BURKHOLDERIA TROPICALIS, A POTENTIAL BACTERIAL INOCULANT TO CONTROL NEMATODES AND IMPROVE SUGARCANE GROWTH

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Abstract

The bacterial genus *Burkholderia* is abundant in the sugarcane rhizosphere. Literature states that some *Burkholderia* strains are nematode and fungal antagonists and/or plant growth promoters. Research was undertaken to obtain indigenous sugarcane-associated *Burkholderia* strains that possess these properties. Preliminary screening produced 13 isolates that are able to paralyse juveniles of *Meloidogyne* spp. This collection of isolates comprised *Burkholderia cepacia* complex, *B. gladioli*, *B. caribensis, B. fungorum* and a new species, *B. tropicalis*. Since *B. cepacia* complex and *B. fungorum* are associated with hospital infections, these species were omitted from further study. *B. gladioli* is often a mild plant pathogen, and was thus also eliminated. *B. tropicalis,* which has never been associated with clinical cases, was found to be an endophyte that enhances maize growth and fixes nitrogen. Consequently, search was made in sugarcane for N-fixing strains of *B. tropicalis* that are antagonistic to plant parasitic nematodes and various plant pathogens such as the smut fungus, *Ustilago scitaminea*, and *Fusarium* spp. Strain LM1-376.8 belongs to *B. tropicalis* and has been isolated from the rhizosphere of sugarcane. It fixes nitrogen and is antagonistic to nematodes, but not to certain pathogenic fungi. A culture medium has been designed for the isolation of more N-fixing *B. tropicalis*. To confirm the identification of *B. tropicalis,* a species-specific PCR was designed. The expected outcome of this research is the selection of strains whose inoculation would benefit sugarcane yield through a combination of anti-nematode, anti-fungal and growth promotion properties.

Keywords: sugarcane, biological control, nematodes, *Burkholderia tropicalis*, nitrogen fixation, maize, endophytic bacteria

Introduction

B. tropicalis is a new species which has been found associated with teosintle, the wild ancestor of maize, and it is still found in remote parts of Mexico in 'Indian' maize fields, where the crop is grown without pesticides or fertilisers. The bacterium densely colonises roots and shoots of the plant and is able to fix nitrogen (Estrada *et al*., 2002). It is hypothesised that this *B. tropicalis*-maize association represents a primitive symbiosis selected along geological time co-evolution, which was preserved during domestication of teosintle into maize, some 7000 years ago, in Mexico.

B. tropicalis is currently being described by a consortium of four teams, including one from the South African Sugar Association Experiment Station (SASEX) at Mount Edgecombe.

The reason for this is that 180 isolates of this new bacterium were obtained around Mount Edgecombe during a survey of dominant bacteria associated with the roots of sugarcane. They were collected with the aim of finding strains with anti-nematode properties that would constitute an alternative to chemical control (Vogel *et al*., 2002). Understanding the role of this bacterium in sugarcane growth, and its possible use in a biotechnology programme, are currently the goals of a collaborative programme between SASEX and two French research organisations, *viz*. the IRD and the CNRS.

Material and Methods

Plants
Untreated maize seeds cv Centena (38B42) were obtained from Pioneer Semences in France. The sugarcane plants studied were chosen from various SASEX field trials in KwaZulu-Natal.

Soil samples
Soil used for greenhouse inoculation experiments with maize was collected in La Côte Saint André (CSA), 70 km from Lyons, a place where maize has been grown continuously for more than 20 years.

Inoculation of maize and cultivation
Maize plantlets were immersed for three to four hours in a bacterial culture of Mexican *B. tropicalis* strains (BM273 or BM16) containing 10^6 CFU/ml, after which they were planted into pots containing 2 kg of CSA soil and placed in a greenhouse.

Isolation of strains
Burkholderia isolates have been obtained on PCAT (Burbage and Sasser, 1982).

DNA extractions
Bacterial genomic DNA was extracted using the Qiagen DNA extraction mini prep kit. Genomic DNA of endophytic bacteria was extracted using the Qiagen plant DNA extraction kit. DNA was extracted from rhizosphere and soil using the MO BIO UltraClean soil DNA isolation kit.

B. tropicalis-specific PCR primers
Burkholderia 16S sequences are available in GenBank. They were aligned (using ClustalX) and searched for *B. tropicalis*-specific regions.

PCR and primers
Specific *B. tropicalis* 16S amplification was performed in 50 µl reaction mixtures containing 5 µl of DNA or colonies, 20 µM of each deoxyribonucleoside triphosphate, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.5 µM of each primer and 2.5 U of Taq DNA polymerase (GIBCO).

