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Improved yield of micropropagated sugarcane following inoculation by endophytic *Burkholderia vietnamiensis*

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Abstract

During a survey of nitrogen-fixing *Burkholderia* associated with sugarcane in Tamil Nadu, some endophytes were isolated on PCAT medium. Isolation was based on the use of the selective PCAT medium. Four isolates were studied, all belonging to the genus *Burkholderia*. One of them, MG43 was consistently more active in reducing acetylene and was identified as *Burkholderia vietnamiensis*. This isolate was used to inoculate micro-propagated sugarcane plantlets in a comparison with two other diazoptrophs, viz. *Gluconacetobacter diazotrophicus*^{T} and *Herbaspirillum seropedicae*^{T}. Inoculated plants and uninoculated controls were used in a pot experiment followed by two field experiments under different rates of nitrogen fertilisers. MG43 and *G. diazotrophicus* performed best in sugarcane, their natural host. Biomass increase due to MG43 inoculation reached 20% in the field. Inoculated plants were heavily colonised by the inoculated bacterium (up to 115,000 CFU g^{-1} root fresh weight). Inoculation by a combinaison of the three strains performed less well than inoculation by a single MG43 suspension. Ecological implications are discussed, as well as the potential of these bacteria to provide a feasible alternative to higher N fertilisers rates in a low input and long term sustainable rural economy.

Introduction

Large increases in agricultural production following the green revolution have been permitted by the development of plant genotypes highly responsive to chemical fertilizers. Nitrogen in modern agriculture is the main limiting factor of crop productivity and, where affordable, increasing rates of chemical fertilizers have been used in the recent decades. But N fertilizers are expensive and recently proved to be ecologically unsafe, which prompted the search for alternatives. Among them the use of biological atmospheric Nitrogen fixation is an obvious choice. The use of N_2-fixing legumes has long become common practice. Free-living nitrogen fixing bacteria belonging to the genera *Azospirillum* (Bally et al., 1983; Dobereiner and Day, 1976), *Gluconacetobacter* (Gillis et al., 1989), *Azoarcus* (Reinhold-Hurek et al., 1993), *Enterobacter* (Bilal et al., 1990) and *Herbaspirillum* (Baldani et al., 1986) appear to be frequent colonizers of important cereal crops and grasses and have been extensively studied (Döbereiner and Urquiaga, 1992; Kennedy and Tchan, 1992; Ueda et al., 1995). As time goes on new N-fixing bacteria are discovered as is the case for the *Burkholderia* genus.

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Nitrogen fixing *Burkholderia vietnamiensis* (Gillis et al., 1995) has been isolated from the rhizosphere of rice in Vietnam (Tran Van et al., 1994). Its detailed beneficial effects were reported by Tran Van et al. (2000). It was the first species of *Burkholderia* reported to fix nitrogen. More recent reports suggest that many species of this genus actually contain diazotroph strains. Some time ago, Hartman et al. (1995) had suggested that "Isolate E", a diazotroph isolated from within sugarcane, sweet potato and rice, also belonged to the genus *Burkholderia*. Baldani (1996) reported that rice plants inoculated with "isolate E" could be colonised extensively by the bacteria and fix substantial amounts of N_2. Estrada et al. (2002) isolated a comparable nitrogen-fixing endophyte from teosintle and maize in Mexico. They were demonstrated to belong to the same *Burkholderia* species, along with sugarcane isolates from South Africa: this species has since been described as *Burkholderia tropica* (Reis et al., 2004). Recently *Burkholderia unamae* has been described as another diazotrophic maize endophyte (Caballero-Mellado et al., 2004).

Application of bacterial inoculants as biofertilizers has resulted in improved growth and increased yield of several cereal crops (Boddey et al., 1986, 1995; Fages 1994; Kapulnik et al., 1981; Kennedy and Tchan 1992; Omar et al., 1992; Pereira et al., 1988; Tran Van et al., 2000). Beneficial effects of these plant growth promoting rhizobacteria (PGPR) have been attributed to biological nitrogen fixation (BNF) (Boddey et al., 1995 Urquiaga et al., 1992) and production of phytohormones that promote root development and proliferation resulting in a more efficient uptake of water and nutrients (Haahtela et al., 1990; Jacoud et al., 1999; Sarig et al., 1992). The inoculation of cereals with diazotrophic bacteria so far has mainly been performed with *Azospirillum* spp., with variable responses to inoculation (Bashan and Holguin 1997; Okon and Labandera-Gonzalez 1994). N savings represent 25–60 kg N ha^{-1} (Boddey et al., 1995; Omar et al., 1992; Tran Van et al., 2000) in rice. In sugarcane, inoculation with a mixture of diazotrophic bacteria and mycorrhizal fungi was shown to be equivalent to half the dose of recommended fertilisers (Muthukumarasamy et al., 1999, Oliveira et al., 2000).

In this study we report (1) the presence of endophytic *Burkholderia* spp. in sugarcane and (2) the beneficial effect of one isolate on micropropagated sugarcane. This is the first report of a natural association between *Burkholderia* spp. and cultivated plants in India.

Material and methods

Culture media

Isolation media were PCAT (Burbage and Sasser, 1982), LGI-P and LGI-M (Reis et al., 1994); BDN (Estrada de los Santos et al., 2001) was used for growing strains before DNA extraction; LGI-P and BMGM were used for *Gluconacetobacter* and *Burkholderia* recovery; JNFb (Kirchhof et al., 1997) was used to recover *Herbaspirillum*.

LGI-P, contains (g L^{-1}): Sucrose, 100 g; K_2HPO_4, 0.2 g; KH_2PO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; Bromothymol blue (0.5% solution in 0.2 M KOH), 5 mL; pH-5.5. No cane juice was added. Crystal sugar of the initial recipe was replaced by sucrose.

