PSEUDOMONAS FLUORESCENS GENETICALLY ENGINEERED TO PRODUCE AN INSECT TOXIN: A CULMINATION OF FIVE YEARS OF COLLABORATIVE RESEARCH

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Abstract

The bacterium *Bacillus thuringiensis* has been used as a microbial insecticide for half a century. However, limitations on its use against a pest such as *Eldana saccharina* Walker are that: a) *B. thuringiensis* is a soil-inhabiting organism and is therefore not persistent on plants, and b) the proteinaceous insecticide is degraded by sunlight when the bacterial cells lyse at the end of their life cycle. Five years ago the SASA Experiment Station and the Council for Scientific and Industrial Research (CSIR) embarked on a collaborative project to transfer genetic material from one bacterium, *B. thuringiensis* which produces an insecticide, to another bacterium *Pseudomonas fluorescens*. *P. fluorescens* occurs ubiquitously on plants and if genetically manipulated to produce the insecticide, the bacteria would be ingested by young foraging eldana larvae outside the stalk, and further damage to the sugarcane would be prevented. Toxicity bioassays carried out in the laboratory indicated that *P. fluorescens* genetically engineered to produce the insecticide was toxic to eldana larvae and a LC_{50} value of 1,86 mg of freeze-dried bacterial powder/ml of insect diet was calculated. The genetically engineered bacterium was found to colonise sugarcane in pots to a satisfactory level for a period of five months, after which time the bacteria could not be recovered from plants. Sugarcane plants in the glasshouse inoculated with the genetically manipulated bacterium were protected from eldana damage when compared with untreated controls. Significantly higher numbers of eldana per stalk and per cent internodes bored per stalk were observed for control plants, when compared with plants sprayed with the genetically engineered micro-organism.

Introduction

The use of synthetic organic insecticides, developed during the last half of this century, may pose risks to human health and can cause environmental problems. Consequently, interest has developed in using alternative means for pest control. One contemporary approach that has received attention is the development of *Bacillus thuringiensis* toxins as biological insecticides.

B. thuringiensis is a gram positive soil bacterium which produces a crystalline inclusion during sporulation. These inclusions consist of proteins exhibiting a highly specific insecticidal activity. Most strains of *B. thuringiensis* are active against larvae of the Lepidoptera group, but some show toxicity against Diptera (Thorne *et al.*, 1986) or Coleoptera species (Krieg *et al.*, 1983).

Upon ingestion by insects, the crystalline inclusion is solubilised in the midgut, releasing proteins called δ-endotoxins. These proteins (protoxins) are activated by larval midgut proteases, and the activated toxins interact with the midgut epithelium causing a disruption in membrane integrity and ultimately leading to insect death (reviewed by Hofte and Whiteley, 1989; Gill *et al.*, 1992).

Eldana saccharina Walker (Lepidoptera: Pyralidae) is a major pest of sugarcane in South Africa (Atkinson *et al.*, 1981). The larvae bore into the stalks of sugarcane and can cause considerable crop loss. A preliminary investigation into the control of eldana using a biological insecticide, Thuricide (a commercial formulation of the bacterium *B. thuringiensis* var. *kurstaki*), indicated that large amounts of the preparation had to be applied frequently, which was uneconomical (*Rutherford, personal communication).

The feasibility of using *B. thuringiensis* for insect control has been increased by advances in recombinant DNA technology. Use of this technology has facilitated cloning of the toxin genes and their expression in plants (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Delannay *et al.*, 1989), and in plant- and soil-inhabiting bacteria (Obukowicz *et al.*, 1986; Waalwijk *et al.*, 1991).

Previous research with locally isolated strains of *B. thuringiensis* indicated high toxicity levels towards eldana in the laboratory (Jacobs, 1989; Black and Snyman, 1991), and because monocotyledonous plants such as sugarcane are difficult to transform using standard techniques, it was decided to introduce the insecticidal toxin gene into a bacterium which has the ability to colonise sugarcane plants.

