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Biochemical and Physiological Characterization of *Burkholderia cepacia* as Biological Control Agent

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ABSTRACT

Burkholderia cepacia is recognized as a biological control agent for the control of plant pathogens. Two isolates of *B. cepacia* BC-S and BC-TM were screened for their potential for antagonism against *Schizophyllum commune* Fr. and *Fusarium oxysporum* f.sp. *lycopersici* respectively. The purpose of the study was to determine the differences in biochemical and physiological characteristics of both isolates of *B. cepacia.* Both isolates grew on NA resulting in the production of yellow colony with entire margin 48 hours after incubation. At five days after incubation, isolate BC-S started to change from yellow to reddish yellow. Isolates BC-S and BC-TM were identified using Biolog® Identification System with similarity of 99% and 100% respectively. Reaction of eight out of 96 carbon sources in the Biolog® kit showed slight difference between BC-S and BC-TM such as L-fucosa, N-acetyl-D-galactosamine, L-alanyl-glycine, D-threhalose, 2-3-butanediol, alpha-hydroxy butyric acid, putrescine, glycil-L-glutamic acid. Pathogenicity test of both isolates on onion bulbs resulted in non soft rot symptoms. The BC-S inhibited the mycelial growth of *S. commune* (70.8%) and *Colletotrichum dematium* (77%); while, BC-TM was more effective in inhibiting the mycelial growth of *F. oxysporum* f.sp. *lycopersici* (62%), *F. solani* (72.5%) and *G. boninense* (66.6%).

Key Words: *Burkholderia cepacia;* Biolog® Identification System; Dual Culture; Pathogenicity test

INTRODUCTION

Conventional agricultural practices have the tendency of applying a large amount of pesticides in order to attain optimum yield when pests presence. The challenge for a productive solution in the application of both biological control agents and limited application of pesticide refer to the sustainable agriculture.

Burkholderia cepacia was first discovered as the causal agent of rotten onion bulbs (Burkholder, 1950). However, this bacterium has emerged to be an important biological control agent for the control of plant pathogen such as *Botrytis cinerea*, the causal agent of blue mold of apple. *B. cepacia* has also been found to control *Schizophyllum commune* Fr. the causal agent of seed rot of oil palm (Janisewiez & Roitmann, 1988; Dikin *et al.,* 2003a) and *Ralstonia solanacearum*, the causal agent of bacterial wilt of tomato. *B. cepacia* has been isolated from the rhizosphere of healthy tomato plants and its biochemical characteristics have been reported (Sfalanga *et al.,* 1999). Suparman *et al.* (2002) reported that an isolate of *B. cepacia* obtained from the rhizosphere of tomato inhibited the spore germination of *Fusarium oxysporum* f.sp. *lycopersici*, the causal agent of Fusarium wilt of tomato.

B. cepacia produced pyrrolnitrin which was the major factor that suppressed the growth of *Rhizoctonia solani*, Rhizoctonia stem rot of poinsettia (Baligh *et al.,* 1999). Other metabolic compounds produced by *B. cepacia* are pyoluteolin, cepabactin, volatile ammonia and siderosphore (Lievens *et al.,* 1989; Meyer *et al.,* 1989).

Evidence that *B. cepacia* is an important human pathogen that causes cystic fibrosis has also been reported (Isles *et al.,* 1984). So far the biotype of *B. cepacia* used as biological control of plant pathogen has been differentiated from the biotype of human pathogen that causes cystic fibrosis on the production bacteriocin and pectolytic enzymes (Gonzalez & Vidaver, 1979).

In general, morphological and biochemical characteristics of *B. cepacia* are yellow colony on NA medium, entire margin, gram negative reaction, motile, non-fermentative, non-fluorescent, oxidase positive, hydrolysis of tyrosine and utilization of citrate, malonate, alanine, asparagines, glutamine, proline, citraconate, gluconate, and azelate. Acid is produced from adonitol, dulcitol, fructose, geraniol, glucose, propylene glycol, rhamnose, sorbitol, arabinose, galactose, glycerol, inositol, lactose, maltose, manitol, mamnose, salicine, sucrose, tartrate, threhalose, and xylose. *B. cepacia* is sensitive to chloramphenicol, nalidixic acid, novobiocin and resistant against spectinomycin, streptomycin, tetracycline, ampicillin and penicillin-G (Sfalanga *et al.,* 1999).