LGIM: similar to the above with only 10 g L^{-1} sucrose

BDN contains (g L^{-1}): peptone, 2; yeast extract, 0.1; K2HPO4, 0.4; KH_2PO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.2; pH 6.5.

BMGM (Estrada de los Santos et al., 2001) contains (g L^{-1}): K_2HPO_4, 0.4; KH_2PO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $Na_2MoO_4 \cdot H_2O$, 0.002; $FeCl_3$, 0.01; bromothymol blue, 0.075; agar, 2.3. pH adjusted to 5.7; autoclaved 20 min at 121 °C; add 40 μg mL^{-1} of filter-sterilised cycloheximide.

JNFb contains (g L^{-1}): malic acid, 5; K_2HPO_4, 0.6; KH_2PO_4, 1.8; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; KOH, 4.5; bromothymol blue, 2 mL of 0.5% in KOH 0.2 N; 1.64% Fe EDTA, 4 mL; vitamin solution, 1 mL; micronutrient solution, 2 mL. pH 5.8.

Vitamin solution (mg L^{-1}): biotin, 100; pyridoxal-HCl, 200.

Micronutrient solution (g L^{-1}): $CuSO_4$, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.12; H_2BO_3, 1.4; $Na_2MoO_4 \cdot 2H_2O$, 1; $MnSO_4 \cdot H_2O \cdot 1.5$.

Reference strains

Gluconacetobacter diazotrophicus (Yamada et al., 1997) strain LMG7603^T (ATCC49037) has been isolated from sugarcane root tissues in Brazil as strain Pal5 of *Acetobacter diazotrophicus* (Cavalcante and Döbereiner, 1988)

Herbaspirillum seropedicae (Baldani et al., 1996) strain LMG6513^T (ATCC35892) has been isolated from surface sterilised rice roots in Brazil (Baldani et al., 1986). This species is also present in sugarcane (Boddey et al., 1995).

Burkholderia vietnamiensis (Gillis et al., 1995) strain LMG10929^T has been isolated by Tran Van et al. (1994) from rice roots in Vietnam.

Isolation and enumeration of Burkholderia *spp.*

Triplicate samples of root, stem and leaves of different cultivars of sugarcane were obtained from fields of different regions of Tamil Nadu state, India. Roots were washed with MgSol (10 mM $MgSO_4 \cdot 7H_2O$) (Estrada de-los-Santos et al., 2001), and vortexed for 3 min under agitation in full strength bleach solution containing sodium hypochlorite (4%). They were rinsed five times for 5 minutes in sterile H_2O. Roots were then rolled on to Luria-Bertani agar plates to verify root surface sterilization.The basal stem portions were cut into small pieces (approximately 5 cm) and surface sterilized by dipping in 95% ethanol and washed five times with sterile water; 1 cm portion from each end was cut with a sterile blade (Gyaneshwar et al., 2001). Samples of root and stems were homogenized with a mortar and pestle in MgSol, serially diluted and 0.1 mL suspension were inoculated into vials containing 5 mL of N-free semisolid LGIM (Estrada et al., 2002) with an initial pH of 6.0. After 72–96 h of incubation vials with white, yellowish surface pellicles were further replicated into fresh LGIM media. Acetylene reduction positive vials were streaked onto PCAT agar plates (pH 5.7) and incubated at 28 °C for seven days for further study.

Characterisation of Burkholderia *spp.*

Pigment production was monitored on nutrient agar and King 'B' media. Colony morphology was examined on PCAT (Burbage and Sasser, 1982) and BMGM agar plates. Microscopic observations were done using a trinocular phase contrast fluorescent microscope (Olympus AX 80T). The type of flagellation and the cell dimension were determined using cells negatively stained with aqueous uranyl acetate 2% (w/v) at 3.5 pH, observed under transmission electron microscopy. Bacterial motility was tested by growth in a semisolid medium containing 0.3% mannitol (motility test medium). Oxidase and catalase tests were determined using commercially available discs (Hi media). Growth was tested using medium BMGM in which the carbon source was replaced by individual carbon substrates (5 g L^{-1}) such as D-glucose, sorbitol, meso-inositol, mannose, glycerol, L-rhamnose, lactose, fructose, L-arabinose, trehalose, L-raffinose, meso-erythritol, galactose, mannitol, cellobiose, xylose, sucrose, starch, maltose; tested organic acids (0.5% w/v) were: adipic, malonic, succinic, oxalic, valeric, fumaric, hippuric, malic, tartaric, alpha-keto glutaric and citric acid. Growth on amino acids was studied in the presence of sorbitol as a carbon source. Studied amino-acids were; L-cysteine, L-glutamic, L-proline, L-trytophane, L-leucine, L-threonine, L-histidine, L-lysine, L-tyrosine, and L-valine.

Nitrogenase activity

Acetylene reduction assays (ARA) were performed in vials containing 10 mL of BMGM semisolid medium, inoculated with single colonies and pre-incubated at 28 °C for 3 days. After the pre-incubation tubes were closed with Suba-Seal rubber stoppers, and acetylene (10% v/v) was injected. Tubes were incubated at 28 °C for 24 h. Acetylene reduction activity was measured using a flame ionisation gas chromatograph (Systronic) equiped with a Poropak Q column. Uninoculated vials were used as negative controls. To determine the ability of individual strains to reduce acetylene on different carbon sources, isolates were incubated on BMGM medium in which the carbon source was replaced by the carbon source to test (5 g L^{-1}). Eight major carbon sources were used: azelaic acid, fructose, glycerol, succinic acid, sucrose, mannitol, malic acid and glucose.

