ASSESSMENT OF SUGARCANE ENDOPHYTIC BACTERIA AND RHIZOSPHERIC *BURKHOLDERIA* SPECIES AS ANTIFUNGAL AGENTS

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Abstract

Naturally occurring bacterial endophytes were isolated from the stalks of six different sugarcane varieties. The highest numbers of bacterial populations were isolated from variety N12 and this variety was used for further investigations. Xylem sap as well as crushed cane sap was used for isolations, but no differences were observed between the type and numbers of species isolated. Endophytic bacterial populations decreased from the lowest to the upper-most internode. The lowest internode was surface sterilized and used to extract bacterial species on four different media (modified PW, TSA, PCAT and *Candida* medium). Most bacterial species were isolated on PW while PCAT medium was used to isolate *Burkholderia* and *Pseudomonas* species. The most common bacterial species isolated from sugarcane stalks were *Pseudomonas* spp., *Zymomonas* spp., *Burkholderia* spp., *Bacillus* spp., *Serratia* spp., *Klebsiella* spp. *and Xanthomonas* spp.

Burkholderia reference strains, local endophytic stalk isolates as well as local *Burkholderia* isolates from the sugarcane rhizosphere were tested for their antifungal activity against sugarcane smut (*Ustilago scitaminea)* and *Fusarium* spp. causing stalk rot. Forty-seven strains inhibited the growth of *Ustilago* while seventy-two strains inhibited the growth of *Fusarium in vitro.* Twenty-one of these bacterial strains inhibited the growth of both *Fusarium* and *Ustilago*.

Keywords: Sugarcane, endophytes, *Burkholderia*, pathogen inhibition

Introduction

Endophytic bacteria are present in most plant species and can latently or actively colonize the plant locally as well as systemically. It has recently been discovered that these bacteria can have beneficial effects on host plants, such as growth promotion and increased resistance against pathogens and parasites (Hallman *et al*, 1998).

The term 'endophyte' in this paper is defined as all bacteria within tissues internal to the epidermis of the sugarcane stalk, with no distinction between 'pathogenic' and 'non-pathogenic' types. This definition is similar to that of Kloepper *et al*. (1992) who described endophytes as all bacteria that colonize the interior of plants, including active and latent pathogens.

Environmental and health concerns about the extended use of pesticides in agriculture necessitate the finding of alternative control approaches for eliminating or controlling pathogens from crops. Biological control of diseases and pests thus need to be investigated. Several authors have reported on the use of bacteria or fungi as biocontrol agents. Raupach and Kloepper (1998) tested plant growth-promoting bacteria (*Bacillus* spp.) for biocontrol against cucumber pathogens, *Colletotrichum orbiculare*, *Pseudomonas syringae* and *Erwinia tracheiphila*. A mixture of *Bacillus* spp as a seed treatment showed intensive plant growth promotion and disease reduction to a level statistically equivalent to a synthetic elicitor applied as a spray. Sung and Chung (1997) (reported in

Raupach and Kloepper, 1998) found that chitinase-producing *Streptomyces* and *Bacillus cereus* used in conjunction with antibiotic-producing *P. fluorescens* and *Burkholderia cepacia* isolates caused suppression of sheath blight in rice.

The endophytic bacteria isolated from sugarcane (*Saccharum* spp hybrids) include diazotrophic (nitrogen-fixing) bacteria such as *Acetobacter diazotropicus*, *Herbasprillum* and *Azospirillum* spp (James and Olivares, 1997; Sevilla *et al*., 2001). *Azospirillum* are not wholly endophytic but are root-associated, soil-dwelling bacteria that are often found within plants (James and Olivares, 1997). Root-associated *Burkhoderia* species have been isolated from different sugarcane varieties in South Africa (Vogel, unpublished). A *Burkholderia* species isolated from maize roots was found to promote maize growth, enhance crop yields and to suppress soil-borne pathogens (Estrada-de-los Santos *et al*., 2001).

The objectives in the present study were two-fold: The first was to determine what endophytes colonise sugarcane stalks, their distribution in the stalk and whether stools in a field of the same variety are equally infected by endophytes. The second objective was to determine whether naturally occurring strains of endophytic- and root-associated bacteria from South African sugarcane varieties have an inhibitory effect on the growth of the fungal sugarcane pathogens, *Ustilago scitaminea* (the causal agent of smut) and *Fusarium* spp. (the causal agent of stem rot). Strains that inhibit the growth of pathogens and also have nitrogen-fixing properties could be inoculated into sugarcane varieties and thereby enhance the growth of the crop in the field.

Materials and Methods

Isolation of enodphytic bacteria from stalks
Undamaged sugarcane stalks of 6 varieties, N12, N14, N16, N17, N23 and NCo376 from different places were washed and surface sterilised. The lowest 3 internodes from each stalk were cut and the ends sealed in molten candle wax. They were then surface sterilised in 10% Jik for 10 minutes. A core sample was then taken from the middle internode, weighed and centrifuged aseptically. After centrifugation the stalk pieces were crushed with a sterilised garlic crusher. Subsamples of 100 µl of sap were plated out onto different growing media, namely, PCAT, TSA, modified PW and *Candida* medium (Atlas, 1997) to isolate bacteria and yeasts present in the cane stalks. Bacterial counts were done 48 hours after incubation of the plates at 30^{o}C.

