Suppression of Bacterial Wilt and Fusarium Wilt by a *Burkholderia nodosa* Strain Isolated from Kalimantan Soils, Indonesia

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A trial was conducted to suppress bacterial wilt of tomato (BWT) caused by *Ralstonia solanacearum* using biocontrol agents (BCAs) isolated from soils in Kalimantan, Indonesia. Five isolates were selected from 270 isolates as better performing BCAs through screening four times using a pumice medium. The isolates selected were identified as *Burkholderia nodosa*, *Burkholderia sacchari*, *Burkholderia pyrocinia* and *Burkholderia terricola* according to 16S rDNA sequences, fatty acid composition and carbon source utilization patterns. Among them, *B. nodosa* G5.2.rif1 had significant suppressive effects on Fusarium wilt of tomato (FWT) and spinach (FWS) as well as BWT. When *B. nodosa* G5.2.rif1 was inoculated into a pumice medium in combination with sucrose, it showed even more stable disease suppression for BWT, but not for FWS. This suppression was considered to mainly occur through competition for nutrients. In two times greenhouse experiments for BWT using pots comparable in size to those used commercially, *B. nodosa* G5.2.rif1 significantly suppressed the disease index by 33–79%, with no inhibitory effects on the growth, yield and quality of tomatoes.

Key words: screening, biocontrol agent, bacterial wilt, enhancement, sucrose

Soil-borne pathogens have been one of the major constraints to crop production. Disease control has been accomplished through the breeding of resistant varieties, crop rotations, cultivation techniques, additions of composts etc., but is largely dependent on the use of synthetic chemical pesticides⁴³. However, there is growing concern over the increased use of such pesticides to support agricultural production because of effects on mainly non-target organisms²³. In addition, leaching into underground water, run-off and volatilization of chemicals can cause environmental pollution³⁷. Therefore, biocontrol agents (BCAs) have attracted much attention as an alternative to the use of chemicals for disease control^{7,23,42}.

BCAs are sustained by various beneficial interactions such as competition for nutrients and space, antibiosis, parasitism and induced systemic resistance^{1,7,29}. Currently, several microorganisms possessing some of these properties are being used as active ingredients in a new generation of microbial pesticides^{1,23,43}.

BCAs are promising agents for the control of soil-borne plant diseases but a major problem is inconsistent colonization and so poor performance^{31,35}. In addition, not all species survive well in soil, because BCAs are not indigenous to the soil or because they poorly respond to diverse soil conditions. Important features in the colonization of soil and root by inoculated microorganisms are their persistence and expression of activity, which result in success of their application⁴⁰. The addition of carbon substrates is considered one effective method to enhance the colonization of BCAs. Carbon sources can increase growth rates of certain types of

rhizosphere microorganisms and thereby affect competition for space and nutrients, resulting in a modified community of microorganisms^{9,15,16,19,41}). Our previous study also found that the addition of organic compounds such as glucose and lysine had a conducive suppressive effect against bacterial wilt of tomato, possibly through modification of the indigenous microbial community²⁶.

Soils from Kalimantan in Indonesia were selected since there have been no reports on the isolation of BCAs from this area, in spite that Kalimantan is well known to be rich in microbial resources. For example, phosphorus-solubilizing microorganisms, nitrogen-fixing bacteria, decomposers and mycorrhizal fungi, have been studied^{3,27,28,34}). The purpose of this study is to screen BCAs from Kalimantan soils that would protect vegetables from soil-borne diseases and to examine a combination with organic carbons that enhance the activity of BCAs. In addition, the effect of the BCAs on the yield and quality of tomato was evaluated.

Materials and Methods

Strains and soils

The pathogens used in this study were *Ralstonia solanacearum* YU1Rif43³⁶, *Fusarium oxysporum* f. sp. *lycopersici* 880621a, race 2 and *F. oxysporum* f. sp. *spinaciae* GF-2. The bacterial strain was cultured overnight at 28°C in one tenth strength nutrient broth (1/10 NB) medium (Eiken Chemical Co., Ltd., Tokyo, Japan). The *F. oxysporum* strains were cultured in Czapeck medium¹⁰) for one week at 28°C on a NR-2 rotary shaker (Taitec Co., Ltd. Japan) at 75 rpm, and bud-cells were obtained by passing the culture through a funnel glass filled with absorbent cotton (Hasegawa Mengyo Co., Ltd., Aichi, Japan).

Histosols (peat) and spodosols (kerangas) were collected from Kalimantan, Indonesia and used as sources of biocontrol agents. For

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the histosol soils, we took 5 samples from different areas in agricultural fields (three fields planted with corn, a field planted with grasses, and a field planted with pumpkin) and 4 samples from different habitats (a burnt forest and a natural forest in Kalampangan, and two secondary forests in Setia Alam). A spodosol soil sample was collected from a secondary forest in the Tangkiling area. Some of the chemical and physical properties of the soils have been reported previously³³.