The bacterium chosen as the recipient for the toxin gene is a fluorescent pseudomonas, which is a gram negative, saprophytic bacterium, capable of colonising both the phylloplane and the rhizosphere of many plants. The advantages of cloning the toxin gene into this bacterium are that it will colonise plant surfaces and produce the insecticidal toxin at the same time. Foraging eldana larvae which ingest sufficient amounts of the genetically engineered bacterium will die before causing further damage to the plant.

Before contemplating the release of the genetically engineered *Pseudomonas fluorescens*, it was necessary to establish certain criteria about the performance of the bacterium, so the following were investigated: (a) toxicity levels to eldana in laboratory conditions, (b) the colonisation ability on sugarcane in pots and (c) the effect of inoculated glasshouse-grown sugarcane plants on eldana.

Materials and methods

Bacterial strains

B. thuringiensis was isolated locally from dead feral eldana larvae and *P. fluorescens* was isolated from the surface of sugarcane leaves, stems and eldana borings according to Jacobs (1989).

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Cloning the δ-endotoxin gene

The B. thuringiensis toxin gene was sequenced, characterised and chromosomally inserted into *P. fluorescens* as reported by Herrera (1992).

Laboratory toxicity bioassays

Insecticidal activity of the toxin-producing pseudomonas was assessed using the bioassay method previously described by Black and Snyman (1991). Two week old eldana larvae were fed on an artificial diet into which different concentrations of freeze-dried pseudomonas bacteria were incorporated. Larvae were incubated for five days at 30°C after which mortality was recorded. Each bioassay was repeated three times and mortality ratings were compared by probit analysis (Finney, 1971).

Colonisation of sugarcane by the genetically engineered pseudomonas

P. fluorescens was grown to stationary phase in nutrient broth (Biolab, SA) containing the antibiotics (all Sigma, USA) rifampicin (100 mg/l), nalidixic acid (100 mg/l) and kanamycin (20 mg/l), for 24 h at 27°C. Single budded sugarcane setts (variety N11) were dipped into a suspension containing the bacteria and methyl cellulose (3% (w/v); Sigma, USA) (Kloepper and Schroth, 1981). The concentration of the initial inoculum was 1×10^9 colony forming units (cfu)/sett.

The stems of sugarcane plants grown in pots in the glasshouse were sampled at monthly intervals, as were stalks from control plants, which were not inoculated with the bacterial suspension. Access to the glasshouse was restricted because the environmental impact of the genetically engineered bacterium had not been assessed. The leaf blades were discarded and the stalk was cut into 5 cm pieces. The stalk segments were placed into glass bottles containing 50-200 ml sterile water and glass beads, and were shaken on a wrist-action shaker for 30 min. The resultant suspension was centrifuged at 8 000 g for 20 min after which the bacterial pellet was resuspended in 2 ml of water. Aliquots were plated onto King's medium B (King *et al.*, 1954) containing the selective antibiotics. Plate counts were carried out after incubation at 27°C for 48 h.

The effect on eldana of sugarcane inoculated with the genetically engineered pseudomonas

Six month old sugarcane plants grown in the glasshouse were inoculated with 200 eldana eggs per plant. The eggs were placed by hand at the base of the stalk, behind a leaf sheath (Nuss and Atkinson, 1983). Watering was withheld for a week before and after inoculation. Stalks were sampled four weeks after egg placement, and the larval numbers and number of internodes bored were recorded.

Results and discussion

Larval bioassays

P. fluorescens genetically engineered to produce the *B. thuringiensis* protoxin was shown to be toxic to eldana larvae in the laboratory. Mortalities ranging from 26-89% were observed when larvae were fed on diet into which the genetically engineered bacteria had been incorporated (Figure 1). The lethal concentration at which 50% of the larvae died (LC_{50}), obtained by probit analysis, was 1,86 mg freeze-dried bacterial powder/ml insect diet.

