ABILITY OF *PSEUDOMONAS FLUORESCENS*, ENGINEERED FOR INSECTICIDAL ACTIVITY AGAINST SUGARCANE STALK BORER, TO COLONISE THE SURFACE OF SUGARCANE PLANTS

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Abstract

A transgenic strain of *Pseudomonas fluorescens*, containing the cry1A(c) gene from a wild type strain of *Bacillus thuringiensis*, was evaluated for its ability to colonise the external surfaces of sugarcane. Plants were inoculated with non-transgenic (parent) and transgenic strains of *P. fluorescens*, separately and in combination, by dipping setts into bacterial suspensions before germination, followed by aerial spraying of shoots with the equivalent suspensions one month later. The populations of bacteria resident on the stalks were sampled by a washing protocol at monthly intervals two to eight months after first inoculation. At 11 months, stalk, leaf and root populations were sampled. Viable bacteria, all fluorescent types as well as the non-transgenic strain and the transgenic strain of *P. fluorescens*, were enumerated by means of plate counts on selective media. In addition, the presence/absence of the cry1A(c) gene was determined by means of a specific PCR procedure using DNA extracted from bacterial washings. Results showed that the stalk surface density of general fluorescent bacteria gradually declined with time, and at 11 months was significant only on the roots. The surface density of the non-transgenic and transgenic *P. fluorescens* strains declined rapidly, and viable cells were recoverable for only three months, although the cry1A(c) gene was detectable by PCR for up to eight months. Data from the experimental material treated with the combination inoculum demonstrated that the non-transgenic *P. fluorescens* strain outcompeted its transgenic counterpart.

Introduction

Eldana saccharina Walker (Lepidoptera: Pyralidae) is an endemic insect species of sugarcane throughout Africa. It has been estimated that an average annual loss of R6 million was caused by eldana damage during the drought years 1980 to 1986 (Smith, 1990). However, damage caused by eldana to sugarcane in recent seasons is estimated to be R60 million per annum (*Leslie, personal communication). Since 1974, research into controlling *E. saccharina* at the SA Sugar Association Experiment Station has been continuous. Studies investigating the biology of *E. saccharina*, varietal resistance, crop management, chemical control and biological control have been conducted (Carnegie, 1983) and are still being pursued.

Bacillus thuringiensis (Bt) is a gram positive bacterium which produces a crystalline inclusion consisting of highly specific proteins which exhibit insecticidal activity to larvae of the Lepidoptera and Diptera (Thorne *et al.*, 1986). Previous bioassays using a locally isolated strain of Bt (Bt 234) indicated high toxicity levels towards eldana in the laboratory (Black and Snyman, 1991). The δ-endotoxin gene from Bt 234 was cloned and transferred into a bacterium (Herrera *et al.*, 1994). The bacterium chosen as the recipient for the toxin gene was a fluorescent pseudomonad, which is a gram negative epiphytic bacterium considered to be capable of colonising both the phylloplane and the rhizoplane of sugarcane (Snyman *et al.*, 1993). The fluorescent pseudomonad with the cry1A(c) gene insert expresses the 133 kD toxin protein and is toxic to eldana larvae in the laboratory (Herrera *et al.*, 1994). Preliminary studies have indicated that control of eldana can be achieved by inoculating plant surfaces with the transgenic bacterium (Snyman *et al.*, 1993).

The aim of this project was to evaluate the colonisation ability of the transgenic and non-transgenic *P. fluorescens* on inoculated sugarcane plants by using both conventional microbiological plate count methods and specific PCR protocols for the detection of the cry1A(c) gene. This report shows that the transgenic and the non-transgenic pseudomonad strains are poor colonisers on the sugarcane stalk surface. In addition, the wild type strain outcompetes the transgenic strain *in vivo*.

Materials and methods

Bacterial strains and culture conditions

Fluorescent bacteria of the pseudomonad group were selectively isolated from sugarcane plants by culture on King's medium B (King *et al.*, 1959). *P. fluorescens* 14, a spontaneous mutant resistant to rifampicin and nalidixic acid, was cultured in the laboratory on King's medium B containing the antibiotics at a concentration of 100 mg/ℓ, and the same medium used for the selective isolation of the *P. fluorescens* 14 strain from plants (Jacobs, 1989). The genetically engineered *P. fluorescens* 14 :: Omegon-Km-cry strain *P. fluorescens* (toxin) was selectively cultured and re-isolated from plants using King's medium B containing nalidixic acid, rifampicin and kanamycin (20 mg/ℓ), as described by Snyman *et al.*, (1993).