Isolation of biocontrol agents

Disease suppressive properties of BCAs were tested first against bacterial wilt of tomato (BWT) and then against Fusarium wilt of tomato (FWT) and Fusarium wilt of spinach (FWS). A total of 270 bacterial isolates were isolated from the soils using a one tenth strength nutrient agar medium (Eiken Chemical Co., Ltd., Tokyo, Japan) supplemented with 0.1 mg L⁻¹ of crystal violet (CV medium¹⁴), since our preliminary study suggested the a frequency of isolates with high biocontrol ability to be highest when CV medium was used as an isolation medium (data not shown). Ten grams of each soil sample was mixed with 90 ml of sterile distilled water and shaken by a Vortex-Genie 2 (Scientific Industries Inc., New York, USA) for one minute. Serial dilutions of 10⁻¹ up to 10⁻⁶ were prepared and the aliquots (0.1 ml) were plated on CV medium. The plates were incubated at 28°C for 3 days and the total number of colonies was calculated as CFUs (colony forming units per gram soil). For each soil sample, 25 isolates were randomly picked. A colony of bacteria was suspended in a tube containing 20% (w/v) glycerol and stored at -85°C until further use.

Screening of biocontrol agents against bacterial wilt of tomato (climatron experiment)

Four times screenings of BCAs were conducted for BWT. In the first screening, for each treatment, the 5 strains (1 ml overnight culture using 1/10 NB per strain) were combined and poured into a vinyl pot (Sakata Seed Co., Yokohama, Japan) 7 cm in diameter and 9 cm in height, containing 65 g of pumice and supplemented with CaCO₃ at a rate of 15 g kg⁻¹ for neutralization. The pumice (Ecoporus[®]; >2 mm 40%, 1 mm to 2 mm 51%, <1 mm 9%) was obtained from Ebara Co., Tokyo, Japan. Six tomato (*Lycopersicon esculentum* Mill cv. Momotaro) seeds (Takii Co., Ltd., Kyoto, Japan) pre-germinated for 2 days were transplanted to the pot by using sterile forceps. The seeds were grown for two weeks in a climatron (LPH200, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan; day:night=12h:12h, 285 µmol m⁻² s⁻¹ (photon flux) at 28°C). Watering was done daily using Otsuka liquid fertilizer A formula (Otsuka Chemical Co., Ltd., Osaka, Japan; pH 3.8, N 260, P 120, K 405, Ca 230, Mg 60, Mn 1.5, B 1.5, Fe 2.7, Cu 0.03, Zn 0.09, Mo 0.03 (mg L⁻¹)). After two weeks, six seedlings with pumice from the old pot were transplanted to a new vinyl pot 9 cm in diameter and 7.5 cm in height containing 55 g of pumice inoculated with *R. solanacearum* YU1Rif43 at a rate of 10⁴ cfu g⁻¹. In this screening, control – (no inoculation of BCAs and the pathogen) and control+(inoculation of only the pathogen) were also prepared. This experiment was done in triplicate. The numbers of wilted plants were recorded at 2-day intervals for 30 days after transplanting to the pot infested with the pathogen on the following basis: 0; no wilt symptoms, 1; 0 to 25% of plant showing wilting, 2; 26 to 50%, 3; 51 to 75%, 4; 76 to 100%. A disease index was expressed as the mean for triplicate pots, each consisting of 6 plants.

In the second to fourth screening, the same method as described above was used, except for the application of BCAs, in which a single strain selected from the first screening was inoculated into each pot.

Identification of BCAs

Strains that showed consistent disease suppression were grown in 1/10 NB for 24 h and DNA was extracted from the bacterial strains with the conventional method²²). The primers (27f) 5'-AGAGTTTGATCCTGGCTCAG-3' and (1378r) 5'-TACAAGGC-

CCGGGAACG-3' were used to amplify the segment of the bacterial 16S rDNA from nucleotides 27 to 1378 (*Escherichia coli* numbering)²¹). PCR was carried out using a basic reaction mixture containing an Ex Taq buffer (Takara, Otsu, Japan), 2.5 mM of each deoxyribonucleotide triphosphate, 2.5 U of Ex Taq (Takara), 0.25 mM of each oligonucleotide primer, and 1 µl of template (purified DNA) in a total volume of 25 µl. The thermocycling conditions consisted of an initial denaturing step at 94°C for 3 min, 30 amplification cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, and a final step at 72°C for 7 min with a GeneAmp PCR System (PCR Thermal Cycler, PERSONAL, Takara). The PCR products were confirmed in electrophoresis using an agarose gel with 0.5 µg ml⁻¹ of ethidium bromide and then, direct sequencing of the PCR products was done by Hitachi Science System, Ltd. (Tokyo, Japan). The phylogenetic position of the sequences obtained was examined using the DDBJ homology search system BLAST (<http://blast.ddbj.nig.ac.jp/top-j.html>).

