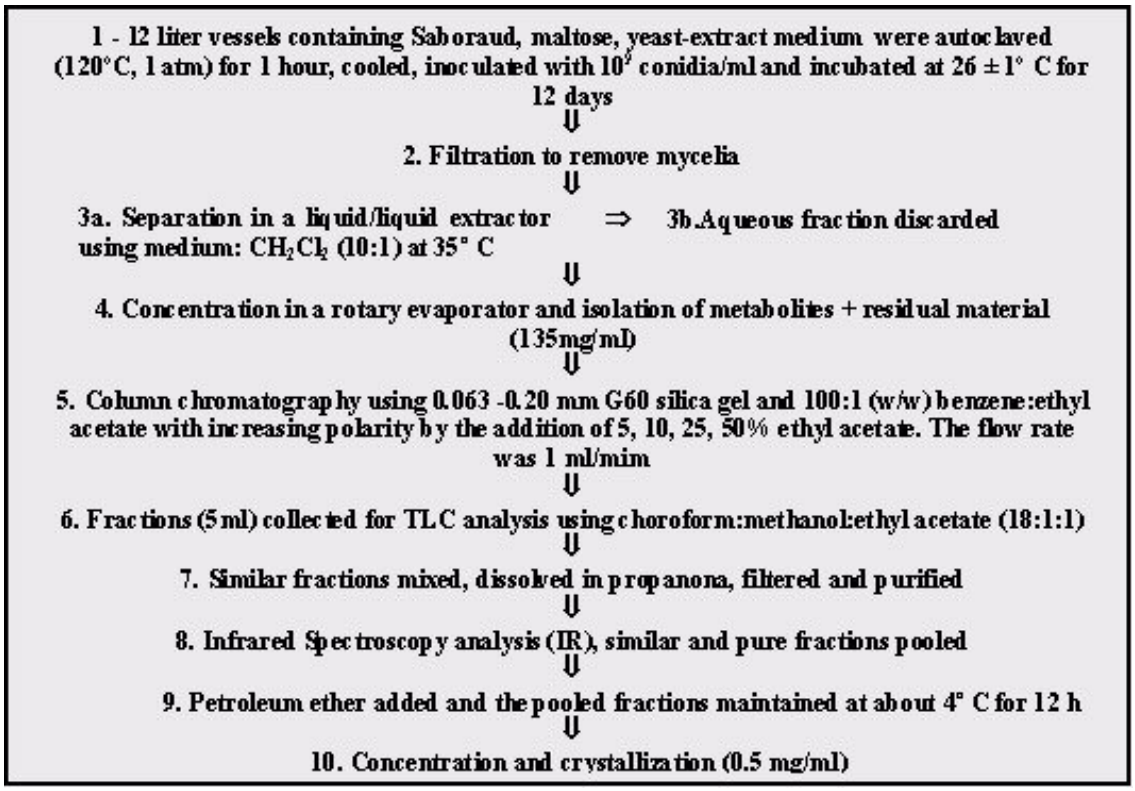

Figure 1 - *NR-tox*$_1$ peptide isolation and purification procedure.

Peptide production and isolation: *N. rileyi* strain SA-86101 was grown in SMY medium as described by Ignoffo et al. (1976) and used to produce a conidial suspension containing about $2.4x10^9$ conidia/ml, which was inoculated into vessels containing 12 liters of SMY broth. Incubation was at 26 ± 1°C for 12 days with constant aeration, after which the culture medium was filtered to remove mycelia and passed through several successive Whatman Nº 1 filter papers. *NR-tox$_1$* was separated in a liquid/liquid extractor using filtered culture medium : dichlorometane (10:1) and concentrated in a rotary evaporator at 35°C, the final fractions containing about 135mg/l of fungal metabolites and other residual material. Metabolites were isolated using G_{60} silica Gel (0.063 to 0.20 mm) column chromatography with a solvent (chloroform :methanol : ethyl acetate, 18:1:1) ratio of 100:1 (w/w), 5ml fractions being collected and analyzed using thin layer chromatography (TLC). Similar fractions were mixed and analyzed by infrared spectroscopy (IR). After successive purification, crystals were obtained, which were maintained at 20°C until needed for chemical and biological analysis.