Large variation of biochemical characteristics among *B. cepacia* obtained from different sources has been reported. They normally occur as *B. cepacia* complex. The group of *B. cepacia* closely related each other occurred naturally in water, soil and plant surface. The complex has nine different genomovars (Tabachioni *et al.,* 2002). Complexity in the characteristics of *B. cepacia* had caused

difficulty in separating *B. cepacia* used as biological control agent from that of other genomovars.

The objectives of the study were to determine the biochemical and physiological characteristics of both isolates of *B. cepacia* BC-S and BC-TM from different sources, as biological control agent and to compare the growth inhibition on selected pathogenic fungi.

MATERIALS AND METHODS

Bacterial isolates. *B. cepacia* (BC-S) was isolated from oil palm seeds infected with *S. commune* and *B. cepacia* (BC-TM) was isolated from the rhizosphere area of tomato plants. Both isolates *B. cepacia* were sub-cultured onto NA and King's B media and incubated at room temperature (26±2°C).

Biochemical and physiological characteristics. Biochemical characteristics of both isolates of *B. cepacia* were obtained using Biolog®Identification System. Fresh cultures of *B. cepacia* (BC-S; BC-TM) on NA medium were streaked on Biolog Universal Growth (BUG) medium. Bacterial isolates were initially determined for their gram reaction, and oxidase test to categorize them into enteric bacteria or non-enteric bacteria. Enteric bacteria are gram negative bacteria and oxidase negative. Non-enteric bacteria are gram negative bacteria and oxidase positive. Bacterial suspension was made in the inoculant's solution (0.1 g Gellan Gum, 4 g NaCl, 0.3 g Pluronic F-68, and 1 L distilled water) with quantification of 63%. Bacterial suspension for biochemical characterization was inoculated into GN micro plate using the 8-channel repeating pipette. Microplate was covered with its lid and incubated at 28-30°C for 24 h to allow the utilization of carbon sources. Reading of the results was directly done after incubation by inserting the microplate into the Biolog's reader apparatus installed with software of Biolog® identification system, for identifying the bacteria up to the species level.

Pathogenicity test of *B. cepacia.* Fresh cultures of both *B. cepacia* isolates on NA medium were suspended in sterilized water (10^8cfu/mL). Four yellow onion bulbs 4-6 cm diameter were inoculated with 1 mL bacterial suspension on each bulb by injection at two opposite locations with a depth of 1-2 cm. Inoculated bulbs were placed on 3 layers of moisten filter paper in a plastic container to ensure high humidity condition. For the control, bulbs were injected with sterilized water. Inoculated bulbs were incubated at 26±2°C for 4 days before observation of rotting symptoms.

Dual culture. PDA medium was prepared in 9 cm diameter Petri dishes for dual culture assay. Bacterial isolates were streaked on PDA medium at 2 cm from the edge of plate. The same plate was inoculated with 4 mm diameter of agar plug from fresh PDA culture of *S. commune* at the centre of the plate. Plates were incubated at room temperature (26-28°C) for 7 days to observe the formation of inhibition zone between the fungus and the bacteria. Growth inhibition of the fungus was measured daily up to 7 days. Each treatment was replicated 4 times. Data was analyzed using the analysis of variance (Dikin *et al.*, 2003b).

RESULTS

Both isolates *B. cepacia* (BC-S & BC-TM) were able to grow on NA medium. The culture colony of both isolates exhibited the same morphological characteristics at 3 days after incubation. The colony characteristics were yellow, circular, convex, entire margin and glistening. The growth of both isolates on King's B agar medium was yellow colonies, circular, entire margin, convex, glistening and non fluorescent under UV light. *B. cepacia* (BC-S) grew well on NA medium and colony colour changed at 7 days after incubation to reddish yellow (Plate 1). The colony colour of *B. cepacia* (BC-S) was reddish yellow with circular, convex, entire margin and glistening at 10 days after incubation.

Characteristics of both isolates on different kind of sugars (Biolog® Kit) showed varied (negative & positive) biochemical reaction. Both isolates of BC-S and BC-TM

Plate 1. Growth of *B. cepacia* (BC-TM) on NA medium (left) and changes in colony colour of *B. cepacia* (BC-S at 7 days after incubation (right)