The utilization of different carbon sources [acetamide, acetic acid, adonitol, aspartic acid, cellobiose, citric acid, D-arabinose, D-fructose, D-glucose, D-raffinose, dulcitol, glutamic acid, lactose, leucine, lysine, maltose, rhamnose, ribose, sorbitol, sucrose, trehalose, xylitol, and xylose (Wako Pure Chemicals Industries, Ltd.)] was tested using an inorganic salt medium (1.0 g NH₄NO₃, 1.0 g KH₂PO₄, 0.5 g MgSO₄, 0.2 g KCl, 10 g each carbon source, and 1000 ml distilled water, pH 7.0).

Fatty acid methyl ester analysis was done using freeze-dried bacterial cells based on the conventional method²⁰). Fatty acid composition was determined by a FID-GC/MS with a 6890 series gas chromatograph and HP 5973 mass selective detector (Hewlett-Packard, Pittsburgh, USA) equipped with a capillary column (DB-23, 0.25 mm ID x 30 m, J&W Scientific) operating with a mass range of m/z 50-500.

Analysis of suppression mechanisms

(1) Competition for substrates

BCAs and the bacterial wilt pathogen were co-inoculated into 1 ml of 1/10 NB and glucose or sucrose medium (the ingredients were 1.0 g NH₄NO₃, 1.0 g KH₂PO₄, 0.5 g MgSO₄ · 7H₂O, 0.2 g KCl, 10 g glucose or sucrose, and 1000 ml distilled water) at initial densities of 5 × 10⁷ cfu ml⁻¹ and 5 × 10⁶ cfu ml⁻¹ for the G5.2rif1 strain and at an initial density of 5 × 10⁴ cfu ml⁻¹ for YU1Rif43. In the control, only the pathogen was inoculated into the media. The population of the pathogen was enumerated by the dilution plate method using 1/10 NA amended with 50 µg mL⁻¹ each of rifampicin (Wako Pure Chemicals Co.) and of polymyxin B sulfate (Wako Pure Chemicals Co.) after 3 days at 28°C.

One milliliter of 1/10 NB at 28°C was co-inoculated with G5.2rif1 strain at an initial density of 5 × 10⁷ or 5 × 10⁶ cfu ml⁻¹ and with the bud-cells of *F. oxysporum* f. sp. *lycopersici* at an initial density of 10⁵ ml⁻¹. After 3 days of incubation at 28°C, the population of G5.2rif1 strain was enumerated using 1/10 NA amended with 50 µg mL⁻¹ of rifampicin and the propagule number of the fungus using Czapeck agar medium amended with 1 g L⁻¹ of triton X-100 (Wako Pure Chemicals Co.) and 300 mg L⁻¹ of streptomycin.

(2) Antibiosis and motility test

Strains of G4.1rif3 and G5.2rif1 were checked for antibiosis towards the bacterial wilt pathogen YU1Rif43 using a dual culture assay on 1/10 NA. The agar plate was overlaid with 5 ml of 1/10 NB added with 0.025 g of agar containing 0.1 ml of overnight culture of YU1Rif43 and then the BCAs were spotted on the medium. Inhibition zones were measured after 3 days of incubation at 28°C. Motility was also tested using a semi-solidified agar (3 g L⁻¹) medium poured into petri dishes⁹). BCAs were applied in the middle of the agar plate, and the diameter of the resulting spot was measured at 2-day intervals.

Disease suppression by BCA

(1) Climatron experiments

Pumice (100g dry basis) was put into a vinyl pot 9 cm diameter, inoculated with YU1Rif43 at a density of 5×10^4 cfu g⁻¹ pumice and supplied with different carbon sources at 0.1 g (xylose and sucrose) or 0.025 g (leucine and lysine). In the case of *Fusarium* diseases, the pumice was inoculated at a density of 10^5 bud-cells g⁻¹ for tomato and at a density of 3×10^5 bud-cells g⁻¹ for spinach. Pumice was added with CaCO₃. Then, six two-day-old tomato seeds were transplanted into the pot. The observation and maintenance of the plants were done in the same way as previously described.