Inoculation of sugarcane plants

Single budded sugarcane setts of variety NCo376 were dipped into bacterial suspensions (10^9 *c.f.u./sett)* containing 3% (m/v) methyl cellulose (Sigma, USA). The following treatments were used: (1) a control (no inoculation), (2) *P. fluorescens* 14, (3) *P. fluorescens* 14 and *P. fluorescens* (toxin) in the same treatment and (4) *P. fluorescens* (toxin). Setts were planted in a soil-compost mixture and germinated in pots in a confined glasshouse. The germinated sugarcane plants were re-inoculated with respective aerial sprays of 10^9 c.f.u./plant one month after the first inoculation.

Enumeration of surface bacteria by means of plate counts

Three samples (whole untrashed stalks) from each treatment were taken at monthly intervals, starting one month after the second (aerial) inoculation with *P. fluorescens* strains

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on the surface of sugarcane plants. After harvesting, the mass of the stalks as well as the surface area were determined. Surface bacteria were harvested by a washing procedure and numbers determined using plate counts as described by Snyman *et al.* (1993). After 11 months the roots, stems and leaves from each treatment were sampled and the bacterial numbers determined in the same way.

Detection of the toxin gene by the polymerase chain reaction (PCR)

To maximise the detection of the cry1A(c) gene fragment, plant washings were pre-cultured in a non-selective nutrient broth at 27°C for 16 h, to multiply bacterial populations. Bacterial DNA (template) was isolated from cultures using the protocol described by Le Gouill and Dery (1991). Specific 22-mer PCR primers were designed, based on the sequence of the *P. fluorescens* cry1A (c) gene insert, to amplify a 543 bp fragment within the coding region. Each specific PCR reaction contained 10 mM Tris- HCl (pH 8,3), 10 mM KCl, 2,5 mM $MgCl_2$, 0,1 mM each of dATP, dCTP, dTTP and dGTP, 0,2 M of each primer, 50-500 ng of template DNA and 1 U Taq polymerase (Boehringer) in a final volume of 15 ul. The Hybaid Omnigene thermal cycler was programmed for one cycle of 3 min at 94°C, 5 min at 60°C and 2 min at 72°C, followed by 35 cycles of 2 min at 94°C, 2 min at 60°C and 3 min at 72°C, and a final cycle of 7 min at 72°C.

Agarose gel electrophoresis of DNA fragments

PCR products were separated by electrophoresis using 2% (m/v) agarose gels run in 0,5 × TBE buffer (45 mM Tris, 44 mM boric acid, 1 mM EDTA (pH 8,0)). Gels were stained in a 1 g/ml ethidium bromide solution for 30 min and destained in 0,5 × TBE for 30 min. DNA fragments were visualised under UV light.

Results

Changes in fluorescent pseudomonad populations following inoculation

The density (c.f.u/cm^2) of total viable fluorescent bacteria on plant stalks over an eight month period is presented in Figure 1. The population density of autochthonous fluorescent bacteria in the uninoculated control treatment was 10 c.f.u/cm^2 and 18 c.f.u./cm^2 after two and four months respectively. The population density in the control treatment decreased to five c.f.u./cm^2 after six months, and remained constant up to eight months. The density of fluorescent bacteria on inoculated plants varied from two to four times higher than those on the control at two months after the inoculation. This indicates that the inoculation of plants was effective (Figure 1). The fluorescent bacterial populations in all treatments declined to about 5 c.f.u./cm^2 at eight months after the inoculation. However, the total number of bacteria isolated from plant surfaces, calculated as c.f.u/plant, increased from 2×10^3 after two months to 1×10^4 at eight months after the initial inoculation *(data not shown). P. fluorescens* strains resistant to rifampicin and nalidixic acid were isolated from all inoculated treatments, but none were isolated from the uninoculated control (Figure 2). *P. fluorescens* strains resistant to rifampicin, nalidixic acid and kanamycin were isolated only from the treatments that were inoculated with transgenic *P. fluorescens* (toxin) (Figure 3). These results confirm the specificity of the selection criteria and suggest the isolated bacteria were derived from the strains used for inoculation. No antibiotic resistant types were detectable by plate counts after three months, indicating that the populations of *P. fluorescens* 14 and *P. fluorescens* (toxin) had declined to extremely low levels. When the roots, stems and leaves from each treatment were sampled after 11 months, fluorescent bacteria were found only on the roots, and did not include the antibiotic resistant strains (i.e. *P. fluorescens* 14 or *P. fluorescens* (toxin)).

