African Journal of Biotechnology Vol. 9(24), pp. 3542-3548, 14 June, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1703
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Characterization of *Burkholderia cepacia* genomovar I as a potential biocontrol agent of *Ganoderma boninense* in oil palm

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Accepted 19 February, 2010

Burkholderia **is an important bacterial genus containing several species of ecological, biotechnological and pathological interest. Bacterial isolate can be gotten from soil, water, plants and even clinics. With their taxonomy undergoing constant revision and the phenotypic similarity of several species, correct identification of *Burkholderia* is difficult. *Burkholderia cepacia* complex (Bcc) consists of nine discrete genomic species and a genetic scheme based on the *recA* gene has greatly enhanced the identification of *B. cepacia* complex species. The objectives of this study were to identify *Burkholderia strain* UPM B3 which was isolated from oil palm roots to the species level based on Biolog® Identification System, and to carry out DNA fingerprinting for strain differentiation as well as differentiate between pathogenic and non-pathogenic human forms. Antagonistic activity of UPM B3 against *Ganoderma boninense* was also evaluated by using dual culture and poison food tests. Genotype characterization was carried out by amplification of the *recA* gene using specific primers, purified using QIA Quick polymerase chain reaction (PCR) purification kit and sequenced. Multiple sequence alignments were performed on closely related sequence accessions using CLUSTAL W software. Result of nucleotide sequencing followed by phylogenetic analysis of the *recA* fragments differentiated both putative and known *Burkholderia* species and all members of the *B. cepacia* complex. Genomovar analysis confirmed that UPM B3, isolated from oil palm roots belongs to genomovar I and has antagonistic activity against *G. boninense* based on *in vitro* dual culture and poison food tests. From the phylogenetic tree, UPM B3 is a specific strain within *B. cepacia* complex species that belong to genovomar I which is associated with strains nonpathogenic to humans. Thus, *B. cepacia* strain UPM B3 has the potential to be used against *G. boninense*, the causal pathogen of basal stem rot (BSR) in oil palm.**

Key words: *Burkholderia cepacia* complex, *recA* gene, UPM B3, basal stem rot, *Ganoderma boninense*.

INTRODUCTION

Oil palm (*Elaeis guinnensis*) is the most important plantation and commercial crop in Malaysia. It is one of the highest yielding plant among the oil producing crops. Basal stem rot (BSR) caused by *Ganoderma boninense*, is a major disease that attacks oil palm (Ariffin et al., 2000; Flood et al., 2003). BSR infection results in crop loss and in severe cases, loses could be up to 45% of the yield (Singh, 1991). The primary control strategies of BSR include cultural, chemical and clean clearing. Unfortunately, the current available control measures are only aimed at minimizing the incidence of BSR in replanting, prolonging the productive life of infected palms, and delaying the progress of *Ganoderma* infection. Biological control has gained much attention in the past decade as a way of reducing the use of chemical products in agriculture.

Endophytic bacteria such as species of *Serratia*, *Pseudomonas*, *Burkholderia*, and *Bacillus* have been shown to be used as biological conrol agent against several fungal and bacterial disease agents (Dorworth and Callan, 1996; Azevedo et al., 2000; Soylu et al.,

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2005). Endophytic bacteria live in the plant tissues without doing substantive harm or gaining benefit other than residency (Kobayashi and Palumbo, 2000; Zaiton et al., 2008). Endophytic bacteria as internal colonizers may compete in the vascular system depriving *Ganoderma* for nutrients and space. *Burkholderia cepacia* strain UPM B3 has been isolated from symptomless palms and its potential as a biocontrol agent against *Ganoderma* has been established (Zaiton et al., 2008). However, the *B. cepacia* complex is a very diverse group of bacteria. It is a group of at least nine closely related species (often called "genomovars") that is currently attracting considerable attention for their extraordinary versatility as plant pathogens, saprophytes, biocontrol and bioremediation agents and also human pathogens. They are also important opportunistic human pathogens that cause infections in patients with cystic fibrosis (Mahenthiralingam et al., 2000). *B. cepacia* complex are naturally abundant in soil, water and on plant surfaces. Some strains are effective biocontrol agents against soil-borne (Milus and Rothrock, 1997), foliar (Joy and Parke, 1994) and post-harvest diseases (Smilanick et al., 1993). It has been reported to inhibit the growth of *Fusarium oxysporum*, *Macrophomina phaseolina*, *Sclerotium roflsii*, *Rhizoctonia solani* and *Pythium ultimum* (Baligh et al., 1991). It can prevent leaf and stem blight caused by the fungus *Alternaria* by inhibiting spore germination. Economically important disease such as blight caused by *Alternaria solani*, *Alternaria brassicae* and *Alternaria brassicola*, which affects the oil-producing plants rape and canola, respectively, can be controlled by *B. cepacia*. The bacterium is also being used to prevent blight of ginseng plants due to *Alternaria panax* (Joy and Parke, 1994) and is effective against the fungus *Aphanomyces euteiches*, which causes root rot in peas, alfalfa and snap beans (King and Parke, 1996). It can prevent *Pythium* diseases of cucumber and peas and *R. solani* stem rot of poinsettia (Cartwright and Benson, 1995). It provides a seemingly environmentally friendly alternative to potent and toxic fungicides, which cannot be broken down in the environment. *B. cepacia* also has been reported to promote growth of maize (Bevivino et al., 1998), enhance crop yield (Chiarini et al., 1998; Tabacchioni et al., 1993), and degrade diverse pesticides (McLoughlin et al., 1992).