Bioassays: The peptide described above was diluted in distilled water to produce solutions containing 1.0, 0.2, 0.1, 0.01, 0.001 and 0.0001 mg/ml and sprayed onto soybean leaves at application rates of 100, 20, 10, 1 and 0.1 μg/cm^2. The leaves were dried in trays for 30 min in a laminar flow chamber, each tray containing a different peptide concentration and application rate. For the bioassay 50, *A. gemmatalis* 3rd instar larva were placed in each tray and incubated at ~25°C under a 12h photoperiod. The mortality rates were observed each day. Control experiments were conducted as above except that leaves were sprayed with distilled water instead of protein solution. Three replicates were made for each treatment and the larvae were kept until they either died or pupated. Data were analyzed using Probit analysis (Sokal, 1958; Finney, 1971) and LC_{50} values calculated.

RESULTS AND DISCUSSION

Chemical analysis showed that the *N. rileyi* metabolite (*NR-tox$_1$*) active against *A. gemmatalis* had a positive ninhidrin reaction, a melting point of 244.4°C and was soluble in water, ethanol and methanol. Infrared and ultraviolet spectral data (Figures 2 and 3) coincided with those expected for an oligopeptide.

Table 1 and Fig. 4 show the insecticidal activity of the isolated peptide on *A. gemmatalis* 3rd instar larvae. The highest mortality rates varied between 82.66% and 80.00 for the 1.0, 0.2, and 0.1 mg/ml peptide concentrations, with no statistically significant differences between them (Table 1), although they were all significantly different to the other concentrations (0.01, 0.001 and 0.0001 mg/ml) which gave significantly lower mortality rates. The 40% mortality rate given by the 0.01 mg/ml concentration was significantly different to the 0.001 and 0.0001 mg/ml concentrations, but significantly lower than the mortality rate given by the higher concentrations.

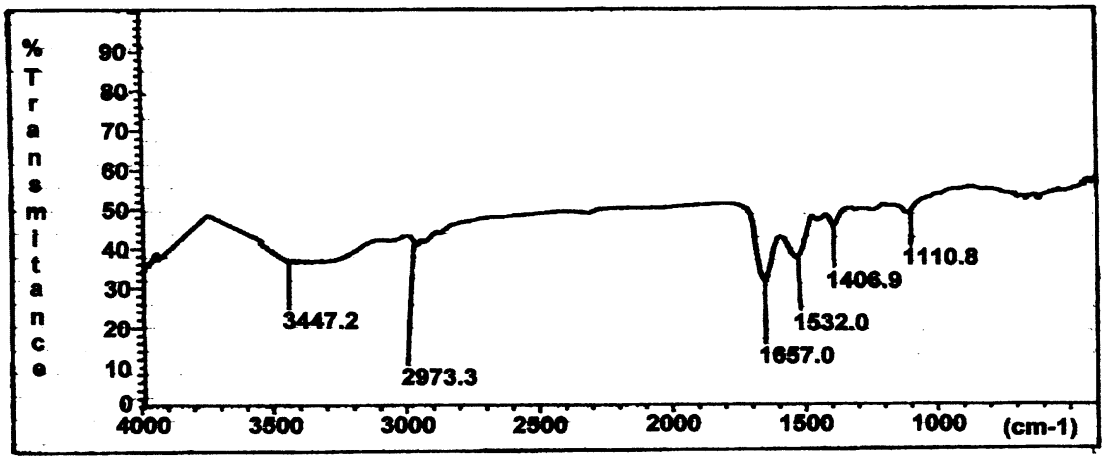

Figure 2 - Infrared spectrograph of a peptide (*NR-tox$_1$*) produced by entomopathogenic *Nomuraea rileyi* active against *Anticarsia gemmatalis.*

