Utilization of Root-Colonizing Bacteria to Protect Hot-Pepper Against *Tobacco Mosaic Tobamovirus*

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Tobacco Mosaic Tobamovirus (TMV) is one of many important viruses infecting Solanaceous plants including hot pepper in Indonesia. To accomplish and improve the effectiveness of virus management, we used root-colonizing bacteria (rhizobacteria) isolated from healthy hot pepper. Eight rhizobacteria isolates were selected and their capacity in enhancing plant growth and inducing systemic resistance (ISR) against TMV in greenhouse trials were evaluated. The rhizobacteria was applied as seed treatment and soil drench. Bacterized-seedling showed a better growth vigor, fitness and a milder symptom than non-bacterized control plants. The protective effect of rhizobacteria was more pronounced after challenging inoculation by TMV, especially for plants treated by isolates I-6, I-16, and I-35. However, TMV accumulation was slightly affected by bacterial treatment. The rhizobacteria might improved ISR by increasing peroxidase enzyme activity but this depends on the species. Based on whole results, isolate I-35 was the potential plant growth promotion rhizobacteria (PGPR). The I-35 was identified as *Bacillus cereus* based on morphological characteristics and nucleotide sequences of 16S r-RNA.

Key words: root-colonizing bacteria, TMV, ISR

INTRODUCTION

Hot-pepper is one of the important crops in Indonesia and also several countries in Asia such as Malaysia, India, Pakistan, Bangladesh, China, and Singapore. Several of the production constraint factors are pests and diseases. The main viral diseases infecting hot-pepper are *Chilli Veinal Mottle Virus* (ChiVMV), *Pepper Veinal Mottle Virus* (PVMV), *Pepper Mottle Virus* (PeMV), *Pepper Severe Mosaic Virus* (PeSMV), and *Cucumber Mosaic Virus* (CMV) (Dolores 1996). In Indonesia, ChiVMV, CMV, TMV, and recently *Geminivirus* are important viruses infecting hot-pepper (Sulyo *et al.* 1995; Duriat 1996; Sulandari 2004). Duriat (1996) reported that TMV infected not only hot pepper, but also infected tomato, tobacco, and egg plant in Indonesia.

TMV is a plant virus spread worldwide and infects many horticulture crops. As a member of *Tobamovirus*, TMV genome contains a single-stranded RNA (ssRNA) with rod-shaped and fairly uniformly sized particles. TMV caused heavy yield losses on tobacco, tomato, and pepper worldwide (Sutic *et al.* 1999; CABI 2005).

Studies in controlling the TMV infection were conducted intensively on tobacco, by using resistant cultivars, cultural control, sanitary method, and biological control by using satellite TMV or by cross protection using avirulent or attenuated strain of TMV (CABI 2005). Recently, Shin *et al.* (2002) reported that they have constructed transgenic pepper successfully by transferring the coat protein (CP) gene of

ToMV (*Tomato Mosaic Virus*) into pepper plant to develop virus-resistant hot-pepper.

Management strategies to control plant viruses in Indonesia were limited on the use of resistant cultivars and culture practice methods. Most farmers rely on chemical insecticides to control the insect vectors. To minimize the use of pesticides and to improve the effectiveness of virus disease control, utilizing of beneficial microbes isolated from plant rhizosphere referred as Plant Growth Promoting Rhizobacteria (PGPR) might offer a promising viral diseases control method. PGPR is defined as root colonizing-bacteria living in the rhizosphere, and distributes on plant root or its close vicinity. Some of these rhizobacteria are beneficial to the plant in direct or indirect way, resulting in a stimulation of plant growth (Bloemberg & Lugtenberg 2001).

PGPR have various ability to induce systemic resistance in plant which provides protection against a broad spectrum of plant pathogens and is reffered as induce systemic resistance (ISR). ISR pathway is induced when plant is challenged by pathogenic organisms (Bloemberg & Lugtenberg 2001). Some PGPR such as *Pseudomonas fluorescens* strain CHAO is effective to control *Tobacco necrosis viru*s (TNV) on tobacco (Maurhofer *et al.* 1994), *P. aeroginosa* strain 7NSK against TMV on tobacco (De Meyer *et al.* 1999), *Bacillus subtilis* IN937b and *B. pumilus* strain SE34 against *Tomato Mottle Virus* (ToMoV) and against CMV on tomato (Murphy *et al.* 2000; Murphy *et al.* 2003). The elevated resistance due to an inducing agent upon infection of pathogen; ISR is expressed upon subsequent or challenge inoculation with pathogen (van Loon 1997; van Loon *et al.* 1998; Ramamoorthy *et al.* 2001).