It is important to identify *B. cepacia* complex at genomovar level for risk assessment. To our knowledge, there was no report of *B. cepacia* complex isolated from oil palm roots that has been identified at the genotype level. Thus, this study was carried out to characterize UPM B3 as potential biocontrol agent against *G. boninense*. The *recA* gene was used in this study to identify the genomovar of UPM B3, in order to provide reference for oil palm microorganism safety assessment. Analysis of *recA* has proven very useful in molecular systematics among closely related bacteria (Eisen, 1995). The *recA* gene was used in a cultivation-independent approach to examine the *Burkholderia* diversity associated with the maize rhizosphere (Payne et al., 2006) whereby, phylogenetic analysis of 188 novel *recA* genes enable clarification of the taxonomic position of several important *Burkholderia* strains and revealed the presence of four novel *B. cepacia* complex *recA* lineages. Antagonistic activity of UPM B3 against *G. boninense* was also evaluated by using dual culture and poison food tests.

MATERIALS AND METHODS

Biolog identification system

UPM B3 was identified to the species level with the Biolog® identification system (Version 4.2). The procedure for identification utilized 96 wells of microplate containing 95 different dried carbon source plus control. Bacteria from 24 h old cultures growing on NA were streaked on Biolog reader universal growth (BUG) medium. The bacteria isolate was initially determined by tetrazolium violet as a colorimetric indicator of the gram reaction and oxidization and categorized as enteric or non-enteric bacteria. Non-enteric bacteria are gram-negative bacteria and positive oxidase. The bacteria were then suspended in the inoculation fluid (0.4% sodium chloride, (NaCl); 0.03% pluronic F-68; 0.01% gellan gum) with quantification of the cell based on transmittance reading. The bacteria suspension was inoculated in a GN biolog 96-well microtiter plate with 150 µl per well. The microtiter plate was incubated at 28-30°C for 24 h, and the resulting pattern of colored wells analyzed using the Microstation™ system and Biolog MicroLog™ software to give the bacteria identification up to the species level.

Genomic DNA extraction from UPM B3

DNA extraction was carried out following the cetyltrimethyl ammonium bromide (CTAB) method (Sambrook et al., 1989). Ten colonies of UPM B3 was inoculated into 10 ml nutrient broth and incubated at 28 ± 2°C overnight. 1 ml of overnight culture was transferred into a 1.5 ml eppendorf tube and centrifuge for 30 s at 13000 rpm. The bacteria cells were collected by discarding the supernatant and resuspended in 567 µl TE buffer (10 mM Tris-HCL, pH 7.4, 1 mM EDTA and 1 l distilled water), mixed well by vortexing. Then 30 µl of 10% sodium dodecyl sulfate (SDS), 3 µl of NAOAC (sodium acetate) pH 5.2, 100 µl of 5 M NaCl and 80 µl CTAB-NaCl were added to a total volume of 780 µl and mixed well before incubating for 10 min in a water bath at 65°C. An equal volume (780 µl) of chloroform/isoamyl alcohol (24:1) was added to the mixture and centrifuged at 13000 rpm for 5 min to separate the phases. The clear supernatant was transferred into a new eppendorf tube and the aqueous DNA layer was again extracted using phenol/chloroform/isoamyl alcohol (25:24:1).