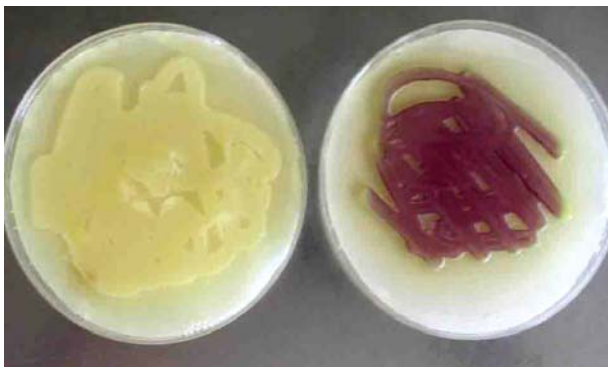
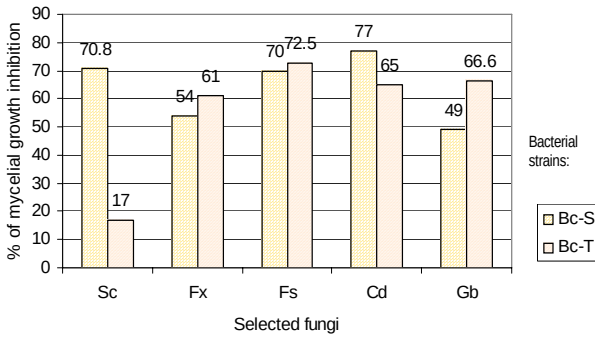


Fig. 1. *B. cepacia* BC-S; BC-TM mycelial growth inhibition of *S. commune* (Sc), *F. oxysporum* f.sp. *lycopersici* (Fx), *F. solani* (Fs), *C. dematium* (Cd) and *G. boninense* (Gb) on PDA medium



were gram negative, oxidase negative and enteric bacteria. Biolog® identification system confirmed that both isolates had similarity to Biolog's standard identification of 99% and 100% respectively. Eight out of 96 biochemical reactions were different between isolates BC-S and BC-TM. Different biochemical reactions of both isolates were on the utilization of L-fucosa, N-acetyl-D-galactosamine, L-alanyl-glycine, D-threhalose, 2-3-butanediol, alpha-hydroxy butyric acid, putrescine and glycil-L-glutamic acid. Detail reaction on each sugar on Biolog kit is presented in Table I.

Pathogenicity of both *B. cepacia* BC-S; BC-TM resulted in symptom production on the internal part of inoculated onion bulbs. Sunken dried lesions appeared on the outside surface of the bulbs around the point of syringe injection. *B. cepacia* is known to cause bulb rot of onion.

Dual culture test on both isolates of *B. cepacia* against pathogenic fungi at 7 days after incubation showed varied percentage of mycelial growth inhibition (Fig. 1). Isolate *B. cepacia* BC-S was significant different ($P \leq 0.05$) with respect to the mycelial growth inhibition of *S. commune* of

Table I. Biochemical characteristics of *B. cepacia* on Biolog® Identification System

Source of carbon	Reaction*)		Source of carbon	Reaction*)	
	BC-S	BC-TM		BC-S	BC-TM
1	2	3	4	5	6
Water	-	-	N-Acetyl-D-galactosamine	+/-	-
I-Erythritol	-	-	I-inositol	+	+
D-Melibiose	-	-	Sucrose	+	+
Acetic acid	+/-	+/-	d-Gluconic acid	+	+
Para-hydroxy phenylacetic acid	+	+	D-Gluconic acid	+	+
Bromo Succinic acid	+	+	L-Alanyl-glycine	+	+/-
L-Histidine	+	+	L-Pyroglutamic acid	+	+
Urocanic acid	+	+	2-Aminocethanol	+	+
Alpha-Cyclodextrin	-	-	N-Acetyl-D-glucosamine	+	+
D-Fructosa	+	+	Alpha-D-Lactose	-	-
B-methyl-D-Glucoside	-	-	D-Trehalose	+/-	-
Cis-aconitic-acid	+	+	D-Glucosaminic acid	-	-
Itaconic acid	-	-	Propionic acid	+	+
Succinamic acid	-	-	L-Asparagine	+	+
Hydroxy-L-Proline	+	+	D-Serine	+	+
Inosine	-	-	2,3-Butanediol	-	+/-
Dextrin	-	-	Adonitol	+	+
L-Fucosa	+	+/-	Lactulose	-	-
D-Psicose	+/-	+/-	Turanose	-	-
Citric acid	+	+	D-Glucoronic acid	+	+
Alpha- keto butyric acid	+	+	Quinic acid	+	+
Glucuronamide	+/-	+/-	L-Aspartic acid	+	+
L-Leucine	-	-	L-Serine	+/-	+/-
Uridine	-	-	Glycerol	-	-
Glycogen	-	-	L-Arabinose	+	+
D-Galactosa	+	+	Maltose	-	-
D-Raffinose	-	-	Xylitol	-	-
Formic acid	+/-	+/-	Alpha-hydroxyButyric acid	+/-	-
Alpha-keto Valeric acid	-	-	D-Sacharic acid	+	+
L-Alaninamide	-	-	L-Glutamic acid	+	+
L-Ornithine	-	-	L-Threonin	-	-
Thymidine	-	-	D,L-alpha-Glycerol Phosphate	-	-
Tween40	+	+	D-Arabitol	+	+
Gentiobiose	+/-	+/-	D-Manitol	+	+
L-Rhamnose	-	-	Methyl piruvate	+	+
D-Galactonic Acid Lactone	-	-	Beta-hydroxyButyric acid	+	+
Alpha-Keto Valeric acid	-	-	Sebacic acid	+	+
D-Alanine	+/-	+/-	Glycyl-L-Aspartic Acid	-	-
L-Phenylalanine	+	+	D.L-Threonine	-	-
Phenyethylamine	+	+	Glucose-1-Phosphate	-	-
Tween 80	+	+	D-Cellobiose	-	-
Alpha-D-Glucose	+	+	D-Mannose	+	+
D-Sorbitol	+	+	Mono-methyl-succinate	+	+
D-Galacturonic Acid	+	+	Gamma-hydroxyButiric acid	-	-
D.L-Lactic acid	+	+	Succinic acid	+	+
L-Alanine	+	+	Glycyl-L-Glutamic acid	+/-	-
L-Proline	+	+	Gamma-Amino Butyric acid	+	+
Putrescine	+/-	-	Glucose-Beta-phospate	+	+
Probability	99	100	Similarity	0.721	0.690
Distance	4.06	4.73	Type	GN-NENT	GN-NENT