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Mechanism of ISR mediated by PGPR is through the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reactions of the host leading to the synthesis of defence chemicals against the challenging pathogen (reveiwed by Ramamoorthy *et al.* 2001). Furthermore, ISR mediated by PGPR is associated with the pathogenesis-related (PR) proteins (Benhamou *et al.* 1996; Viswanathan & Samiyappan 1999a), synthesis of phytoalexin and other secondary metabolites (Van Peer *et al.* 1991), and the increasing activity of pathogenesis-related peroxidase and chitinase protein (Viswanathan & Samiyappan 1999a, b; Ramamoorthy *et al.* 2002). It showed that the use of PGPR is one of promising approaches in controlling plant viruses.

In Indonesia, the availability of hot-pepper resistant cultivars against either pest or disease are limited. To improve the effectiveness of management of viral diseases, the utilization of beneficial microorganisms such as rhizobacteria needs to be explored. Studies on PGPR in Indonesia as a bio-control agent to control pathogens especially plant viruses were not explored intensively. Exploration of beneficial rhizobacteria eliciting ISR and utilize them more frequently than chemicals, will be useful in Indonesia agriculture. Hence, the objective of this project was to select the ISR eliciting rhizobacteria protecting hot pepper against TMV.

MATERIALS AND METHODS

Rhizobacteria Isolates. Rhizobacteria were isolated from healthy rhizosphere of hot pepper cultivated at Darmaga, Bogor, West Java, Indonesia and was cultured on Tryptic Soy Agar (TSA, Difco, USA). Eight isolates rhizobacteria were used: I-1, I-6, I-8, I-16, I-25, I-35, II-5, II-10, and were evaluated based on their ability to enhance plant growth and their ability to protect hot-pepper against TMV infection.

Identification of Rhizobacteria. The potential candidate as a PGPR was identified using Microbact Kit (Medvet Science Pty, Ltd. Australia). Further identification was combined with sequencing the 16S r-RNA. The primers were specific for prokaryote 16S-rRNA with the forward primer 63f (5'-CAGGCCTAACACATGCAAGTC-3') and the reverse primer 1387r (5'-GGGCGGWGTGTACAAGGC-3') as described by Marchesi *et al.* (1998).

The homology and similarity of the nucleotide sequences were analyzed usingWU-Blast2 software providing by EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute).

TMV Inoculum. The TMV was propagated on tobacco plant (*Nicotiana tabacum*). After being dusted gently by Carborundum 600 mesh (Nacalai Tesque, Japan) the plant was inoculated by infected pepper leaves sap. Infected tobacco leaves were harvested at 10-14 days after infection, then stored in freezer at -80 °C for further experimental use.

Growing Conditions and Rhizobacteria Treatment. The experiments were conducted in a greenhouse to evaluate the rhizobacteria ability as PGPR to protect hot pepper plants against TMV. Hot pepper seeds (*Capsicum annuum* L. var. TM 999) were soaked in different rhizobacteria suspension (10^9 cfu/ml) as treatments for 4 hours, and control seeds were soaked in sterile water. Seeds were then directly sown to sterile growth medium (soil type Latosol: cow dung manure = 2:1), without fertilizer application, and watered with tap water routinely.

Two weeks after seedling, plants were transplanted into pots. A week after transplanting, 1 ml (10^9 cfu/ml) of rhizobacteria suspension was added to pots as soil drench treatment.

Plants were grown in greenhouse with humidity and temperature depends on the natural conditions. The experimental design used in the experiments was randomized complete design with six plants per treatment and three replicates.

Virus Inoculation. Plants were mechanically inoculated with infected plant sap (1:10 w/v) in phosphate buffer pH 7.0 (Merck, Germany) at two weeks post transplanting to the pots. The first two leaves on each plant were gently dusted with Carborundum 600 mesh (Nacalai Tesque, Japan) prior to rub-inoculation with sap containing TMV.

Evaluation of Plant Growth Characters. To examine the effect of rhizobacteria on the plant growth characteristics, each plant height was measured from soil line to shoot apex taken one day prior to inoculation with TMV and eight week post inoculation (wpi). Another growth characteristics were number of flowers/fruits (taken as single measure) at 6-8 wpi and fresh weight of tissues were counted on each plant at the end of experiments. The growth characters data obtained from three replicates.