This step was repeated 3 times and the supernatant pooled. The clear supernatant was transferred into new eppendorf tube and 400 µl of isopropanol was added to precipitate the nucleic acid. Finally the DNA was washed with 200 µl of 75% of cooled ethanol and dried at room temperature (28 ± 2°C) before dissolving in 100µl sterile distilled water (SDW) and kept at - 20°C for further analysis. Electrophoresis was run to identify the nucleic acids after DNA extraction in 1% of agarose gel in 1% of TBE (Tris base, boric acid, 0.5 M EDTA solution, 1 l ddH_2O and pH 8.0). The products were mixed with loading dye buffer (MBI fermentas) in 5:1 ratio and subjected to electrophoresis at 70 v for 1 h and 45 min. DNA ladder 100 bp (MBI fermentas) was used as a marker. The gel was stained in ethidium bromide solution and the bands visualized and photographed using BioRad Gel Doc 2000.

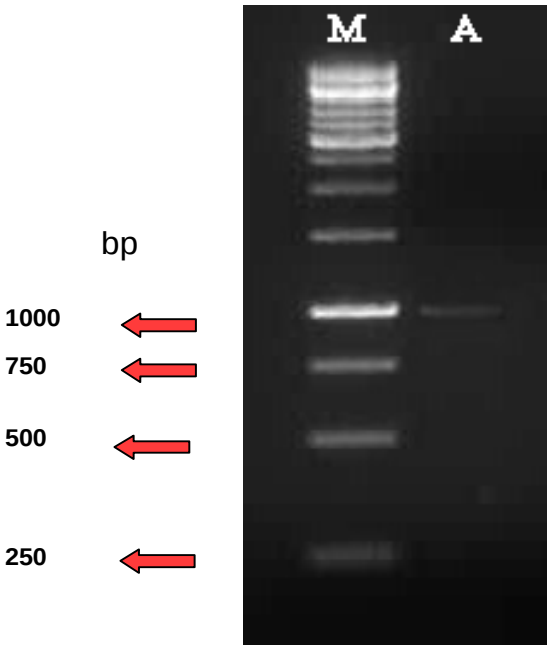

Figure 1. Amplification of *recA* gene for (A) *Burkholderia cepacia* strain UPM B3. (M) Size of DNA ladder.

Nucleotide sequence analysis

The polymerase chain reaction (PCR) products was purified using a commercial kit (QIA Quick PCR purification kit (Qiagen, Valencia, CA), according to manufacturer's instruction. After purification, the PCR products were sequenced. The *recA* gene sequences were aligned using BioEdit software versions 7.0.8 (http://www.mbio.nscu.edu/bioEdit/bioEdit) and searched for sequence similarity to other sequences which are available in the NCBI database at http://www.ncbi.nih.gov using basic local alignment search tool (BLAST) algorithm. Multiple sequence alignments were performed on the selected closely related sequence accessions available using CLUSTAL W software (http://workbench.sdsc.edu/).

PCR amplification

Oligonucleotide primers BCR1 (5'-tgaccgccgagaagagcaa-3', 19 mer) and BCR2 (5' ctcttcttcgtccatcgcctc-3', 21 mer) were used (Mahenthiralingam et al., 2000). Amplification reaction for UPM B3 was performed in 25 µl of total volume containing 2 µl of DNA as a template, 2.5 µl of 10x PCR buffer (Fermentas), 1.5 µl of 25 mM $MgCl_2$ (Fermentas), 0.6 µl of 10 mM dNTPs, 0.2 µl primer oligonucleotides, 0.2 µl *Taq* polymerase (Fermentas) and 17.8 µl of sterilized distilled water. The amplification was performed in a thermal cycler (Biometra®,T3 thermocycler) (Syngene, UK) programmed for pre-denaturing of 3 min at 94°C, 30 cycles of 1 min at 94°C (denaturing), 1 min at 58°C (annealing), 2 min at 72°C (extension). After a final extension of 10 min at 72°C, the samples were cooled to 4°C.

Phylogenetic analysis

Phylogenetic analysis was done based on the nucleotides of *recA* gene using draw tree software provided by the Biology Workbench Program (http://workbench.sdsc.edu/).