Thuricide (the commercial preparation of *B. thuringiensis*) and the unaltered *B. thuringiensis* preparations have LC_{50} values of 0,08 and 0,06 mg/ml respectively. The above LC_{50} values indicate that these preparations are more toxic to

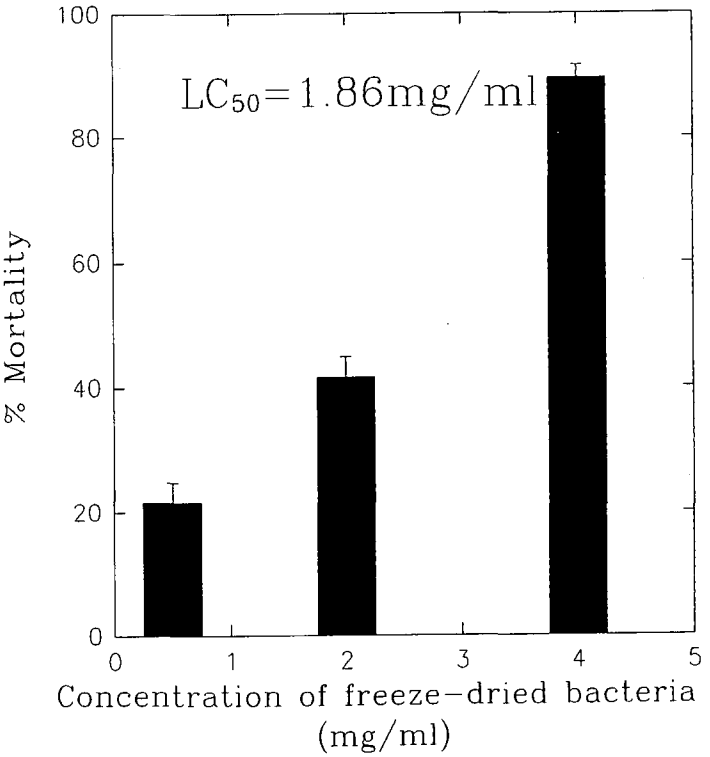

FIGURE 1 Percentage eldana mortality observed after five days when the genetically engineered *P. fluorescens* was bioassayed in the laboratory. (n=3) (SE are represented by lines above bar).

eldana in the laboratory than the insecticidal-producing pseudomonas. However, the difference observed under laboratory conditions may not reflect the performance of the genetically altered bacterium in the field, where potentially, this organism can give inoculated plants better protection from insect attack than the unaltered *B. thuringiensis*. Due to stringent legislation regulating the release of genetically engineered micro-organisms, this hypothesis has not been tested. Consequently, the efficacy of the toxin-producing pseudomonas on eldana infested sugarcane plants in pots was monitored in glasshouse conditions.

Colonisation ability of the toxin-producing pseudomonas

The genetically altered *P. fluorescens* bacteria were isolated from sugarcane stalks, which germinated from setts dipped in a bacterial suspension, over a six-month period. The numbers of bacterial cfu remained stable for the first three months (10^3-10^4 cfu/g fresh weight). Bacterial numbers decreased at months four and five until at month six, no bacteria could be isolated from stalks (Figure 2).

There are several possible reasons why no bacteria were isolated from sugarcane stalks after the sixth month:

(1) the toxin-producing pseudomonas bacteria may be outcompeted by other micro-organisms on the phylloplane. This is supported by the observation that wild type pseudomonas could be isolated from the soil on King's medium B. The genetically engineered organism has to produce an extra protein, the toxin, and it may therefore not be as competitive as normal;

(2) the toxin gene appears to be stable in laboratory conditions, but may be unstable on plant surfaces when exposed to environmental conditions and it may be lost over time. The use of a technique such as the polymerase chain reaction (PCR) (Carozzi *et al.*, 1991) may be employed to detect regions of DNA that have been lost during the bacteria's life cycle;