(2) Greenhouse experiments

Tomato plants were grown in a greenhouse in two years: the first cultivation was done from September, 2006 to March, 2007 and the second cultivation from February, 2007 to July 2007. The first trial included four treatments: C- (without the pathogen), C+ (with the pathogen), G5.2rif1 (with the pathogen and strain G5.2rif1), G5.2rif1+sucrose. Twelve two-day-old tomato seeds were transplanted on September 7th, 2006 into a 0.05 m² pot (1/2000 a Wagner pot, Fujimonoto Science Technology Inc., Japan) containing 3.5 kg of pumice (equivalent to 7 L in volume) with 52.5 g of CaCO₃ and inoculated with strain G5.2rif1 and the pathogen at densities of 5×10^6 cfu g⁻¹ and 5×10^4 cfu g⁻¹, respectively. The pots were put on a heater mat (Takii Co.) to maintain the temperature in the pumice above 15°C. The twelve seedlings were thinned to 3 after one month, then to one after two months and cultivated up to 25 weeks after inoculation (WAI). Sucrose was added into each pot at 3.5 g pot⁻¹, equivalent to 1 g kg⁻¹ pumice, every two weeks from just after transplanting.

In the second cultivation, three treatments were prepared the same as far the first cultivation, except for G5.2rif1+sucrose. Tomato seeds germinated on February 15th, 2007 in a small biodegradable pot (Jiffy-Garden Series, Sakata Seed Co.) 8 cm in diameter and containing 100 g of pumice. For the treatment with G5.2rif1, the bacterium was inoculated into the pot at a rate of 5×10^6 cfu g⁻¹ at the same day. After seven weeks of cultivation in a greenhouse, the seedlings were transferred on April 5th, 2007 to a 0.05 m² pot containing 3.5 kg of pumice added with 52.5 g of CaCO₃ and inoculated with strain G5.2rif1 and the pathogen at densities of 5×10^6 cfu g⁻¹ and 5×10^4 cfu g⁻¹, respectively. For each treatment, five replicate pots containing two plants were prepared. After 3 weeks, two plants per pot were thinned to 1 plant and then cultivated up to 13 weeks after transplanting (WAT).

Watering was done using the Otsuka liquid fertilizer A formula two times a day to adjust the water content to around pH2. Fifty percent of the concentration of the liquid fertilizer was used until the first flowering and then 75% until the initial enlargement of fruits. The disease incidence was monitored every week and biocontrol efficacy was calculated using the following formula: biocontrol efficacy = [(disease incidence of control - disease incidence of treatment group) / disease incidence of control] × 100. At harvest, the quality and quantity of tomato fruits were determined.

(3) Measurement of tomato quality

Electric conductivity (EC) and sugar and vitamin C contents of the tomato flesh were determined to evaluate tomato quality. Slices of tomato fruit (3.0 g) were mixed with 15 ml of distilled water. After vigorous shaking for one minute on a Vortex-Genie 2 (M&S Instruments Inc., Tokyo, Japan), EC values were measured by a Twin Compact Meter (Horiba Ltd., Kyoto, Japan). For the determination of sugar content, one drop of the tomato homogenate was put on the prism of a Hand Refractometer (N-10E type, Atago Co. Ltd., Tokyo, Japan). For the determination of vitamin C, 10.0 g of the tomato flesh was added to 10 ml of 5% metaphosphoric acid (Wako Pure Chemicals Industries, Ltd.) and homogenized by a Mill mixer FM-50 (Sun Co., Osaka, Japan). An aliquot (1.0 ml) of the homogenate was dispensed into a 1.5 ml tube and centrifuged at 15000 $\times$ g for 1 minute and the supernatant was used for the determination of vitamin C using the vitamin C test kit (Merck, Germany) with a

reflectometer RQflex plus 10 (Merck, Germany). The measurements were done in six and five replicates in the 2006 and 2007 cultivations, respectively, which consisted of one to four tomato fruits per replicate.

Results

Screening and characterization of biocontrol agents for bacterial wilt of tomato

In the first screening, 54 combinations each of which contained 5 strains were tested for bacterial wilt of tomato and 9 combinations showed more than 50% reduction of disease incidence. In the second screening, individual strains were tested and 6 out of 45 strains suppressed bacterial wilt of tomato significantly (less than 50 % of disease index (DI), Table 1). Since it was found that the C13, G4 and G5 strains were mixtures of two strains, they were carefully purified again and further tested for the ability to suppress disease. In the third and fourth screenings, three and six isolates suppressed DI by more than 50%, respectively.