Specific PCR detection of the transgenic P. fluorescens (toxin)

The specific 543 bp PCR fragment (toxin gene) was amplified from stalk samples inoculated with the transgenic bacterium up to eight months after treatments but was not detected from root, stem or leaf samples at 11 months. No PCR fragments were amplified from the control treatment

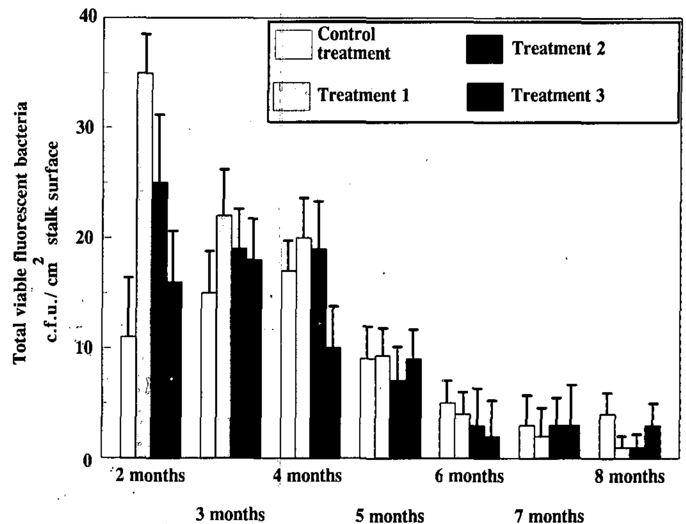

FIGURE 1 Surface density of total viable fluorescent bacteria isolated from sugarcane stalks using plate counts (King's medium B agar) over an eight month period. Treatments: control (no inoculum); treatment 1 (*P. fluorescens* 14 inoculum); treatment 2 (a mixed *P. fluorescens* 14 and *P. fluorescens* :: Omegon-Km-cry), 1:1 ratio; treatment 3 (*P. fluorescens* :: Omegon-Km-cry). All values represent a mean and a standard deviation at 95% confidence levels.

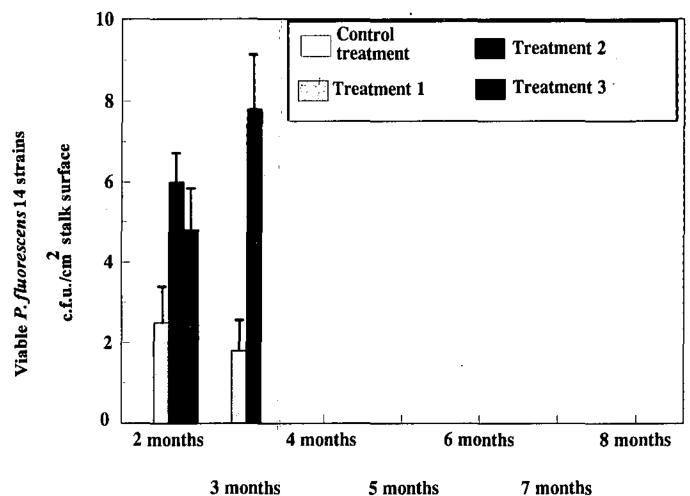

FIGURE 2 Selective enumeration of all *P. fluorescens* strain 14 isolated from sugarcane stalks using plate counts (King's medium B with 100 mg/l rifampicin and nalidixic acid) over an eight month period. Treatments: control (no inoculum); treatment 1 (*P. fluorescens* 14 inoculum); treatment 2 (a mixed *P. fluorescens* 14 and *P. fluorescens* :: Omegon-Km-cry), 1:1 ratio; treatment 3 (*P. fluorescens* :: Omegon-Km-cry). All values represent a mean and a standard deviation at 95% confidence levels.