Results

Diagnosis of B. tropicalis
A sequence was found corresponding to positions 456-474 of the *Escherichia coli* 16S gene (Brosius *et al*., 1981) which is found only in GenBank 16S accessions corresponding to eight *B. tropicalis* strains. Based on it, the following PCR direct primer was defined:

5' TCCCTGGTCCTAATATG 3'

A BLAST search in GenBank retrieved only *B. tropicalis* accessions among bacterial DNA. A complementary reverse primer was designed in a 16S region specific for the *Burkholderia* genus (Pallud *et al*., 2001). BurkhoR corresponds to positions 1240-1256 of the *E. coli* 16S gene (Brosius *et al*., 1981):

5' CAACCCTCT GTTCCGA 3'

A BLAST search in GenBank retrieved only *Burkholderia*. With *B. tropicalis* strains, these primers give an 800 bp amplicon. These primers have been tested on a range of *Burkholderia* isolates and appear to be specific for *B. tropicalis*, which is very useful in a bacterial genus which has a very low diversity in the 16S gene. They gave the expected amplicon with all eight *B. tropicalis* strains tested, but not with eight *B. cepacia* complex strains, nor with strains representing six other species of the genus.

Effect of B. tropicalis *on maize growth*
B. tropicalis could not be found in seeds of several cultivars of maize, either Mexican or French. In French soils no isolates belonged to *B. tropicalis*. Two Mexican Indian maize cultivars grown on a French soil did not show any *B. tropicalis* in their roots. Consequently, an inoculation experiment was organised in a greenhouse using maize inoculated with two strains isolated from Indian maize in Mexico, BM16 and BM273. The effect of inoculation on the growth of maize was measured in two experiments. The two strains had an obvious positive effect on plant growth, especially on shoot growth (Table 1).

Table 1. Effect of inoculation on plant growth, measured as fresh weight of roots and shoots. Results in brackets are percentage increases over the respective controls. D7 = plants 7 days old, D15 = plants 15 days old.

Fresh weight (g)	Roots			Shoots		
	Control	BM273	BM16	Control	BM273	BM16
Experiment A, D15	3,5	5,6 (+60%)	3,8 (+8%)	2,8	5,1 (+82%)	4,0 (+43%)
Experiment B, D7	1,2	1,9 (+58%)	1,8 (+50%)	0,8	1,7 (+112%)	1,7 (+112%)
Experiment B, D15	2,9	4,3 (+48%)	4,1 (+41%)	2,4	5,1 (+112%)	4,6 (+92%)

Colonisation of maize by inoculated B. tropicalis
Presence of *Burkholderia* was assessed by inoculating dilutions of plant tissue macerates to PCAT, followed by PCR check of individual colonies. The inoculated strains densely colonised roots of maize and, occasionally, shoots also. In France no *B. tropicalis* have ever been found in non-inoculated controls.

The total number of *Burkholderia* was not changed significantly by inoculation, although inoculated strains represented a very high proportion of the *Burkholderia* present (around 80%). Strains were recovered from surface sterilised organs, showing that colonisation was partly endophytic, and this was confirmed by PCR on DNA extracted from plant organs (Figure 1).

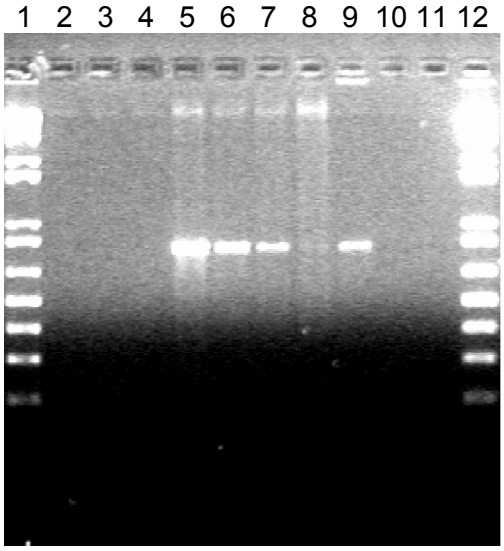

Figure 1. *B. tropicalis*-specific PCR on DNA extracted from different plant parts. Lanes 1 and 12: molecular weight standards; lane 5: surface sterilised roots; lane 6: unsterilised roots; lane 7: surface-sterilised shoots; lane 8: unsterilised shoots; lane 9: strain BM273; lane 10: strain TVV75 (*B. vietnamiensis*); lane 11: control without DNA.
The plant was inoculated by strain BM273.

B. tropicalis and sugarcane

The name *B. tropicalis* was first used in 1997 by Baldani *et al*. It was used also for strain Ppe8 16S sequence deposited in GenBank in 2001. This strain had been isolated from surface sterilised sugarcane stalks in Brazil. In an official description of the taxon which is in preparation, this strain will be proposed as the type strain of the new species.

In Mount Edgecombe, a survey has been conducted of sugarcane-associated *Burkholderia*, with the objective of selecting a strain with anti-nematode activity and plant growth stimulation properties (nitrogen fixation). The survey showed that species of *Burkholderia* represented 2-7% of culturable bacteria in the soil, and 17-25% in the rhizosphere. Of the 980 *Burkholderia* isolates classified using amplified ribosomal DNA restriction analysis (ARDRA), 20% of the root isolates were members of the *B. cepacia* complex. A majority belonged to *B. caribensis* or *B. fungorum*, and 27% were *B. tropicalis*. The latter had a tendency to be more abundant where nematode damage was lower (Vogel *et al*., 2002). There was no indication of the presence of endophytic *B. tropicalis*.