In vitro antagonistic activity of *B. cepacia* UPM B3 against *G. boninense*

Dual culture test: *G. boninense* (UPM 36B) was obtained from the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. A 5 mm diameter agar disc taken from the five day-old potato dextrose agar (PDA) culture of *G. boninense* was placed centrally in nutrient agar plate. Then, 0.01 ml of UPM B3 suspension (10^7 cell/ml) was streaked 3 cm away from *G. boninense* disc. The ability of UPM B3 to inhibit the growth of *G. boninense* was assessed after seven days' of co-incubation at 28 ± 2°C by measuring the radius of the *G. boninense* colony in the direction towards the bacterial colony (R2). The data were later transformed into percentage inhibition of radial growth (PIRG) in relation to the radial growth of G*. boninense* in the control plate (R1) using the formula (Jinantana and Sariah, 1997):

PIRG (%) = [(R1 - R2) / R1] X 100

All tests were conducted in five replicates and repeated twice, in a completely randomized design (CRD).

Poison food test

This test detects the production of non-volatile, diffusible inhibitors produced by UPM B3 in standing liquid culture, either as antibiotics, enzymes or other form of inhibitors. UPM B3 was inoculated in 250 ml nutrient broth and maintained at 28 ± 2°C in the dark for seven days. The culture was then centrifuged at 10000 rpm for 5 min, the supernatant was collected and the pellet discarded. The supernatant was filtered through a 0.25 µm membrane filter in sterile conditions. The filtrate was incorporated into sterilized double strength potato dextrose agar (PDA) in ratio 2:1; 20 ml of the amended agar was poured into each Petri plate and allowed to solidify. A *G. boninense* mycelial plug was centrally inoculated in each of the plate. Non-amended PDA was used as the control. The diameter of the mycelial growth of *G. boninense* was measured over seven days. The antagonistic activity was expressed as PIRG (Jinantana and Sariah, 1997) in relation to the mycelia growth of *G. boninense* in the control plate. The experiment was conducted with five replicates and repeated twice, in a completely randomized design.

RESULTS

Identification of bacterial isolate using biolog reader system

UPM B3 is a non-enteric bacterium that was confirmed with the biolog® identification system and identified as *B. cepacia* based on 100% probability and similarity index of 0.84.

PCR amplification of *recA* gene

Genomic DNA was successfully extracted from UPM B3 using the CTAB method. Expected product size for PCR amplication of *B. cepacia* should be in the range of 1043 bp (Figure 1). Primers BCR1 and BCR2 were used for

Table 1. Strains of *Burkholderia* c*epacia* from BLAST result utilized for a phylogenetic analysis of *recA* sequences.

Gen bank accession numbers	Genomovar	Strain	E value*
AF143788.1	I	ATCC17759	0.0
AF456023.1	I	LMG 14087	0.0
DQ989509.1	I	M297	0.0
AF143787.1	I	CEP509	0.0
AY769897.1	I	KBC-3	0.0
EU079005.1	I	6RT68	0.0
EU079004.1	I	4ST9	0.0
EF602578.1	I	ESR63	0.0
AY769896.1	I	KBC-2	0.0
EU079011.1	I	5RT161	0.0
EU079010.1	I	6SP128	0.0
EU079006.1	I	6RP83	0.0
AF143786.1	I	ATCC25416	0.0
EU079009.1	I	6RT141	0.0
EU079008.1	I	3ST4	0.0
EU079007.1	I	5RT136	0.0
AF456062.1	I	IST431	0.0

* Expected value.

amplification of *recA* gene of *B. cepacia.* PCR primers BCR1 and BCR2 were designed from homologous sequences at the 5' and 3' ends of the *recA* open reading frame. These primers amplified a single 1 kb amplicon from all strains representative of the *B. cepacia* complex. The identification of the 1 kb fragment was subsequently confirmed to be *recA* by direct nucleotide sequence analysis of PCR products.

BLAST result

ATCC17759, M297, LMG14087 and CEP509 had 99% similarity with UPM B3 and the rest of the accessions as listed in the NCBI Genbank used for comparison with UPM B3 gave 98% similarity based on BLAST result as shown in Table 1. The sequence of *B. cepaci*a UPM B3 has been submitted to NCBI Genbank. The Genbank accession numbers is GQ183952.

Phylogenetic analysis

Phylogenetic analysis carried out using sequence of *recA* gene resulted in the phenogram for *B. cepacia* (Figure 2). UPM B3 was grouped together with strains belonging to genomovar I; ATCC17759, CEP509, M297, and LMG 14087. However, LMG14087 was obtained from wound swab and the rest were isolated from plant rhizospheres.