Fatty acid analysis

The MIDI-FAME technique was used to determine the cellular fatty acid profiles of the isolates (MGRB, MG43) and the *B. vietnamiensis* type strain (TVV75). Isolates were grown overnight on trypticase soy agar plates. One loopful of fresh cells was harvested and transferred to a screw cap culture tube. One mL of saponification reagent was added. Tubes were tightly sealed with a teflon-lined screw cap, and vortexed for 5–10 s. The tubes were placed in water bath at 100 ± 2 °C for 5 min. They were then removed from the boiling water bath and cooled slightly, vortexed for 10 s and incubated in a water bath for an additional 25 min. After a total of 30 min of saponification in the water bath the samples were placed under tap water. Each tube received 2.0 mL of methylation reagent, was tightly capped and vortexed for 10 s. The tubes were placed in a water bath at 80 ± 1 °C for 10 min. after what samples were cooled at room temperature. Then, 1.25 mL of extraction reagent was added into the tubes. The tubes were centrifuged and the bottom phase was removed using pipetman. Finally 3.0 mL of base reagent was added and this was placed again for 5 min in a laboratory centrifuge. The upper solvent phase was removed and transferred to vials for fatty acid analysis.

Isolation of total DNA

Isolates and type strains were grown in BDN medium (Estrada et al., 2001). Cultures were incubated at 28 °C for 24 h and centrifuged at 12,300 g. Pellets were washed with TE buffer, then resuspended in 10 mL of TE (1X), 3 mL of 5% SDS in TE (1X) and 3 mL of proteinase K 2.5 mg mL^{-1}. This was then incubated at 37 °C for one hour. The clear lysates were extracted with phenol:chloroform:isoamylic alcohol (25:24:1). DNA was precipitated with the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ethanol to the supernatant. Dried pellets were dissolved in 1× TE buffer.

PCR-amplification of the nif genes

The sequences of PCR primers for *nif* D were nif DF883 5-ATSGARTWCAACTTCTTCGG-3 and nif DR1337 5-ARTCCCAIGAGTGCATYTGIC GGAA-3 (where I represents inosine, R represent A or G, S represent C or G, W represent A or T and Y represent C or T) according to Ueda et al. (1995). Amplification reactions were performed in a total volume of 25 µl. The reaction mixture contained: 2.5 µl 10× PCR buffer, 2.5 µl of 2 mM each of dATP, dCTP,dTTT and dGTP; 3 µl of each forward and reverse primer (30 ng), 1 µl of template DNA (10 ng) and 0.3 µl of (3 unit μl^{-1}) *Taq* polymerase; final volume was made 25 µl using milli-Q water. The step-up PCR procedure included denaturation at 95 °C for 3 min, 52 °C for 1 min, and 72 °C for 1 min, followed by 30 cycles of 95 °C for 1 min, 54 °C for 30 sec and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplification products were electrophoresed on 1.5% agarose gel in 1× TBE buffer.

PCR-amplification and 16S rRNA sequencing

The 16S rRNA gene sequences were determined by PCR amplification (Kwon et al. 2003) and direct sequencing (Hiraishi, 1992). For the phylogenetic analyses, related 16S rRNA gene sequences within the genus *Burkholderia* were included. 16S rDNA sequences were aligned by using the MEGALIGN program of DNASTAR. An evolutionary distance matrix was generated as described by Jukes and Cantor (1969). The evolutionary tree for the datasets was inferred from the neighbor-joining method of Saitou and Nei (1987) by using the neighbor-joining program of MEGA version 2.1 (Kumar et al., 2001).

RecA amplification and RFLP

*Rec*A was amplified using primers BCR1 and BCR2 (Mahenthiralingam et al., 2000). Amplicons were restricted with *Hae*III and patterns compared with patterns of type strains.

Sugarcane micropropagation

Micropropagated sugarcane seedlings were produced from apical meristem of two different commercially important cultivars, Co 86032 and Co 86027, following the method described by Sreenivasan and Sreenivasan (1984). Fully developed seedlings were obtained in about 90 days using modified MS liquid and solid media under axenic growth conditions (Murashige and Skoog, 1962). Callus initiation, multiplication of aerial shoots,

multiplication of roots were obtained by accordingly modifying the concentrations of growth hormones in the basal medium. Uniform size seedlings were individually transferred to 50 mL test tubes containing 15 mL of modified MS medium (Reis et al., 1999), where N and all other salts were reduced to one-tenth of the original concentration and no plant hormones were present. Three days after transfer, only those tubes with no apparent contamination were used for inoculation with diazotrophs. Serial dilutions of random samples were transferred on BMGM medium to ensure that no diazotrophic bacteria were initially present on the plantlets. A 0.1 mL of respective strain suspension containing 10^8 cells mL^{-1} was inoculated into tube containing micropropagated plants. Plants were maintained at 28 °C with 60 μmol m^{-2} s^{-2} illuminance for 14 h day^{-1}. After seven days of inoculation, they were subjected to primary and secondary hardening. After primary hardening, seedlings were maintained under 50% light intensity. When the plants reached a height of 75–100 mm, they were used for pot and field experiments.

Sugarcane pot experiment

Micropropagated seedlings of culivar Co 86032 were used for pot experiments. Soil used was the upper layer of a farm soil, pH 7.5. 50 L plastic containers, filled with 40 kg of thoroughly homogenized soil were used. This experiment had nine treatments and six replicates in a randomised complete block design (RCBD). The treatments were:

– four levels of inoculation : MG43, *G. diazotrophicus* ATCC49037, a combination of them, uninoculated control.
– two levels of N fertilizers : recommended rate (280 kg N ha^{-1}) and half this rate (140 kg N ha^{-1}).
– an uninoculated control without N

The containers were maintained and irrigated by natural rainfall and tap water, if necessary. Phosphorus and potassium were applied at the rate of 65 and 115 kg ha^{-1} for all treatments. Bacterial populations from root tissues was estimated by the MPN technique using three replicates. *G. diazotrophicus* counts were done on medium LGIP where they form a typical subsurface pellicle; isolates were obtained from this pellicle by streaking on LGI agar plates supplemented with 50 mg L^{-1} yeast extract. Their identification was confirmed by specific PCR using primers AC and DI (Sievers et al., 1998). *Burkholderia* were counted on BMGM followed by streaking on PCAT for confirmation. Leaf N was determined by the microKjeldahl method (Humphries, 1956) every month on the fourth and fifth leaf from the top. After six months, plants were uprooted and root and shoot biomass measured.