Table 2. Distribution of anti-nematode properties in different species of *Burkholderia*.

Species	No. of strains tested	% active against *Meloidogyne*	% rating 3
B. cepacia complex	81	79	73
B. graminis	36	31	73
B. gladioli	34	47	63
B. caribensis/fungorum	74	27	60
B. tropicalis	77	19	53

A simple *in vitro* test was designed to compare the effect of these *Burkholderia* isolates on nematodes. *Meloidogyne* juveniles in water received a suspension of each bacterial strain, and were observed for motility after three, six and nine hours of contact. More than half of the tested strains produced paralysis in nematodes, and were scored 1, 2 or 3 according to whether the paralysis lasted at least three hours, six hours or more than nine hours, respectively (Table 2).

The most active strains were also evaluated for their ability to fix atmospheric nitrogen and antagonism against pathogenic fungi. This provided a list of isolates with a good potential for sugarcane inoculation (Table 3). Unfortunately, most active strains belonged to the *B. cepacia* complex, which contains strains pathogenic to humans. It was decided not to pursue research on these. To a lesser degree, *B. gladioli* and *B. fungorum* have occasionally been reported in opportunistic infection of humans, and were thus also discarded. This left a strain of *B. tropicalis* that was able to paralyse nematodes and fix atmospheric nitrogen. Unfortunately, this strain was not inhibitory for either smut or *Fusarium* (Table 3).

Table 3. Isolates combining interesting properties in addition to being good antagonists of *Meloidogyne*. Potential pathogenicity to humans, antagonism to smut (*Ustilago scitaminea*), antagonism to *Fusarium* and ability to fix atmospheric nitrogen (nd = not determined).

Strain	Species	Potential pathogen	Smut antagon.	*Fusarium* antagon.	Fixes N
T16.1B	*B. cepacia* complex	+	partial	+	nd
OAF.2B, GMCP.4B, N8.1, N8.2, N8.4, N8.6, N8.7	*B. cepacia* complex	+	+	+	nd
OAC.1B	*B. gladioli*	+	+	partial	nd
TR30.6B	*B. fungorum ?*	+	+	+	nd
LM1-N16.8	*B. fungorum*	+	+	+	nd
TR30.8B	*B. fungorum*	+	+	partial	nd
LM1-376.8	*B. tropicalis*	-	-	-	+

This result justifies the continued search for a good sugarcane inoculant among *B. tropicalis*. Research is in progress to design culture media that provide high frequency isolation of *B. tropicalis*. Among the possibilities under investigation is the use of rhamnose-based media. This simple sugar is assimilated by most *B. tropicalis* strains and is never used by strains belonging to the *Burkholderia cepacia* complex.

Discussion

In maize, the new species *B. tropicalis* is thought to be an ancient symbiotic partner, lost during exportation of maize to Europe but still able to bring about a significant improvement in plant productivity when inoculated into seeds. Inoculation of field grown maize has not yet been performed, but should not meet major regulatory difficulties as *B. tropicalis* is only very distantly related to potential human pathogens.

From the survey of bacteria that could be used as an inoculant for sugarcane, it was found that members of the *B. cepacia* complex were the most efficient in combatting nematodes and fungal diseases.

Unfortunately, it is not possible at present to distinguish between harmful and harmless members of the complex, and registration of a product based on a *B. cepacia* complex strain is unlikely. The only strain with some prospect is LM1-376.8 of *B. tropicalis*, which is a good antagonist of *Meloidogyne* and fixes nitrogen.

From what has been seen in isolates of other *Burkholderia* species, it seems likely that a more intensive survey of *B. tropicalis* in local sugarcane fields would produce strains with the same properties as LM1-376.8, but with antagonism against fungal diseases of sugarcane. It is nevertheless anticipated that, if *B. tropicalis* was able to establish a primitive symbiosis with maize, the same may have happened with sugarcane and endophytic *B. tropicalis* will be found in this plant as well. This hypothesis is supported by the accidental finding of the endophytic strain Ppe8 by Baldani *et al*. (1997) in Brazil. The absence of such endophytes in modern sugarcane fields in KwaZulu-Natal may well be the consequence of repeated hot water treatment of seedcane setts to decrease the incidence of ratoon stunting disease (RSD), caused by the endophytic pathogenic bacterium *Leifsonia xyli* subsp. *xyli*. Although hot water treatment does reduce the incidence of RSD, it might also have the negative effect of counter-selecting a beneficial endophytic *B. tropicalis*. Various sources of untreated sugarcane are being surveyed for such endophytes. If successful, this research could provide bacterial strains with the potential to decrease damage from parasitic nematodes in roots, while increasing resistance of shoots to fungal diseases and improving the nitrogen nutrition of the host plant.

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