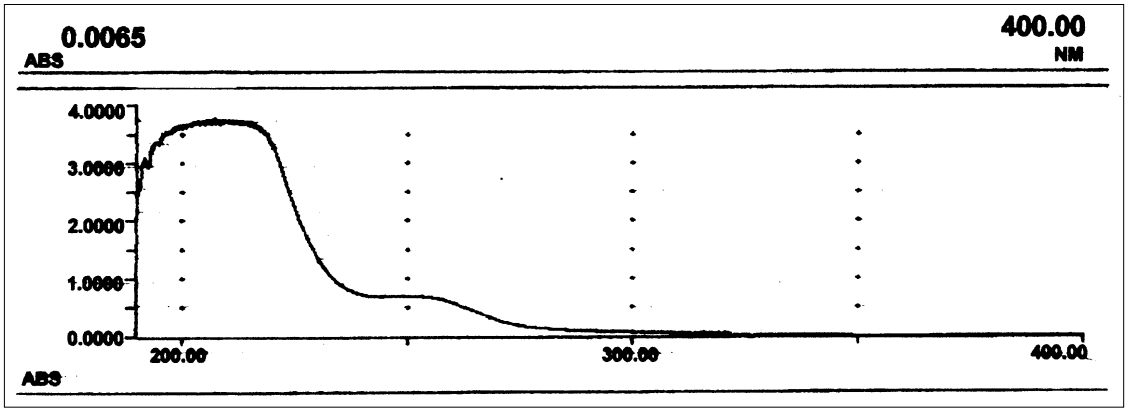

Figure 3. Ultraviolet spectrograph of a peptide (*NR-tox$_1$*) produced by entomopathogenic *Nomuraea rileyi* active against *Anticarsia gemmatalis.*

Mortality data submitted to linear regression resulted in the equation: Y = 6,81176 + 1,01382 * LOGx which gave the estimated LC_{50} value as 0.0163 mg/ml with a 95% confidence interval of 0.0100 to 0.0266 (p=5%) These results for the *N. riley* peptide agree with those reported by YE, et al., (1993) who demonstrated that purified peptides extracted from fungal culture media showed insecticidal activity against *B. mori, Prodenra litura* and *Pieris rapae* larvae. Mohamed and Nelson (1984) reported that *N. riley* crude extracts caused 42, 48 and 72 h mortality rates of 23.3, 44.5 and 68.9% for *H. virensens* larvae and 28.7; 53.8 and 78.3% for *H. zea* larvae.

Table 1 - Percentage mortality and mean lethal time for *A. gemmatalis* 3rd instar larvae exposed to different concentrations of (*NR-tox$_1$*) peptide.

(*NR-tox$_1$*) peptide (mg/ml)	% Mortality (0± SD)[1]	Lethal time (days)
1.0	82.6 ± 9.2a[2]	6.8
0.2	81.0 ± 6.3a	7.7
0.1	80.0 ± 8.4a	7.8
0.01	40.0 ± 3.5b	8.8
0.001	10.0 ± 1.5c	9.1
0.0001	2.0 ± 0.4c	10.0

[1] Mean ± Standard deviation

[2] Numbers followed by the same latter present no statistical differences (Duncan's test, P ≤ 0,05).

The insecticidal activity of the *N. rileyi* peptide described in this paper demonstrated similar toxicity to that of other metabolites produced by entomopathogenic fungi such as *B. bassiana*, which produced beauvericin and the enniatin complex, both of which were effective against *Calliphora erythrocephala* and *Aedes aeggypti* larvae (Grove and Pople, 1980). In a study on the effect of beauvericin on *Spodoptera exigua* larvae, Boucias et al. (1994) showed that 86% of treated larvae exhibited tetanic paralysis within 6 hours of treatment and mortality rates of about 26% after 24h, 45% after 36h and 73% after 72h. The entomopathogenic fungus *M. anisopliae* also produced toxins, known as destruxins, which have been shown to be toxic to the larvae of *A. aegypti, Galleria mellonella, Delia antiqua, Cetonia aurata, Oryctes rhinoceros, Choristoneura fumiferana, Schistocerca gregaria, Periplaneta americana* and *Aedes albopictus* (Crisan, 1971;

Poprawski et al., 1995; Fargues, et al., 1985; Fargues et al., 1986; Huxman et al., 1989; Kopecky et al., 1995; James et al., 1995; Brousseau et al., 1996).