Sugarcane field experiments

Two field experiments were conducted on clay soils at Kavidandalam and Orakatpettai, S.V. Sugar mills area, Tamil Nadu state, using micropropagated seedlings of cultivars Co 86032 and Co 86027. Applied fertiliser rates were 280, 115 and 65 kg ha^{-1} for N, P and K. These trials had six treatments in four replicates in a randomised block design with a total area of 24 cents. Each plot was one cent in area (6 m^2, approximately). Irrigation was by natural rainfall and borewell waters. The six treatments were:

– four modalities of inoculation: MG43, *G. diazotrophicus* ATCC49037, *Herbaspirillum seropedicae* ATCC35892, a combination of them. Applied N fertilizer was 140 kg N ha^{-1} (50% of recommended rate).
– two uninoculated controls: recommended rate of N (280 kg ha^{-1}) and half of the recommended N (140 kg ha^{-1}).

Counting of bacterial population and leaf N content analysis were as above. *Herbaspirillum* were counted on semi-solid medium JNFb where they form a subsurface pellicle. On solid JNFb medium with 1 g L^{-1} yeast extract, green centered colonies were obtained. Aerial parts were harvested after 12 months to calculate the yield.

Results

Isolation of diazotrophs

The populations of N_2 fixing *Burkholderia* spp. were higher in root samples as compared to stem

and leaves. On LGIM agar plates, colonies were round, yellowish, mucoid, smooth, and translucent with entire margins. Colonies on nutrient agar and King's B medium were white, smooth without pigment (Gillis et al., 1995). Colonies on PCAT agar plates were white, round, smooth, convex with entire margins. A total of 41 isolates were obtained on PCAT, of which four presented a consistently high acetylene reduction activity. The most active strain was MG43, isolated from sugarcane surface sterilised roots of cultivar Co 86032 in Tirupathur.

Identification and properties

The presence of 16:0 3-OH in fatty acids suggested that isolates belonged to the *Burkholderia* genus (Viallard et al., 1998). The 16S gene sequence gave a 100% match with *B. vietnamiensis* LMG10929 (Figure 3). Moreover, the *Hae*III restriction pattern of *rec*A (Mahenthiralingam et al., 2000; Vermis et al., 2002) showed a complete similitude with *B. vietnamiensis* (Figure 2). All isolates could reduce acetylene ; PCR amplification of *nif*D showed that isolates MG43, MG87, MG32 and MG44 and type strain *B. vietnamiensis* produced the expected 450 bp amplification products (Figure 1). It is a confirmation that these isolates are nitrogen fixers.

Sugarcane pot trial

Increasing N fertiliser from 0 to 140 and 280 in uninoculated controls increased yield from 877 to 1851 and 1977 g, showing that N was the limiting factor in this pot experiment. The monthly evolution of root populations (Table 1) indicated that the populations of *Burkholderia* reached 10^5 CFU per g fresh root tissues and remained stable for 180 days after inoculation; *G. diazotrophicus* populations were comparable up to day 120 and then gradually declined. When the two bacteria were inoculated together, *G. diazotrophicus* also declined, but *Burkholderia* kept the same numbers as when inoculated alone. Without inoculation N-fixing bacteria are virtually absent at the beginning and appear progressively, except in the full N dose treatment (Table 1). The mean leaf N content and total biomass (Tables 2–3) are increased by inoculation. Both strains perform well alone or in combination without N fertilisation: on an average, N content of leaves is significantly increased by 43%; total plant biomass is increased by 42%; a combination of the two strains gives the same result (Table 3).

Sugarcane field trials

In the field experiments also, N was the limiting factor of yield: in uninoculated controls yield increased with N fertiliser rates. In the two field experiments, MG43 inoculation significantly increased yield as biomass by 20 and 19% (Tables 5–7). *Gluconacetobacter* was less efficient (13–16%), followed by the mixed inoculum (14%), then *Herbaspirillum* (5–12%). N contents of leaves were consistently increased by all inoculation treatments, and mixed inoculation

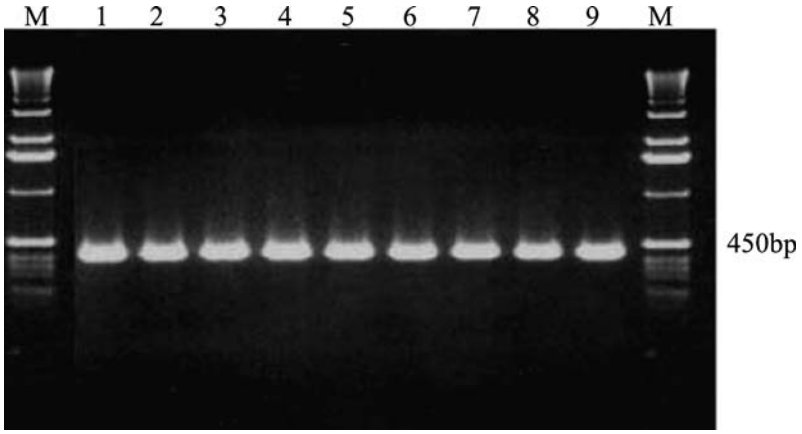

Figure 1. nifD gene amplification products of some isolates. 1: MGRB, 2: MGK2, 3: MGK3, 4: MG87, 5: MG4R, 6: MG43, 7: MG44, 8: MG32, 9: *B. vietnamiensis* LMG10929.