Antagonistic activity of *B. cepacia* strain UPM B3 against *G. boninense*

B. cepacia strain UPM B3 inhibited the mycelial growth of *G. boninense* based on the *in vitro* dual culture and poison food tests with PIRG values of 78 and 88%, respectively (Figure 3). It gave a clear inhibition zone of 9.25 mm after seven days of co-incubation on nutrient agar The UPM B3 parasitized mycelia of *G. boninense* from the inhibition zone could not be recovered when transferred to fresh PDA plate, and thus confirmed their potential as biocontrol agents against *G. boninense.*

DISCUSSION

B. cepacia was first described in 1949 by Walter Burkholder, as the causal agent of bacterial rot of onions (Burkholder, 1950). Later, it was proposed as *B. cepacia* complex in the genus *Burkholderia* and it consist of nine genomovars. Based on biolog reader system, the bacteria UPM B3 was identified as *B. cepacia*; however it could not be differentiated between the pathogenic and nonpathogenic human forms. Thus molecular approaches were carried out to differentiate the pathogenic and nonpathogenic forms within *B. cepacia* complex. Based on the *recA* gene sequence, it was confirmed that UPM B3 was categorized under genomovar I of *B. cepacia* complex. The *recA* gene has proven very useful for the

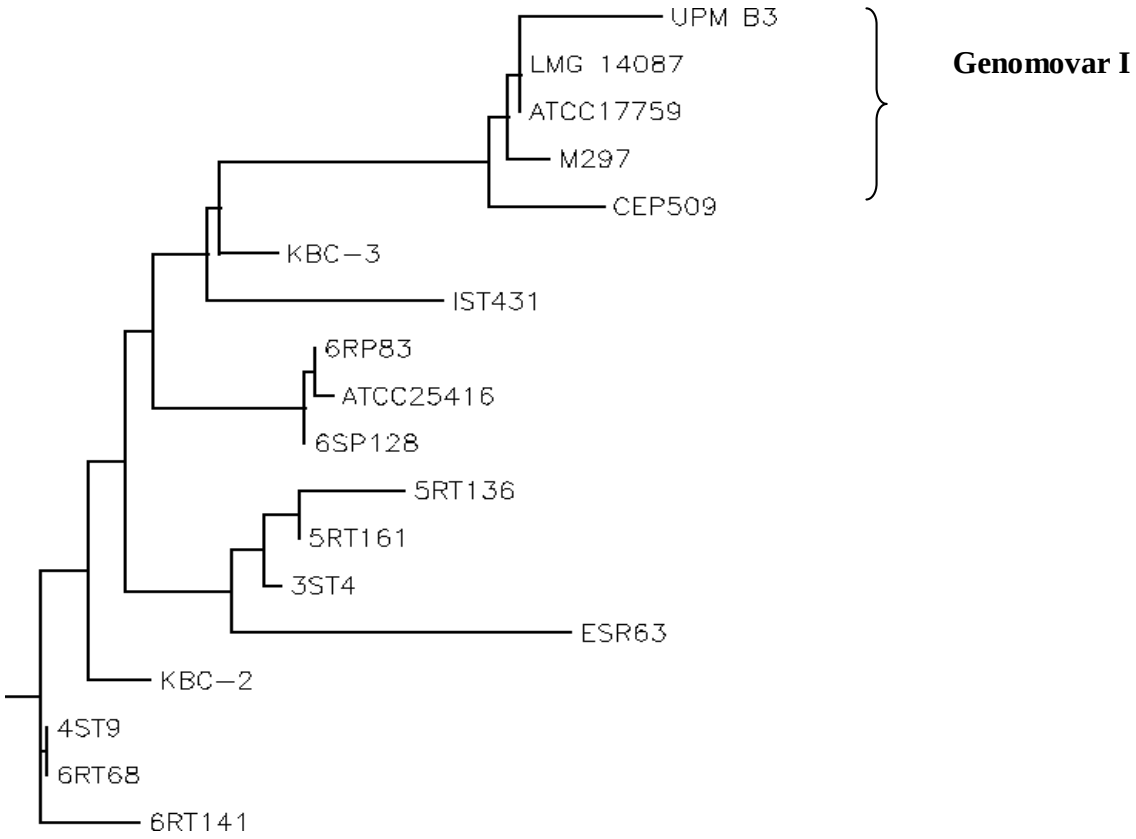

Figure 2. PHYLIP rooted tree: Phenogram for *Burkholderia cepacia* strain UPM B3.