Some properties of the strains C13.1, G4.1, G5.1 and G5.2 were shown (Table 2) and they were nearest to *Burkholderia sacchari*, *Burkholderia pyrracinia*, *Burkholderia terricola* and *Burkholderia nodosa* (Fig. 1)

The following substrates are utilized by C13.1: adonitol, cellobiose, citric acid, D-arabinose, D-fructose, D-glucose, D-raffinose, D-xylose, lactose, leucine, lysine, maltose, rhamnose, sorbitol, sucrose and xylitol. The following carbon sources are not utilized: acetamide, acetic acid, aspartic acid, dulcitol, glutamic acid, ribose and trehalose. Strain G4.1 showed the following substrate utilization profile: positive (acetamide, adonitol, cellobiose, D-arabinose, D-fructose, D-glucose, D-raffinose, dulcitol, D-xylose, lactose, leucine, lysine, maltose, sorbitol, sucrose, trehalose and xylitol) and negative (acetic acid, aspartic acid, citric acid, glutamic acid, rhamnose and ribose). Strain G4.2 had quite a similar profile to G4.1 (data not shown). Strain G5.1 utilized adonitol, cellobiose, D-glucose, D-raffinose, rhamnose, sorbitol, sucrose and xylitol and but not acetamide, acetic acid, aspar-

Table 1. Suppression of bacterial wilt of tomato by BCAs isolated from Kalimantan soils.

Screening times	Disease index		
	2 nd	3 rd	4 th
Control+	100 a	97 a	61 a
C12	35 b	47 c	28 ab
C13	17 b	—	—
C13.1	—	61 b	11 b
C13.2	—	69 b	38 ab
E20	19 b	68 b	39 ab
G3	21 b	81 a	39 ab
G4	22 b	—	—
G4.1	—	65 b	6 b
G4.2	—	36 c	11 b
G5	40 b	—	—
G5.1	—	38 c	13 b
G5.2	—	61 b	6 b

Disease index at 30 d after inoculation is shown: 0, all healthy, 100: all dead. Values with the same letters are not significantly different (P=0.05) according to the protected Fisher's LSD test.

Table 2. Identification of biocontrol agents and analysis of the suppression mechanisms.

Strain	Identification a	Similarity	Origin	Antibiosis	Competition	Motility
C13.1	<i>Burkholderia sacchari</i>	100%	Agricultural peat	–	nd	nm
G4.1	<i>B. pyrrocinia</i>	100%	Burn forest	+	+	m
G4.2	nd	nd	Burn forest	+	+	m
G5.1	<i>B. terricola</i>	100%	Burn forest	–	+	nm
G5.2	<i>B. nodosa</i>	100%	Burn forest	–	+	m

a, identification according to 16S rDNA sequencing and fatty acid analysis.

Nd, not determined; m, motile; nm, nonmotile; +, antibiosis/competition; –, no antibiosis/competition

Fig. 1. Phylogenetic tree based on the 16S rDNA gene sequence analysis of peat soil bacteria scale bare indices 10% sequences divergence. Strain names are preceded by their Gen Bank accession numbers and the date of sample collection is written in parentheses. DNA sequences of some control strains were obtained from the DDBJ database.

Table 3. Competition between *B. nodosa* G5.2rif1 and *R. solanacearum* YU1Rif43 in different media (nutrient broth, glucose and sucrose media).

Treatment	Population density of YU1Rif43 after 3 d incubation in		
	Nutrient broth ($\times 10^8$ cfu ml ⁻¹)	Glucose ($\times 10^6$ cfu ml ⁻¹)	Sucrose ($\times 10^7$ cfu ml ⁻¹)
Pathogen alone (5×10^4 cfu ml ⁻¹)*	5.6 a	2.7 a	5.4 a
Pathogen+G5.2rif1 (5×10^7 cfu ml ⁻¹)	1.6 b	0.1 b	2.5 b
Pathogen+G5.2 (5×10^6 cfu ml ⁻¹)	2.3 b	0.4 b	4.3 b

*(): the initial density of the isolate. Values with the same letters are not significantly different ($P=0.05$) according to the protected Fisher's LSD test.

tic acid, citric acid, D-arabinose, sucrose, dulcitol, D-xylose, glutamic acid, lactose, leucine, lysine, maltose, ribose and trehalose. The following substrates were utilized by strain G5.2: cellobiose, D-arabinose, D-glucose, D-raffinose, D-

xylose, lactose, leucine, lysine, maltose, rhamnose, sorbitol, sucrose, trehalose and xylitol, while the following were not utilized: acetamide, acetic acid, adonitol, aspartic acid, citric acid, D-fructose, dulcitol, glutamic acid and ribose.

Table 4. Competition between *B. nodosa* G5.2rif1 and *F. oxysporum* f.sp. *lycopersici* 880621a, race 2 (FOL) in nutrient broth.