Isolation of root-associated bacteria
Root–associated bacteria were isolated as described by Vogel *et al*. (2002), from varieties NCo376, N8, N12, N21 and N16.

Fungal sources
The *Fusarium* isolate was obtained from sugarcane bored by the stalk borer, *Eldana saccharina*. *Ustilago scitaminea* spores from a smut whip were incubated on PDA plates and single sporidia were subcultured and used in the bioassays.

Identification of endophytic bacteria from stalks
Sequencing of the intergenic spacer region on the *rrn* operon of bacteria (van Antwerpen, 1999) as well as the BIOLOG identification system were used to identify the bacteria.

Inhibition assays
Eighty-four endophytic bacterial strains isolated from the stalk, three hundred and twenty five root-associated bacterial isolates of various sugarcane varieties and thirty-nine *Burkholderia* reference strains (received from Jacques Balandreu) were spot inoculated onto PW medium and incubated for 3 days at 30^{o}C. The bacteria were then killed by chloroform fumigation for 45 minutes and the

bacterial culture was washed off with sterile water and the plates left to dry for 2 hours. Streptomycin (2.0 mg/0.5ml) and penicillin (1.3 mg/0.5ml) were added to the plates to prevent the growth of any other contaminating bacteria and left to dry. The plates were overlaid with smut sporidia and incubated for 48 hours and observed for the appearance of inhibition zones. For the *Fusarium* inhibition assays the bacteria and the fungus were plated onto PW medium simultaneously and checked for inhibition zones after 3 days.

Results and discussion

Bacterial endophytes

In an initial experiment where six different sugarcane varieties were used to isolate endophytic bacteria, it was found that variety N12 contained the most species and numbers of bacteria, followed by variety N16. It was decided to concentrate on variety N12 for future studies. The most common bacterial genera isolated from sugarcane stalks were *Pseudomonas* (15%), *Xanthomonas* (15%), *Klebsiella* (15%), *Zymomonas* (4%), *Bacillus* (4%), *Serratia* (0.3%), *Burkholderia* (0.2%) and 7 other unidentifeid bacterial species (46.5%). Other bacteria that occur naturally in sugarcane (especially in the leaf), are species of *Herbaspirillum* and *Erwinia* (van Antwerpen and McFarlane, 1999). The xylem-inhibiting bacterium *Leifsonia xyli* subsp. *xyli* (the causal agent of Ratoon Stunting Disease) is also a common inhabitant of sugarcane but it is slow growing and difficult to isolate. In this study only fast-growing non-pathogenic bacteria were tested as biological control agents.

Distribution of bacteria in the stalk

It was found that the number of bacteria decreased from the bottom (2665 bacteria/ml) to the top (99 bacteria/ml) of the cane stalk. The reason for this might be because bacteria enter the plant through the roots or through the cut ends of planting material. More bacteria were isolated from internodes immediately above ones with a boring or crack. Damage to the root system by nematodes may also increase the incidence of bacteria in the plant. For this study the second internode from stalks without any damage were used for bacterial isolations. It was also found that not all stools carried the same load of endophytes, with some stools poorly colonised (0-100 bacteria/ml) whilst others were heavily colonised (2000-7000 bacteria/ml).

Inhibition assays

Eighty-three bacterial isolates from stalks of N12, 325 *Burkholderia* root-associated isolates from various varieties and 39 *Burkholderia* reference strains were used to determine inhibition of *Ustilago* and *Fusarium* growth. Forty-seven strains inhibited the growth of *Ustilago* whilst seventy-two strains inhibited the growth of *Fusarium in vitro.* Twenty-one of these inhibited the growth of both the *Fusarium* and *Ustilago*. Most of these belonged to the *Burkholderia cepacia* complex. Inhibition zones caused by some of the isolates can be seen in Figure 1.

Figure 1. Inhibition of *Fusarium* (left) and *Ustilago* sporidia (right) by endophytic bacteria.

We have shown, for the first time, that endophytic bacteria are common in sugarcane in South Africa. We have also shown, for the first time, that some of the endophytic bacteria and some of the bacteria recovered from the roots of cane are able to inhibit the growth of two important fungal pathogens of sugarcane. Gosic *et al* (2002) also recorded inhibition of phytopathogenic fungi by endophytes. Their bacteria were recovered from maize and sunflower.

Future work will concentrate on those endophytes best able to colonise the stalks of sugarcane and on the identity of the compound(s) responsible for the inhibition. Bacterial isolates that inhibit the growth of pathogens will also be assessed for their ability to fix nitrogen as well as for the influence they have on the plant parasitic nematode communities associated with sugarcane.

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