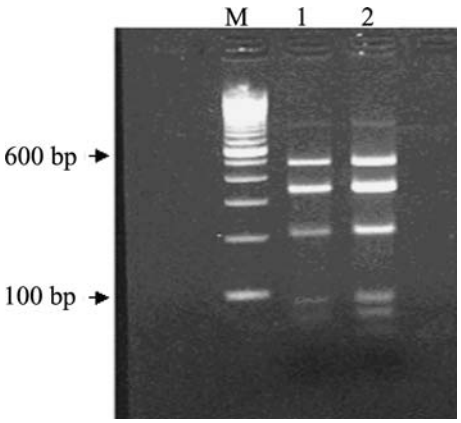

Figure 2. *Rec*A PCR-RFLP pattern obtained for strain MG43. Lane1: *Burkholderia vietnamiensis*^{T} LMG10929, lane 2: MG43 and M: 100 bp molecular weight standard.

had the maximum effect on this parameter (+70%), exceeding the effect of a doubling in N fertiliser rate.

All inoculated bacteria colonised their host plant as in the pots. Colonisation by *Gluconacetobacter* again is transitory and its numbers decreased after two (first experiment) or three months (second experiment). *Burkholderia* decreases slightly after five months. *Herbaspirillum* numbers are high and constant throughout the two experiments. When co-inoculated the three bacteria have the same ability to colonise as when used separately.

Discussion

Isolation

Viallard et al. (1998) reported that all strains of *Burkholderia* spp. grew on PCAT, while *Ralstonia* strains did not grow. This medium is not completely specific for *Burkholderia* and some gamma-proteo bacteria episodically grow in it (Pallud et al., 2001). Conversely there are a few strains of *Burkholderia*, which are unable to grow on PCAT, but it is exceptional and limited to a few collection strains. Only one species, *B. dolosa*, has been showed recently to be completely unable to grow on PCAT (Vermis et al., 2004). To the best of our knowledge, all *B. vietnamiensis* strains grow on PCAT.

Endophytism

This seems to be the first report about *B. vienamiensis* as a sugarcane endophyte. In the initial isolation of *B. vietnamiensis* from rice roots (Tran Van et al., 1996) no attempt was made at localising the bacteria: as whole roots were macerated, the isolate could come either from the rhizoplane or from root internal tissues. More recently, Caballero-Mellado et al. (2001) definitely found this species as an endophyte in maize.

Effects on growth

Inoculation of micropropagated sugarcane plants has been attempted a few times and has already showed interesting effects on resulting plants. Muthukumarasamy et al. (1999) and Oliveira et al. (2002) had reported pot experiments. The current paper is the first report of field experiments using in vitro inoculated micropropagated sugarcane plants.

Muthukumarasamy et al. (1999) inoculated a mixture of diazotrophs and mycorrhizal fungi to micropropagated sugarcane and obtained an effect equivalent to half the recommended rate of nitrogen fertilizers under pot conditions. In the present study, individual inoculation of *B. vietnamiensis* MG43 gave an effect higher than increasing fertiliser from half to full recommended rate.

In the present study, individual inoculation of *B. vietnamiensis* MG43 and *G. diazotrophicus* gave a higher cane yield as compared to combined inoculation. Oliveira et al. (2002) also reported a pot experiment in which the combined inoculation of *G. diazotrophicus, A. amazonense* and *Burkholderia sp.* gave a lower effect as compared to a mixture of *Herbaspirillum spp.*

Colonisation

Colonisation of plants is effective (10^5 CFU g fresh tissues^{-1}) and durable with *Burkholderia vietnamiensis* MG43. It is similar to maize colonisation by *Burkholderia tropica* (Estrada et al., 2002) but somewhat lower than previously reported sugarcane rates of colonisation by *Burkholderia* (10^7 CFU g fresh tissues^{-1}, Robertson et al., 2001).