Treatment	Population density of FOL after 3 d incubation ($\times 10^3$ cfu ml ⁻¹)
FOL alone (10^5 cell ml ⁻¹)*	80 a
FOL+G5.2 (5×10^7 cfu ml ⁻¹)	6 b
FOL+G5.2 (5×10^6 cfu ml ⁻¹)	8 a

*(): the initial density. Values with the same letters are not significantly different ($P=0.05$) according to the protected Fisher's LSD test.

Table 5. Effect of *B. nodosa* G5.2rif1 and carbon sources on the disease index of bacterial wilt of tomato, and Fusarium wilt of tomato and spinach in small-pot experiments.

	BWT		FWT	FWS	
	1st ^x	2nd ^x	1st ^z	1st ^x	2nd ^y
Control+(Pathogen alone)	65a	67a	83a	50a	78a
+G5.2rif1	11b	44b	24b	47b	
+Sucrose	61a				
+G5.2rif1+sucrose	6cd	0b	89a		
+G5.2rif1+leucine	33b				
+G5.2rif1+lysine	15bc				
+G5.2rif1+xylose	17bc				

BWT=bacterial wilt of tomato; FWT=Fusarium wilt of tomato; FWS=Fusarium wilt of spinach. Disease index are the values at x=30 d after inoculation (dai); w=24 dai; y=40 dai and z=46 dai. Values with the same letters are not significantly different ($P=0.05$) according to the protected Fisher's LSD test. 1st and 2nd indicate repeated experiments.

Strain C13.1 showed C16:0 (29.4%), C16:1 (29.6), C17:0 cyclo (3.5%), C18:1 ω 7c (20.5%), and C19:0 ω 8c cyclo (3.9%). Strain G4.1 showed C14:1 (18.1%), C14:0-3OH (5.9%), C16:0 (31.5%), C16:1 (19.6%), C18:0 (1.5%), C18:1 ω 7c (14.3%), and C19:0 ω 8c cyclo (3.3%). Strain G5.1 showed C14:0 3OH (5.5%), C14:1 (24.7%), C16:0 (22.4%), C16:1 ω 7c (13.2%), C17:0 cyclo (3.0%), C18:1 ω 7c (23.2%) and C19:0 ω 8c cyclo (3.7%). Strain G5.2 showed C14:0 3-OH (7.7%), C16:0 (41.5%), C16:1 (22.4%), C17:0 cyclo (2.9%), C18:1 ω 7c (19.6%) and C19:0 ω 8c cyclo (3.6%).

Competition for substrates

When *R. solanacearum* YU1Rif43 was inoculated into 1/10 NB, glucose and sucrose media simultaneously with *B. nodosa* G5.2rif1, the population of YU1Rif43 significantly

Table 6. Effect of *B. nodosa* G5.2rif1 and sucrose on the disease index of bacterial wilt of tomato and population density of the BCA and the pathogen in a small-pot experiment

Treatment	Disease index	Population density of (log cfu g ⁻¹ root)	
		YU1Rif43	G5.2rif1
Control+(Pathogen alone)	100 a	8.6 a	–
+G5.2rif1	67 ab	5.3 b	4.7 b
+Sucrose	89 a	8.6 a	–
+G5.2rif1+sucrose	50 b	6.4 b	6.4 a

Disease index was measured at 30 days after seeding/inoculation and the population density at 24 days. Values with the same letters are not significantly different ($P=0.05$) according to the protected Fisher's LSD test.

decreased in the presence of the competitor (Table 3). The number of *F. oxysporum* f. sp. *lycopersici* 880621a also significantly decreased in the presence of *B. nodosa* G5.2rif1 (Table 4).

Biocontrol ability of BCA

When carbon sources (sucrose, xylose, leucine and lysine) were added into a pumice medium in combination with *B. nodosa* G5.2rif1, addition of all the substrates markedly decreased BWT (Table 5). The combination with sucrose was most effective and the efficacy was confirmed a further two times (2nd in Table 6 and Table 7). The population density of the pathogen was significantly lower in the presence of *B. nodosa* G5.2rif1, while the population density of *B. nodosa* G5.2rif1 was higher with sucrose (6.4 log cfu g⁻¹ root) than on single inoculation (4.7 log cfu g⁻¹ root) (Table 6). In contrast, the combination of sucrose and *B. nodosa* G5.2rif1 did not suppress FWS, while the single inoculation of *B. nodosa* G5.2rif1 significantly suppressed FWS (Table 5).