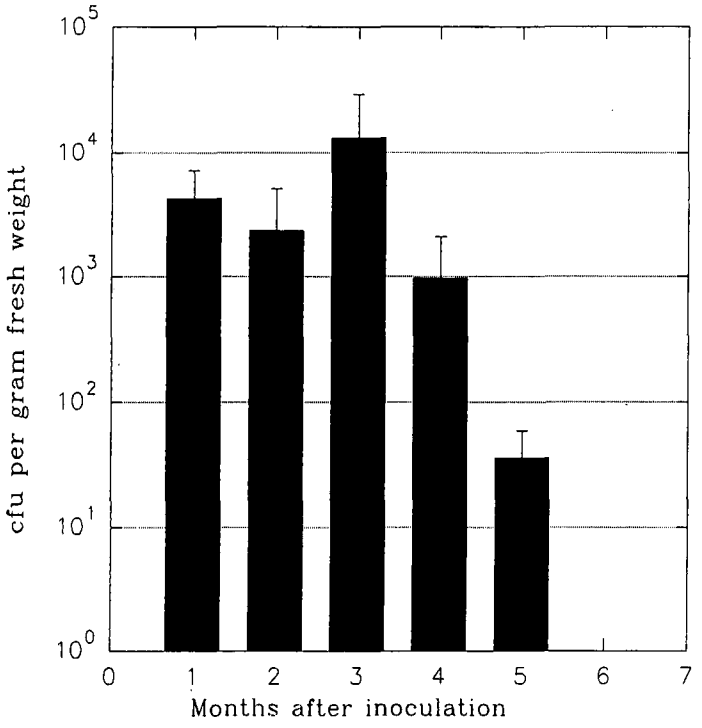

FIGURE 2 Colonisation of sugarcane stalks by the genetically engineered *P. fluorescens* over six months. Stalks were sampled at monthly time intervals on KMB containing the antibiotics rifampicin and nalidixic acid (both at 100 mg/l) and kanamycin (20 mg/l). (n=12) (SD at the 95% confidence level).

(3) it is possible that the bacteria were present at levels lower than detectable by the sampling method employed.

Further glasshouse trials need to be carried out to provide more conclusive evidence for the above assumptions.

The effect of pseudomonas inoculated plants on eldana

No bacteria could be recovered from six month old plants, so they were re-sprayed with a bacterial suspension (2×10^9 cfu; 100 ml/pot) two weeks prior to being inoculated with eldana eggs. The plants were examined for eldana damage after four weeks. Apparently, control plants were heavily infested with eldana, with frass evident at the base of most stalks, indicative of eldana activity.

Control plants had significantly more eldana, and internode damage per stalk and per pot, than the plants sprayed with the insecticide-producing pseudomonas. There were three times as many eldana larvae in the control pots than in the treated ones, with eldana numbers of 3,6 and 0,9 larvae (or pupae) per pot, respectively (Table 1). Similarly, there was a three times difference in the amount of damage recorded per pot, with 33,5% of the internodes being bored in control pots, whereas only 10,9% internodes were bored in the treated pots.

Bacteria were isolated from plants which were reinoculated after six months with the genetically manipulated pseudomonas. Plate counts indicated that both the genetically engineered bacteria and wild type pseudomonas bacteria were present on the surface of plants in the glasshouse.

Table 1

A comparison of eldana numbers and stalk damage between control sugarcane plants and those sprayed with the genetically engineered pseudomonas. (n=27 for pots, n=46 for sprayed stalks, n=58 for control stalks) (SD at the 95% confidence level)

	No. eldana per pot	% internodes damaged per pot	No. eldana per stalk	% internodes damaged per stalk
Control	3,6 ± 1,3	33,5 ± 8,6	2,2 ± 0,8	30,0 ± 7,3
Treatment	0,9 ± 0,5	10,9 ± 4,5	0,6 ± 0,2	12,5 ± 4,9

Conclusions

The toxin gene was successfully transferred from *B. thuringiensis* to *P. fluorescens*, where it now resides on the chromosome. The glasshouse trial reported here supports laboratory evidence that the genetically engineered pseudomonas is capable of killing eldana larvae. The sugarcane plants in the glasshouse sprayed with the insecticidal-producing pseudomonas two weeks before eldana inoculation, showed very low eldana levels and plants were not as severely damaged as untreated control plants.

It may be necessary to conduct further glasshouse trials to try to improve bacterial detection, and also to determine whether low levels of bacteria are able to prevent eldana infestation of pseudomonas inoculated sugarcane in the glasshouse. The CSIR are attempting to increase toxin expression in the pseudomonas, by placing the toxin gene under the expression of a stronger promoter (the tac promoter; Ge *et al.*, 1990) and this will require additional glasshouse trials.

The protection offered by the genetically engineered pseudomonas is very encouraging, but at present, it still does not offer long term protection against eldana infestation.

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