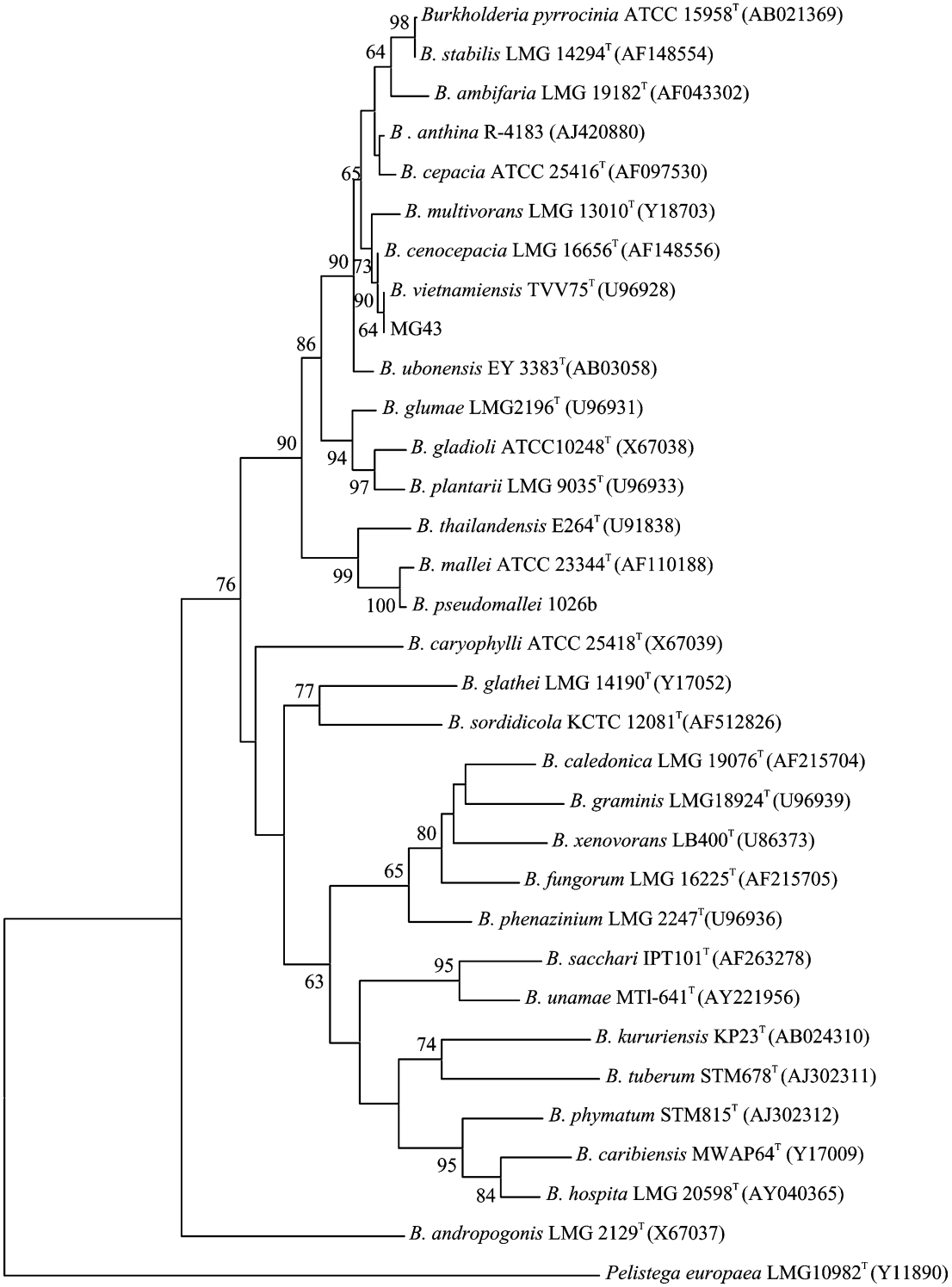

Figure 3. Phylogenetic position of strain MG43 within the genus *Burkholderia* on the basis of 16S rRNA gene sequences. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987), and the 16S rRNA gene sequence of *Pelistega europaea* LMG10982^T (Y11890) was used as the outgroup. The numbers at the nodes indicate the levels of the bootstrap support based on a neighbor-joining analysis of 1000 resampled data sets. The bootstrap values below 60% were not indicated.

Table 1. Sugarcane pot trial. Numbers of diazotrophic bacteria (10^4 g^{-1} root fresh wt): total (in LGIM), *G. diazotrophicus* (in LGIP) and *Burkholderia* (in PCAT) in micropropagated sugarcane variety Co 86032

Treatments	Days after planting				
	60	90	120	150	180
0 kg N ha^{-1} control[a]	0.01	0.11	0.21	0.40	0.40
Id + *G. diazotrophicus*	11.5	11.5	11.5	7.35	7.35
Id + *B. vietnamiensis* MG 43	11.5	11.5	11.5	11.5	11.5
Id + combined inoculum (*G.d* + MG 43)	11.5	11.5	11.5	4.35	4.35
G. d + *B. v* MG 43	11.5	11.5	11.5	11.5	11.5
140 kg N ha^{-1} control[a]	0	0	0	0.11	0.21
Id + *G. diazotrophicus*	11.5	11.5	11.5	7.35	4.35
Id + *B. vietnamiensis* MG 43	11.5	7.35	11.5	11.5	11.5
id + combined (*G.d* + MG 43) *G. d*	11.5	11.5	11.5	4.35	4.35
MG 43	11.5	11.5	11.5	11.5	11.5
280 kg N ha^{-1} control[a]	0	0	0	0	0

All plants received the equivalent of 65 and 115 kg ha^{-1} of P and K respectively.
Arithmetic zero represents value beyond detection limit (10^4 g^{-1} fresh wt).
[a]Seedlings inoculated with autoclaved cells.

Table 2. Sugarcane pot trial, N content of leaf samples in variety Co 86032 grown for six months with 65 and 115 kg ha^{-1} of P and K respectively. mg g^{-1} fresh wt. Means of 6 replicates

Treatments	Days after planting					
	60	90	120	150	180	Mean
0 kg N ha^{-1} control[a]	ND	2.20 d	4.51 c	4.27 c	3.81 b	3.69 d
Id + *G. diazotrophicus*	6.42 b	5.35 bc	6.06 b	5.95 b	3.78 b	5.28 c
Id + *B. vietnamiensis* MG 43	6.40 b	5.14 c	5.88 b	5.67 b	4.41 b	5.27 c
Id + combined (*G.d* + MG 43)	8.82 a	5.49 bc	5.70 b	6.02 ab	3.92 b	5.28 c
140 kg N ha^{-1} control[a]	ND	5.39 bc	6.09 b	5.70 b	5.91 a	5.77 bc
Id + *G. diazotrophicus*	ND	6.19 a	6.09 b	6.09 ab	6.05 a	6.10 b
Id + *B. vietnamiensis* MG 43	ND	5.84 bc	5.95 b	6.37 ab	6.16 a	6.08 b
Id + combined (*G.d* + MG 43)	ND	6.05 b	5.70 b	6.09 ab	6.30 a	6.03 b
280 kg N ha^{-1} control[a]	4.79 c	7.14 a	7.31 a	6.75 a	6.30 a	6.87 a
F-value	22.85	39.77	15.73	16.68	53.31	22.70
CV (%)	24.35	24.72	12.75	12.24	22.0	14.84

When followed by different letters within a column, results differ at 5% level (Tukey test).
[a]Seedlings inoculated with autoclaved cells.