In the greenhouse experiments, no inhibitory effects were found on the growth, yield and quality of tomato for a single inoculation of G5.2rif1 or a combination of *B. nodosa* G5.2rif1 with sucrose (Table 7). In 2006, the tomato yield was approximately 1600 g plant⁻¹ in the control –, *B. nodosa* G5.2rif1 and *B. nodosa* G5.2rif1+sucrose treatments, while it was only 800 g plant⁻¹ in the control+treatment and the differences were highly significant. This result was confirmed in the 2007 greenhouse experiment, in which tomato yield

Table 7. Effect of *B. nodosa* G5.2rif1 on the disease index of bacterial wilt of tomato and quality of tomato fruit in greenhouse experiments

Treatment	Disease index	Height* (cm)	Yield (g/plant)	EC (mS/cm)	Sugar (%)	Vit. C (mg/100g fruit)
<i>First time cultivation 2006 Sep to 2007 Mar</i>						
No pathogen (Control–)	0 c	171	1557 a	1.1	7.1	34
Control+(Pathogen alone)	62 a	153	883 b	1.0	7.3	33
+G5.2rif1	42 b	134	1783 a	1.2	7.5	33
+G5.2rif1+sucrose	13 c	172	1351 a	1.1	7.4	33
<i>Second time cultivation 2007 Feb to 2007 Jul</i>						
No pathogen	0 c	119	849 a	1.5	7.9	26
Control+	35 a	116	347 b	1.6	8.4	26
+G5.2rif1	10 b	122	675 a	1.6	8.7	25

EC, electric conductivity; Vit., vitamin C; * at 25 and 13 weeks after transplanting in 2006 and 2007, respectively. Values with the same letters are not significantly different ($P=0.05$) according to the protected Fisher's LSD test.

was about 760 g plant⁻¹ in the control – and *B. nodosa* G5.2rif1 treatments, but 340 g plant⁻¹ in the control+treatment. In the measurement of the quality of tomato, there were no significant differences in the EC values, or sugar and vitamin C contents of tomato fruits among the control –, *B. nodosa* G5.2rif1 and *B. nodosa* G5.2rif1+sucrose treatments in either year.

Discussion

Among 270 crystal violet tolerant bacterial isolates from soils of Kalimantan, Indonesia, five strains (C13.1, G4.1, G4.2, G5.1 and G5.2) showed good suppression of bacterial wilt of tomato caused by *R. solanacearum* in small pot experiments. The strains C13.1, G4.1, G5.1 and G5.2 were phylogenetically closest to *Burkholderia sacchari*, *B. pyrrocinia*, *B. terricola*, and *B. nodosa*, respectively. We checked by 16S rDNA sequences in 4 species but we did not sequence one isolate (G4.2), because in term of colony and substrate utilization of G4.1 and G4.2 isolates were similar. Fatty acid methyl ester profiles of strains C13.1, G4.1, G5.1 and G5.2 were compared with those of related *Burkholderia* species such as *B. sacchari*⁴⁾, *B. pyrrocinia*³²⁾, *B. terricola*¹¹⁾, *B. nodosa*⁶⁾, *B. ferrariae*³⁸⁾, *B. unamae*⁵⁾ and *B. kururiensis*⁴⁴⁾. The fatty acid profiles of strains C13.1, G4.1 and G5.1 were similar to those of *B. sacchari*, *B. pyrrocinia* and *B. terricola*, respectively. Strain G5.2 was phylogenetically closest to *B. nodosa*, but it was also close to *B. ferrariae* (Fig. 1). C17:0 cyclo is major fatty acid in *B. ferrariae*³⁸⁾, but not in *B. nodosa*⁶⁾. Thus, the fatty acid profile of strain G5.2 was considered to be more similar to that of *B. nodosa*. Carbon source utilization patterns supported these identifications: 70% homology between G5.2 and *B. nodosa*, 27% between G5.2 and *B. ferrariae*, 71% between G5.1 and *B. terricola*, 85% between G4.1 and *B. pyrrocinia*, and 60% between C13.1 and *B. sacchari*.

de Souza and Raaijmakers⁸⁾ reported that *B. pyrrocinia* produces pyrrolnitrin which is a broad spectrum and chlorinated phenylpyrrole antibiotic first isolated from *B. pyrrocinia*. In this study, *B. pyrrocinia* G4.1 also showed antibiosis against *R. solanacearum*. *B. pyrrocinia* belongs to the *B. cepacia* complex and *B. cepacia* has been recognized as an important opportunistic human pathogen, especially among cystic fibrosis patients³⁰⁾, although strains belonging to *B. cepacia* have often been identified as BCAs³³⁾. *B. pyrrocinia* and *B. terricola* have the ability to suppress plant disease^{24,25)}. But there are no reports for the remaining 2 species *B. sacchari*, and *B. nodosa* as biocontrol agents. Goris *et al.*¹¹⁾ reported that a bacterium belonging to *B. terricola* has genes for degrading 2,4-dichlorophenoxyacetic acid (2,4-D). Brämer *et al.*⁴⁾ reported that *B. sacchari* strain IPT101, isolated from a sugar-cane plantation soil in Brazil, was a producer of polyhydroxyalkanoates from sucrose and other carbon sources. *Burkholderia nodosa*, a new member of the genus *Burkholderia*, was originally isolated from woody legumes native in Brazil⁶⁾. In some culture collections, such as American Type Culture Collection, (ATCC), Collection de l'Institut Pasteur (CIP) and Deutsche Sammlung Von Mikroorganismen und Zellkulturen (DSMZ), all strains belonging to the species *B. sacchari*, *B. terricola* and *B.*