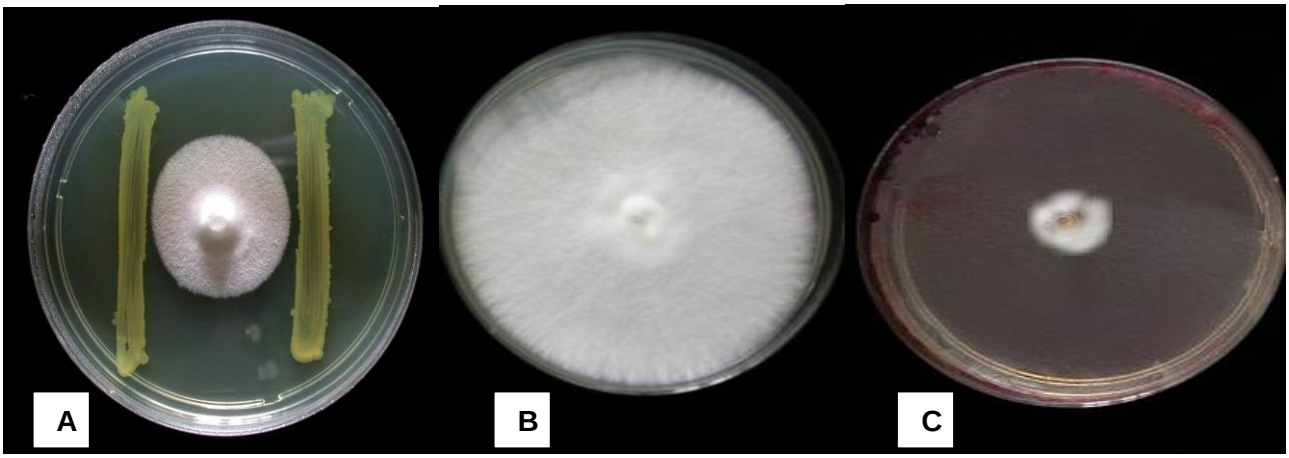

Figure 3. Antagonistic activity of UPM B3 against *G. boninense* in the dual culture: (A) and Poison food test, (B) *G. boninense* in control plate, and (c) G. *boninense* in culture flitrate (at 7 days incubation).

identification of *B. cepacia* complex species, with phylogenetic analysis of sequence variation within the gene enabling discrimination of all the nine current species within the genus (Mahenthiralingam et al., 2000). Primers designed for the original *recA*-based approach, BCR1 and BCR2, are specific only to members of the *B. cepacia* complex and fail to amplify this gene from other *Burkholderia* species. Genomovars II and III of *B. cepacia* complex had the highest isolation frequency from clinics of America, Canada and Europe, and genomovar III is the most virulent of the *B. cepacia* complex strains (Chiarini et al., 2006).

Members of genomovars I, III, VII and IX had been confirmed as having good biocontrol ability, especially

effective against *Pythium* and *Rhizoctonia* (Milus and Rothrock, 1997; Cartwright and Benson, 1995). Strains belonging to genomovar III from the maize rhizosphere could facilitate plants to resist fungal diseases (Bevivino et al., 1998; Zhang and Xie, 2006). However, the isolation frequency of genomovar III from clinic is also very high; it was considered to be a hazardous human opportunistic pathogen. Therefore, whether the application of genomovar III has any risk implications to human is still being disputed. Most of the strains from non-clinical sources belong to genomovar I (*B. cepacia sensu stricto*), V (*Burkholderia vietnamiensis*) and VI (*Burkholderia dolosa*). *B. cepacia* which has been shown to have potential as an agent for both bioremediation and biocontrol, is also being considered as a plant-growth promoting rhizobacterium. *B. cepacia* can also antagonize and repress many fungal plant pathogens such as *Fusarium*, *Pythium*, *Rhizoctonia*, *Cylindrocarpum*, *Botrytis* and *Alternaria* (Reddy, 1997). The commercial development of *B. cepacia* as a biological control agent for plant pathogens and as growth promoter has been of interests to many agriculturists. In this study, UPM B3 has shown strong antagonistic activity *in vitro* tests with significant ability to inhibit the mycelial growth of *G. boninense*, and has demonstrated suppressive effect against *Ganoderma* infection in oil palm seedlings (Zaiton et al., 2008). BSR is a major disease of oil palm in Malaysia, and therefore, of great economic importance to the Malaysian oil palm industry. Introducing UPM B3 to the roots in order to control the disease is to manipulate the indigenous communities of the roots, in a manner which can lead to enhanced suppression of *Ganoderma*. However, field studies must be done to confirm the efficacy *in vivo*. There is a need to determine the required population density of UPM B3 to apply and the best method of application. The effects of agrochemicals on UPMB3 for good management of BSR by the bacteria also need to be investigated.

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