Herbaspirillum seropedicae also appears an efficient coloniser, confirming previous reports (Oliveira et al., 2002; Boddey et al., 2003).

Gluconacetobacter diazotrophicus ATCC490 37 has a different behaviour: numbers diminish after a while, in spite of sugarcane being its host-plant. Oliveira et al. (2002) already mentionned difficulties to establish this bacterial species in high numbers onto micropropagated sugarcane. Nevertheless some caution should be exerted when estimating colonisation with MNP data and molecular methods should be employed to confirm the low figures found for *Gluconacetobacter*.

Specificity

The two best-performing strains (*Gluconacetobacter diazotrophicus* and *Burkholderia vietnamiensis* MG43) have been isolated from sugarcane. Host specificity is practically unknown in non-symbiotic N_2-fixation but results like this point to the

Table 3. Sugarcane pot trial, effect of inoculation of *G. diazotrophicus* and *Burkholderia* MG43 on the biomass and leaf N content of micropropagated sugarcane variety Co 86032 after 180 d of planting.

Treatments	Biomass (g)			Leaf N[b] (mg g dry wt^{-1})
	Root	Shoot	Total	
0 kg N ha^{-1} control[a]	55.83 d	821.66 c	877.50 c	3.69 d
Id + *G.d*	89.16 cd	1143.33 bc	1250.83 bc	5.28 c
Id + *B. v* MG 43	106.66 cd	1139.16 bc	1250.83 bc	5.27 c
Id + combined (*G.d* + MG 43)	121.66 bcd	1116.66 bc	1246.66 bc	5.28 c
140 kg N ha^{-1} control[b]	165.83 abc	1685.33 a	1851.66 a	5.77 bc
Id + *G. diazotrophicus*	183.33 ab	1744.16 a	1928.33 a	6.10 b
Id + *B. vietnamiensis* MG 43	202.50 a	1779.16 a	1981.66 a	6.08 b
Id + combined (*G.d* + MG 43)	140.00 abc	1354.16 ab	1494.16 ab	6.03 b
280 kg N ha^{-1} control[b]	190.83 ab	1798.33 a	1977.50 a	6.87 a
F-Value	9.89	12.23	12.44	22.70
CV (%)	30.47	40.95	29.86	14.84

When followed by different letters within a column results differ at 5% level (Tukey test). Means of six replicates.
[a]Seedlings inoculated with autoclaved cells.
[b]Means of six months.

Table 4. Sugarcane field trial 1, at Kavidandalam. Numbers of diazotrophic bacteria (10^4 g^{-1} root fresh wt): total (in LGIM), *G. diazotrophicus* (in LGIP), *Burkholderia* (in PCAT) *Herbaspirillum seropedicae* (in JNFB) in micropropagated sugarcane variety Co 86032

Treatments	Days after planting					
	30	60	90	120	150	180
140 kg N ha^{-1} control[a]	2.12	2.12	4.09	4.09	4.59	4.59
140 kg N ha^{-1} + *G. diazotrophicus*	11.5	11.5	7.35	7.35	4.09	2.12
140 kg N ha^{-1} + *H. seropedicae*	11.5	11.5	11.5	11.5	11.5	11.5
140 kg N ha^{-1} + *B. vietnamiensis* MG 43	11.5	11.5	11.5	11.5	11.5	11.5
140 kg N ha^{-1} + combined (*G.d* + *H.s* + MG 43) *G.d*	11.5	11.5	7.35	4.59	2.12	1.15
B. v	11.5	11.5	11.5	11.5	11.5	7.35
H. s	11.5	11.5	11.5	11.5	11.5	11.5
280 kg N ha^{-1} control[a]	0	0	0	0	0	0

All plants received the equivalent of 65 and 115 kg ha^{-1} of P and K respectively.
Arithmetic zero represents value beyond detection limit $< 10^4$.
[a]Seedlings inoculated with autoclaved cells.

possibility of an adaptation to the host plant. It thus seems unadvisable to use an isolate on a plant other than its own host (Balandreau and Roger, 1996).

Mechanism

In the first field experiment, inoculation brings about an increase in yield higher than increasing N fertilizers by 140 kg ha^{-1}. In the second field experiment inoculating *Herbaspirillum seropedicae* is equivallent to increasing N fertilisers by 140 kg ha^{-1}, while other bacteria have a still stronger effect. It confirms previous findings (Omar et al., 1992) showing that N_2-fixing bacteria have an effect on N nutrition of the plant, only when N is the actual limiting factor. Nevertheless, without using ^{15}N or nif$^-$ mutants (Oliveira et al., 2002; Sevilla et al., 2001), one cannot estimate the actual contribution of nitrogen fixation to the observed effects.

It is likely that rhizosphere adapted bacteria simultaneously use several mechanisms to increase the size of their niche and the supply of plant derived carbon compounds, thus indirectly increas-

Table 5. Sugarcane field trial 1: N content of leaf samples and biomass in variety Co 86032 grown for 12 months. Inoculation with *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae* and *Burkholderia* MG43

Treatments	N Content						Final shootbiomass
	Days after planting						
	60	120	180	240	300	Mean	
140 kg N ha^{-1} control[a]	2.45 c	1.64 b	3.25 b	4.27 cd	4.13 a	3.14 d	158.87 d
140 kg N ha^{-1} + *G. diazotrophicus*	4.02 b	2.03 b	4.62 a	4.69 bc	4.30 a	3.93 c	185.52 b
140 kg N ha^{-1} + *H. seropedicae*	3.04 c	1.96 b	4.62 b	4.20 cd	4.48 a	3.66 c	178.75 bc
140 kg N ha^{-1} + *B. vietnamiensis* MG43	4.13 b	1.92 b	3.11 a	4.34 d	4.37 a	3.57 c	191.00 a
140 kg N ha^{-1} + combined (*G.d* + *H.s* + MG 43)	10.22 a	2.24 b	4.79 a	4.97 b	4.44 a	5.33 a	181.45 b
280 kg N ha^{-1} control[a]	2.45 c	4.37 a	5.00 a	5.60 a	4.37 a	4.35 b	163.12 c
F-value	233.61	50.65	12.36	25.88	0.22	15.22	16.31
CV (%)	63.24	40.92	20.75	11.32	11.03	15.28	1.38

N content results are expressed in mg g−1 and are the means of 6 replicates. Biomass in Mg ha^{-1}.
When followed by different letters within a column results differ at 5% level (Tukey test)
[a]Seedlings inoculated with autoclaved cells.