*) + means positive reaction; - means negative reaction; +/- means borderless.

70.8% while isolate BC-TM was 17% of the mycelial growth inhibition. In contrast, isolate BC-TM was significantly different (P≤0.05) on the mycelial growth inhibition of *F. oxysporum* f.sp. *lycopersici* of 61% and BC-S was 54% growth inhibition.

Both isolates of *B. cepacia* was also significantly different (P≤0.05) on the mycelial growth inhibition of *F. solani, C. dematium* and *G. boninense. B. cepacia* isolate BC-S was higher mycelial growth inhibition on *C. dematium* than isolate BC-TM. However, BC-TM has higher mycelial growth inhiboition on *F. solani* and *G. boninense* compared to isolate BC-S.

DISCUSSION

B. cepacia from different sources such as oil palm plantation and tomato cultivations showed varied reactions of physiological and biochemical tests. *B. cepacia* was recognized to be a complex association of species due to varied biochemical reactions and also sources of isolates from different environments.

The *B. cepacia* complex consists of several species of closely related species. The species of *B. cepacia* has been split into 8 genomovars, including five named species (Parke & Sherman, 2001). *B. cepacia* complex was also recognized on the basis of differences between biological control strain and human pathogenic strains. Biochemical characteristics of both isolates on carbon source of Biolog's Identification showed slightly different reaction. Both isolates BC-S and BC-TM are believed to be from the same group of biological control agents but with different genetic sequences, although DNA sequences of *B. cepacia* of both isolates have not yet been proven. The same physiological characteristics of both isolates of BC-S and BC-TM had also been reported by Tsuchiya *et al.* (1995). They reported that *B. cepacia* isolated from lectuce was not able to suppress mycelial growth of *F. oxysporum* f.sp. *radicis-lycopersici* compared to *B. cepacia* isolated from soybean as well as cabbage. Heungens and Parke (2000) confirmed that differences in suppression of selected fungi by *B. cepacia* were in relation to their differences in the ecology of the two fungi.

Burkhead *et al.* (1994) reported that *B. cepacia* strain B37w inhibited the growth of a bioherbicide fungus, *Colletotrichum truncatum.* This fungus is commonly used for the control of weed hemp sesbania (*Sesbania exaltata*). Other applications showed that *B. cepacia* strain B37w suppressed *F. sambuinum*, the causal agent of dry rot of potato.

B. cepacia isolates BC-S and BC-TM grew well on NA medium and changes the colony colour at 5 days after incubation. The similarity of both isolates (BC-S & BC-TM) in the Biolog's identification system was 99% and 100% respectively based on the characterization of biochemical and physiological tests. Both isolates, BC-S and BC-TM were able to suppress pathogenic fungi

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