SHORT COMMUNICATION

EVALUATING THE INHIBITORY ACTIVITY OF SYNTHETIC ANTI-MICROBIAL PEPTIDES AGAINST *USTILAGO SCITAMINEA*, *FUSARIUM VERTICILLIOIDES* AND *ELDANA SACCHARINA*

RAMGAREEB S and RUTHERFORD R S

South African Sugarcane Research Institute, Private Bag X02,
Mount Edgecombe, 4300, South Africa
sumita.ramgareeb@sugar.org.za stuart.rutherford@sugar.org.za

Abstract

Varieties of sugarcane that show resistance to *Ustilago scitaminea* are more often than not susceptible to *Eldana saccharina* and *vice versa*. In addition, recent evidence suggests that *Fusarium verticillioides*, commonly found colonising stalk tissue surrounding borings, positively influences the survival, growth and fecundity of *E. saccharina*. Sugarcane varieties engineered with enhanced resistance to *U. scitaminea*, *F. verticillioides* and *E. saccharina* are potentially of great value to this industry. The aim of this study was to investigate the anti-microbial and insecticidal activities of synthetic cationic anti-microbial peptides as a prelude to a possible transgenic approach. Members of four classes of anti-microbial peptides (D4E1, GR7, REV4, PON-G1) were selected as candidates for *in vitro* testing against *U. scitaminea* (smut), *F. verticillioides* (fusarium) and *E. saccharina*. The Ponericin PON-G1 was found to be most effective against both fusarium and smut with low concentrations of 1.7 μM and 0.1 μM, respectively, reducing the growth of these pathogens by 50% after a 48 hour incubation period. The Indolicidin REV4 was not able to reduce fusarium growth by 50%, even at the highest concentration tested (52.9 μM or 100 μg/ml); however, inhibitory activities were recorded against smut and *E. saccharina*. Preliminary tests suggest that exposure of neonate *E. saccharina* larvae to REV4, PON-G1 or GR7 results in a reduction in larval growth rate. Investigations were also done on the potential synergistic inhibition of these three peptides against fusarium. It was found that when REV4 was combined with PON-G1, it was able to enhance the action of this peptide such that a much lower concentration was required to elicit the same effect (50% reduction in growth) obtained when PON-G1 was tested individually. Further work on the synergistic inhibition of REV4, PON-G1 and GR7 against smut and *E. saccharina* are ongoing.

Keywords: Indolicidin, Ponericin, Magainin, Cecropin, *Eldana saccharina*, *Fusarium verticillioides*, *Ustilago scitaminea*

Introduction

The potential of peptides, derived from living organisms, to control microbial pathogens has been investigated in recent years. Much of this research has focused on peptides belonging to four classes (Cecropins, Magainins, Indolicidins and Ponericins). In addition to anti-microbial activities, Ponericins have been shown to possess insecticidal activity, whereas Indolicidins are known to inhibit chymotrypsin protease activity, the main activity found in the mid-gut of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Cecropins and Magainins have anti-microbial activities against a wide range of fungal and bacterial species. Research has shown that it is possible to express these peptides in transgenic crops and thereby achieve

improved resistance to pathogens (Li *et al*, 2001; Ponti *et al*, 2003). Although this approach has been investigated for tobacco (Li *et al*, 2001), to date no such work has been reported for sugarcane. The aim of this study was to evaluate the inhibitory effect of four anti-microbial peptides (D4E1, GR7, REV4, PON-G1) against *Fusarium verticillioides* (fusarium), *Ustilago scitaminea* (smut) and *E. saccharina*. Peptides showing anti-microbial and insecticidal properties might be considered in future transgenic work on sugarcane.

Materials and Methods

An isolate of fusarium that is beneficial to *E. saccharina* (i.e. increases larval growth and survival) (McFarlane and Rutherford, 2005) was grown on cornmeal agar (17 g/L) that contained chloramphenicol (50 mg/L) and streptomycin (100 mg/L). Smut spores were collected in 10 ml of sterile H_2O that contained 5 µl of Tween 20. A single smut sporidial mating strain was isolated onto potato dextrose agar (PDA, 39 g/L) medium that was supplemented with chloramphenicol (50 mg/L).

Fusarium and smut cultures were inoculated into 5 ml of Mueller-Hinton broth (MHB, 17.5 g acid hydrolysate of casein, 3 g beef extract, 1.5 g dextrose) and incubated overnight on a shaker (27 °C). The respective cultures were diluted (2-7 x 10^5 CFU/ml) using MHB, and 100 µl of the diluted culture was dispensed accordingly into two polypropylene multi-well plates. A 10X concentrated stock of the peptides (D4E1, GR7, REV4 and PON-G1) was prepared in 0.1% acetic acid and 2% bovine serum albumin (BSA). A range of peptide concentrations was tested (100, 64, 32, 16, 8, 4, 2, 1, 0.5, 0 µg/ml). The multi-well plate was incubated at 27 °C for 48 hours in a high humidity chamber. After 48 hours the respective MHB cultures were mixed, and a representative sample for each peptide and concentration range was plated onto PDA. The inoculated plates were incubated at 30 °C for 48 hours, after which the colony counts for fusarium and smut sporidia were recorded. For each peptide tested the CFU/ml was plotted against concentration and the MIC_{50} (Minimum Inhibitory Concentration of peptide that reduced microbial growth by 50% when compared with the control, after 48 hours) and MMC (Minimum Microbiocidal Concentration of peptide that completely prevented microbial growth, after 48 hours) values were calculated using the equations that best fit the data obtained from three experiments.