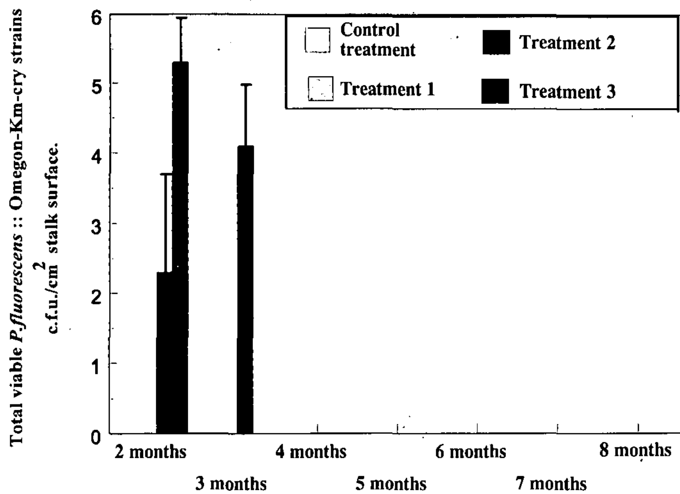

FIGURE 3 Selective enumeration of the *P. fluorescens* :: Omegon-Km-cry strain from sugarcane stalks using plate counts (King's medium B containing 100 mg/l rifampicin, 100 mg/l nalidixic acid and 20 mg/l kanamycin) over an eight month period. Treatments: control (no inoculum); treatment 1 (*P. fluorescens* 14 inoculum); treatment 2 (a mixed *P. fluorescens* 14 and *P. fluorescens* :: Omegon-Km-cry), 1:1 ratio; treatment 3 (*P. fluorescens* :: Omegon-Km-cry). All values represent a mean and a standard deviation at 95% confidence levels.

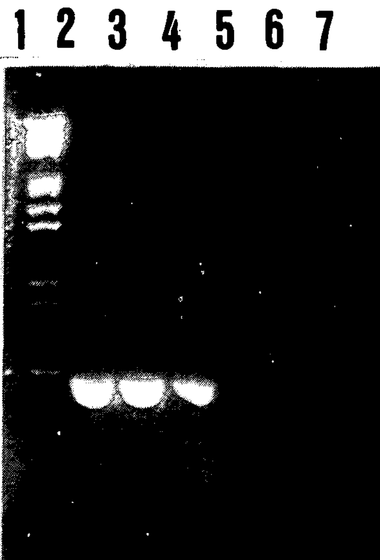

FIGURE 4 Agarose gel electrophoresis of the toxin gene amplification products after specific PCR. DNA was isolated from bacteria cultured from inoculated plant washings. Lanes: (1) M.W. marker λ-EcoR1/Hind III; (2) positive control (*P. fluorescens* :: Omegon-Km-cry chromosomal DNA); (3) mixed *P. fluorescens* 14 and *P. fluorescens* :: Omegon-Km-cry, 1:1 ratio; (4) *P. fluorescens*:: Omegon-Km-cry treatment; (5) *P. fluorescens* strain 14 treatment; (6) control treatment (no inoculum); and (7) no template control.

or the plants inoculated with *P. fluorescens* 14 at any stage (Figure 4). The results indicate that the transgenic pseudomonad was still present on the surface of sugarcane plants at very low levels up to eight months after the initial inoculation.

Competition between the parental and the transgenic P. fluorescens

In order to evaluate the potential for *P. fluorescens* (toxin) to compete against *P. fluorescens* 14, the PCR technique was used to determine the ratio of transgenic to non-transgenic

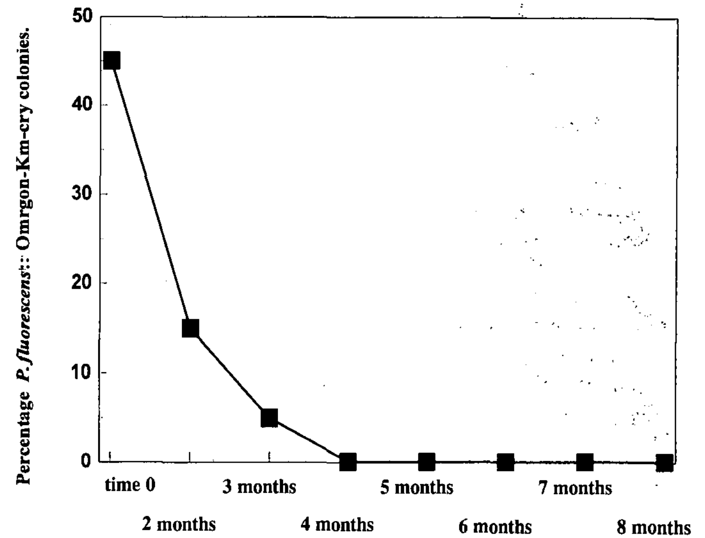

FIGURE 5 Competition between the non-transgenic and the transgenic *P. fluorescens* strains in the mixed treatment over a period of eight months.