Table 6. Sugarcane field trial 2, at Orakatpettai: numbers of diazotrophic bacteria (10^4 g^{-1} fresh wt) in LGIP (*Gluconacetobacter diazotrophicus*), PCAT (*Burkholderia* MG43) and JNFB (*Herbaspirillum seropedicae*) media in micropropagated sugarcane variety Co 86032 grown with 65 and 115 kg ha^{-1} of P and K respectively

Treatments	30	60	90	120	150	180
140 kg N ha^{-1} control[a]	1.15	2.12	4.09	4.09	4.59	4.59
140 kg N ha^{-1} + *G. diazotrophicus*	11.5	11.5	11.5	7.35	7.35	4.59
140 kg N ha^{-1} + *H. seropedicae*	11.5	11.5	11.5	11.5	11.5	11.5
140 kg N ha^{-1} + *B. vietnamiensis* MG43	11.5	11.5	11.5	11.5	11.5	7.35
140 kg N ha^{-1} + combined (*G.d* + *H.s* + MG 43) (*G. d*)	11.5	11.5	11.5	11.5	4.59	4.09
(*B. v*)	11.5	11.5	11.5	11.5	11.5	7.35
(*H. s*)	11.5	11.5	11.5	11.5	11.5	7.35
280 kg N ha^{-1} control[a]	0	0	0	0	0	0

Arithmetic zero represents value beyond detection limit $< 10^4$ or absent,
[a]Seedlings inoculated with autoclaved cells.

ing the growth of their host-plant. N_2-fixation and hormone production are not exclusive of each other and might concur to the adaptation of bacteria, probably with other unknown mechanisms.

The local isolate MG43 of *Burkholderia vietnamiensis* has the highest potential. Omar et al. (1992) have already shown that inoculating rice seeds with a N_2-fixing bacterium was most efficient with a local strain (*Azospirillum brasilense* NO40), as compared with *A. brasilense* Sp7 which significantly decreased yield (Heulin et al., 1991). This could be the result of by-passing the initial competition phase in the rhizosphere colonisation by imposing, from the start, the most adapted strain. Inoculation interest would not be to introduce a "miracle" bacterium, but only to boost a local component of the soil microflora, insuring that the plant will be readily colonised by the "best" bacterium, instead of wasting exudates on a diversity of soil bacteria with diverse levels of efficiency (Heulin et al., 1989).

Prospect

Sugarcane inoculation with *B. vietnamiensis* thus appears as a possible option when the cost of inoculation is lower than the cost of saved N fertiliser (around 140 kg ha^{-1} in the reported field experiments).

Nevertheless, using a *Burkholderia* strain as an inoculum is not without risk. This genus contains several species which have been involved in opportunistic infections and/or in the "cepacia syndrome" a dramatic necrotizing pneumonia

Table 7. Sugarcane field trial 2: N content of leaf samples and biomass in variety Co 86032 grown for 12 months. Inoculation with *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae* and *Burkholderia* MG43. N content results are expressed in mg g^{-1} and are the means of 6 replicates. Biomass in Mg ha^{-1}

Treatments	N Content						Final shoot biomass
	Days after planting						
	60	120	180	240	300	Mean	
140 kg N ha^{-1} control[a]	2.55 c	2.03 c	4.02 b	4.51 bc	3.95 a	3.41 d	146.87 d
140 kg N ha^{-1} + *G.diazotrophicus*	4.23 b	2.20 c	4.34 b	4.62 bc	4.20 a	3.91 c	170.77 b
140 kg N ha^{-1} + *H seropedicae*	3.11 b	2.17 c	4.13 b	4.27 c	4.09 a	3.55 c	153.55 c
140 kg N ha^{-1} + *B. vietnamiensis* MG43	4.30 c	1.64 c	4.20 b	4.90 b	4.09 a	3.82 c	175.07 a
140 kg N ha^{-1} + combined (*G.d* + *H.s* + MG 43)	11.41 a	3.46 b	5.60 a	5.00 b	4.44 a	5.98 a	167.50 b
280 kg N ha^{-1} control[a]	2.55 c	4.72 a	6.19 a	5.91 a	4.41 a	4.69 b	154.27 c
F-value	409.41	44.03	15.51	17.11	1.29	16.72	27.42
CV (%)	67.09	36.90	20.0	12.11	8.30	12.32	6.37

Monthly data are means of six replicates.
When followed by different letters within a column results differ at 5% level (Tukey test)
[a]Seedlings inoculated with autoclaved cells.

which affects patients with Cystic Fibrosis (Isles et al., 1984). The relevant species are *B. cenocepacia, B. multivorans* and *B. stabilis*. They all pertain to a group of nine species called the "*Burkholderia* cepacia complex" or Bcc (Vandamme et al., 1997). These species are closely related and difficult to identify (Coenye et al., 2001). Unfortunately *B. vietnamiensis* is a Bcc member. There might be difficulties in registering a strain such as MG43, unless Bcc pathogenicity genes are clearly identified and shown to be non transferable and absent from MG43.

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