Results and Discussion

The anti-microbial and anti-fungal properties of four peptides were evaluated against fusarium and smut (Table 1). Results showed that the Ponericin, PON-G1, was most effective in inhibiting the growth of both fusarium and smut. Low PON-G1 concentrations of 1.7 µM and 0.1 µM were required to reduce the growth of fusarium and smut sporidia, respectively, by 50% when compared with the control. Of note, REV4 (0.6 µM), was able to reduce the growth of smut by 50%, but not that of fusarium, even at the highest concentration tested (100 µg/ml = 52.9 µM) (Table 1).

The peptides PON-G1, REV4 and GR7 were selected for further work, since preliminary tests suggest that exposure of neonate *E. saccharina* larvae to these peptides results in a reduction in larval growth rate. Also, it has been discovered by other researchers that REV4 is able to protect another synthetic peptide (a Magainin) from protease degradation (Li *et al*, 2002). To establish whether a similar effect was possible in the present study, the potential synergistic action of these three peptides was also investigated.

Table 1. The effect of four peptides on the inhibition of *Fusarium verticilloides* and *Ustilago scitaminea*.

Pathogen	Peptide	Peptide class	MIC_{50} (μM)	MMC (μM)
F. verticilloides (fusarium)	D4E1	Cecropin	9.5	30.4
	GR7	Magainin	18.9	34.4
	REV4	Indolicidin	>52.9 (max tested)	>52.9
	PON-G1	Ponericin	1.7	7.1
U. scitaminea (smut)	D4E1	Cecropin	0.2	0.5
	GR7	Magainin	0.3	1.7
	REV4	Indolicidin	0.6	1.1
	PON-G1	Ponericin	0.1	0.3

MIC_{50} = Minimum Inhibitory Concentration of peptide that reduced microbial growth by 50% when compared with the control, after 48 hours. MMC = Minimum Microbiocidal Concentration of peptide that completely prevented microbial growth, after 48 hours. The MIC_{50} and MMC values were calculated using the equations that best fit the data obtained.

The three peptides (PON-G1, REV4 and GR7) were tested against fusarium individually and in combination (Table 2). It was again evident that REV4 has little or no inhibitory effect against fusarium. However, when PON-G1 was combined with REV4, a much lower PON-G1 concentration (0.5 μM) was required to reduce growth by 50%, suggesting that REV4 is able to protect PON-G1 and thereby maximise its inhibitory effect against fusarium. A similar effect was noted when PON-G1 was combined with GR7. When tested in combination, 0.5 μM PON-G1 and 0.7 μM GR7 were able to inhibit the growth of fusarium by 50%. However, when these peptides were tested individually, double the concentration (1.2 μM PON-G1 and 1.5 μM GR7) was required for the same inhibitory effect (Table 2). Investigations into the synergistic inhibition of PON-G1, REV4 and GR7 against smut and *E. saccharina* are ongoing.

Table 2. Evaluating the synergistic inhibition of three peptides against *Fusarium verticilloides*.

Pathogen	Peptide	MIC_{50} (μM)	MMC (μM)
F. verticilloides (fusarium)	**P**ON-G1	1.2	3.1
	REV4	>52.9 (max tested)	>52.9
	GR7	1.5	8.4
	P + **R**	0.5 + 0.8*	2.5 + 4.2*
	P + **G**	0.5 + 0.7*	4.7 + 13.9
	R + **G**	2.7 + 2.3	17.2 + 15.1
	P + **R** + **G**	1.3 + 2.1 + 1.9	2.4 + 4 + 3.5*

MIC_{50} = Minimum Inhibitory Concentration of peptide that reduced microbial growth by 50% when compared with the control, after 48 hours. MMC = Minimum Microbiocidal Concentration of peptide that completely prevented microbial growth, after 48 hours. The MIC_{50} and MMC values were calculated using the equations that best fit the data obtained. *Synergistic inhibition = two or more peptides acting together to produce an inhibitory effect greater than the sum of the two agents acting separately.

REFERENCES

Li Q, Lawrence CB, Xing HY, Babbit RA, Bass WT, Maiti IB and Everett NP (2001). Enhanced disease resistance conferred by expression of an antimicrobial magainin analog in transgenic tobacco. *Planta* 212: 635-639.

Li Q, Lawrence CB, Davies HM and Everett NP (2002). A tridecapeptide possesses both antimicrobial and protease-inhibitory activities. *Peptides* 23: 1-6.

McFarlane SA and Rutherford RS (2005). *Fusarium* species isolated from sugarcane in KwaZulu-Natal and their effect on *Eldana saccharina* (Lepidoptera: Pyralidae) development *in vitro*. *Proc S Afr Sug Technol Ass* 79: 120-123.

Ponti D, Mangoni ML, Mignogna G, Simmaco M and Barra D (2003). An amphibian antimicrobial peptide variant expressed in *Nicotiana tabacum* confers resistance to phytopathogens. *Biochem J* 370: 121-127.