P. fluorescens strains on the surface of sugarcane stalks inoculated with the mixed treatment. A 0,1 ml aliquot from each non-selective broth culture derived from stalk washings was plated out onto King's medium B containing 100 mg/l rifampicin and nalidixic acid for the selection of both *P. fluorescens* strains. Separate DNA extractions were performed on 20 individual randomly selected colonies and the toxin specific fragment was amplified using the PCR technique. The presence of the 543 bp fragment positively identified a transgenic pseudomonad colony. The percentage of *P. fluorescens* (toxin) colonies was 45% at the time of inoculation (Figure 5). The percentage of colonies containing the toxin fragment decreased to 15% at two months and 5% at three months after inoculation. All subsequent samples contained less than 5% of cells with the specific 543 bp fragment. The data suggests that the non-transgenic pseudomonad outcompetes the transgenic pseudomonad *in vivo*.

Discussion

The transgenic *P. fluorescens* containing the cry1A(c) insert had been shown previously to protect sugarcane plants against eldana damage for two weeks after inoculation (Snyman *et al.*, 1993). However, in this study the plate counts suggest that the transgenic bacterium was present on the surface of sugarcane plants for a period of only three months after inoculation. The results indicate that both the non-transgenic and the transgenic pseudomonad strains are poor colonisers of the surface of sugarcane plants. In addition, since the population density of naturally occurring fluorescent bacteria of the pseudomonad group decreased over time and no cells were detected in washings from stalk surfaces after 11 months, it seems evident that these bacteria are not colonisers of the phylloplane of mature sugarcane plants. The data suggest that fluorescent pseudomonads are better colonisers of the rhizoplane.

In this study a specific PCR technique has been developed which could be used to detect the presence of the cry1A(c) gene in the transformed pseudomonad. The PCR results obtained showed that the transformed bacterium was present on the plant surface in very low numbers for up to eight months, thereby demonstrating the power and sensitivity of the PCR technique compared with plate count methods.

Eldana infestations only occur once the crop is about six months old, and the damage caused by larvae increases as

the age of the crop increases (Atkinson, 1978). Therefore, to be a successful biocontrol agent, the transgenic bacterium would have to colonise mature sugarcane stalks and the bacterium should not be outcompeted by other epiphytic microorganisms on the surface of sugarcane plants. However, the results show that the *P. fluorescens* (toxin) strain does not survive on the stalk surface for a period longer than eight months. Furthermore, the PCR data indicate that the transgenic bacteria compete poorly with the non-transgenic *P. fluorescens* strain on the stalk surface. It is evident that *P. fluorescens* strain 14 is not a good candidate for the integration of the cry1A(c) gene to control eldana damage in sugarcane, as the transgenic strain cannot offer long term protection against eldana damage in mature sugarcane plants.

The successful control of insect damage in other crops by the insertion of a toxin gene from *Bacillus thuringiensis* into plant colonising bacteria has been reported. The cry1A(c) gene has been integrated into the endophyte *Clavibacter xyli* and this bacterium has been shown to offer long term protection to maize plants against European corn borer (Lampel *et al.*, 1994). Furthermore, a strain of *Rhizobium* containing the δ-endotoxin gene has been shown to protect pea and white clover plants against damage caused by *Sitona larvae (Skot et al.,* 1990). It is possible that the integration of the toxin gene into a more efficient sugarcane colonising bacterium could offer long term protection to sugarcane against eldana damage. Therefore, the potential use of a sugarcane endophyte as the recipient of the cry1A(c) gene is also being investigated (Herrera *et al.*, 1994). However, since the recent development of protocols for the transformation of sugarcane plants, greater success might be obtained if the cry1A(c) gene was integrated into the sugarcane genome. The PCR techniques to detect the cry1A(c) gene, developed in this study, could be useful in these research programmes.

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