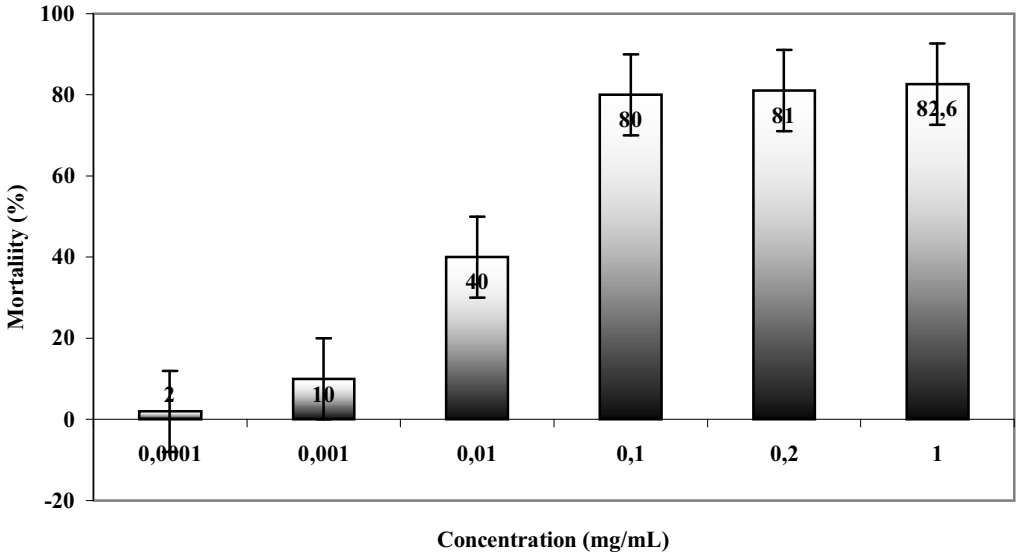

Figure 4 - Mortality of the soybean caterpillar *A. gemmatalis* as a function of peptide concentration (mg/ml).

Although the antimicrobial and insecticidal activity of *N. riley* metabolites is well documented, the chemical structure and mechanism of action of these metabolites are as yet undefined. Such metabolites are important potential instruments to encourage the use of entomopathogenic fungi in pest control, p romoting an integrated approach to pest control. These metabolites could be used as insecticides or antibiotics, with some having the potential for large-scale production for insect control in the field. However, perhaps the most important use of insecticidal metabolites of entomopatho-genic fungi is in the investigation of insect mortality genes and the use of this knowledge to produce more virulent fungal strains. Insertion into plants of genes for insecticidal metabolites, generating plants less susceptible to insect attack, is an important area for future research.

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RESUMO

Este trabalho objetivou determinar a CL_{50} de um peptídeo produzido pelo fungo *N. rileyi*, para larvas de 3º ínstar de *A. gemmatalis*. O peptídeo foi produzido através de fermentação aerada, em meio SMY, sob condições controladas por 12 dias. O metabólito foi purificado, utilizando-se de sistemas de cromatografia. Os cristais obtidos foram então armazenados a baixa temperatura para posterior análise da atividade biológica. Os experimentos foram conduzidos em 3 repetições, com tratamentos que consistiram de soluções com 0,0001; 0,001; 0,01; 0,1; 0,2 e 1,0 mg/mL. Através de análise de regressão chegou-se a equação da reta Y = 6,81176 + 1,01382 * LOGx e um valor para CL_{50} de 0,0163 mg/mL.

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