nodosa were originally isolated from soil but none from medical specimens, suggesting the potential application of these species in fields. For further experiments, strain G5.2 was selected and a rifampicin resistant mutant was obtained, since strain G4.1 which also showed good suppression of BWT like G5.2 belonged to the *Burkholderia cepacia* complex.

The inoculation of *B. nodosa* G5.2rif1 into a pumice medium decreased the wilt symptoms of not only BWT, but also FST and FSW (Table 1 and Table 5). The reduction of bacterial wilt by *B. nodosa* G5.2rif1 was 33–84% in the climatron experiments. The protective effects of this BCA were almost the same as in previous reports, e.g. BCAs reduced the disease in banana, eggplant and tomato by 36–95%²⁾, in tomato by 66–94%¹²⁾, and 27–82%⁴⁵⁾. We further tested the efficacy of *B. nodosa* G5.2rif1 to BWT in greenhouse experiments, in which *B. nodosa* G5.2rif1 significantly increased the yield of tomato both in 2006 and 2007 experiments and significantly decreased BWT in 2007, compared to that in the control inoculated with the pathogen alone (Table 8). Since *B. nodosa* G5.2rif1 did not have any negative effect on plant height and sugar content and EC value of the tomato fruits, it was considered a good biocontrol agent.

Burkholderia nodosa G5.2rif1 did not show inhibition of to *R. solanacearum* and *F. oxysporum* f. sp. *lycopersici* 880621a (Table 2), suggesting that antibiosis was not involved in the suppression. Kamilova *et al.*¹⁷⁾ reported that *Pseudomonas fluorescens* 1751 which does not possess antifungal activities, such as lipase, β -glucanase, cellulase, chitinase, autoinducer, biosurfactant, hydrogen cyanide, and indole-3-acetic acid, reduced root rot symptoms caused by *F. oxysporum* f. sp. *radicis-lycopersici* through competition for nutrients and niches. In this study, the population density of *R. solanacearum* YU1Rif43 was significantly decreased by the co-inoculation of *B. nodosa* G5.2rif1 into all the media used (1/10 NB, glucose and sucrose media) (Table 3), suggesting that competition for nutrients was at least one of the main mechanisms by which G5.2rif1 suppressed bacterial wilt disease. Suppression of plant diseases through competition for nutrients between BCA and a pathogen has been reported. Van Dijk and Nelson³⁹⁾ reported that *Enterobacter cloacae* suppressed *Pythium ultimum* sporangium germination and damping-off through competition for nutrients. Kamilova *et al.*¹⁸⁾ found that a BCA, *Collimonas fungivorans*, controlled tomato foot and root rot (TFRR) mainly through competition for nutrients and niches, rather than mycophagous activity. In this study, the contribution of induced systemic resistance was not tested as a suppressive mechanism of strain G5.2rif1 and thus remains to be further evaluated.

In this study, to enhance the suppressive properties of the BCAs, different kinds of organic compounds were selected based on two criteria; utilizability by the BCAs but not by the pathogen and economical considerations. The addition of leucine, lysine, xylose (substrates not utilized by the pathogen) or sucrose (utilizable by the pathogen), along with *B. nodosa* G5.2rif1, markedly suppressed BWT (Table 5). Among them, the sucrose treatment was the most suppressive, although the addition of sucrose alone was not suppressive, suggesting that *B. nodosa* G5.2rif1 utilized sucrose more efficiently and developed its population and thereby

suppressed BWT. The efficacy of sucrose in combination with *B. nodosa* G5.2rif1 was further confirmed in a greenhouse experiment (Table 7), in which a significant reduction of disease incidence was observed with G5.2rif1+sucrose compared to that obtained with a single inoculation of G5.2rif1. In contrast, a combination of *B. nodosa* G5.2rif1 with sucrose did not show enhanced suppression for FWT and FWS (Table 5). Hoffland *et al.*¹³ explained that disease susceptibility is highly pathogen-specific and is probably dependent on differences in resource requirements of the pathogen, or the sensitivity of the pathogen to plant resistance reactions, or both these factors.

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