Disease Assessments. Disease severity rating was made by using the following rating scale on the leaves adopted from Murphy *et al.* (2003): 0 = no symptoms, 2 = mild mosaic symptoms, 4 = severe mosaic symptoms, 6 = mosaic and deformation, 8 = severe mosaic and severe deformation, and 10 = severe mosaic and deformation with stunted growth. Disease severity rating evaluation was performed with mock inoculated plants of treatment as a standard.

Accumulation of TMV in foliar tissues were determined by double antibody sandwich Enzyme-linked immunosorbent assay (DAS-ELISA). Leaves were sampled at 2 and 4 wpi by collecting of the youngest leaflet from young non-inoculated leaves. ELISA procedure are carried out as manufacture's recommendation (DSMZ; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany).

TMV accumulation was quantitatively measured by using ELISA reader at 405 nm. Positive samples was considered for the presence of TMV when absorbance value was twice of accumulation of healthy control samples.

Extraction and Quantification of Peroxidase Enzyme Activities. To test the effect of bacterized-treatment on plants, peroxidase (PO) enzyme activity was measured by using spectrophotometer. Extraction and quantification of PO enzyme activities were conducted at 1 week post inoculation (wpi) according to method described previously (Hammerschmidt *et al.* 1982) with minor modification. Half gram of composite samples of each treatment was added with 1.5 ml of 0.1 M phosphate buffer pH 7.0 (Merck, Germany) at 4 °C and ground in mortar. The sap was put in the 1.5 ml tubes, then centrifuged at 16,000 g for 15 minutes and the supernatant was used as the enzyme source.

The PO enzyme activity was quantified after addition of 1.5 ml of 5 molale pyrogallol and 0.5 ml of 1% hydrogen peroxide (H_2O_2) into the supernatant. The reaction mixture was incubated at room temperature and the absorbance was counted using spectrophotometer at 420 nm with interval of 30 seconds for 3 minutes. The enzyme activity was expressed as a change in absorbance (min^{-1} mg^{-1} protein). The total protein was measured by using Bradford reagent with bovine serum albumin (BSA; Sigma Aldrich, USA) as a standard. PO enzyme was extracted from leaf samples of each treatment as composite samples from three experiments.

Data Analysis. All data were analyzed by analysis of variance (ANOVA) and the treatment means were separated by using Duncan's Multiple Range test (DMRT) (p = 0.05) using SAS software version 6.13 (SAS Institute, Gary, NC, USA).

RESULTS

Plant Growth Characteristics in Response to Rhizobacteria and TMV. Four tested bacterial isolates (I-6, I-8, I-16, and I-35) showed their ability to enhance plant growth, while plant height was slightly different between bacterized-treated and non-bacterized control plants. Bacterized-treated plants showed vigor, fitness and leaf size visually greater than non-bacterized control plants (Damayanti, unpublished data). The differences were more visible when bacterized plants challenge inoculated with TMV. At 8 wpi, plants treated with isolates I-16, I-25, and I-35 showed significantly different (p = 0.0016) in height and vigor than those of non-bacterized control plants, while plant treated with I-1, I-8, and II-10 did not showed any difference with non-bacterized control plants respectively (Figure 1).

Number of flower and fruits of healthy bacterized-plants fewer than control plants, however the flowers of control plants were fallen off severely lead the number of fruits fewer than bacterized plants. When plants challenge inoculated with TMV, bacterized-plants still could produce more flowers/fruits greater than non-bacterized control plants (Figure 2).

The fresh weight of healthy bacterized plants within some treatment tend to be higher, however the difference was not significant (p = 0.5756). The fresh weight difference was showed by plants treated with I-35 and I-16, respectively. Similar results with addition I-25 were shown after plants challenge inoculated with TMV (Figure 3).

The results showed that some bacterial treatments able to induce plant growth. Furthermore, some of bacterial treatment could maintain better plant growth characters than non-bacterized control plants even when infected by TMV (Figure 1, 2, 3).

Diseases Assessments. The incidence of TMV range from 66.7-100% with initial mosaic symptom presence in control plants at 4-5 dpi, whereas bacterized-plants mostly remained symtompless at that time especially plants treated with I-6, I-16, and I-35. The bacterized-plants exhibited phenotype mosaic symptom at 10-14 dpi with symptom less severe than control plants, indicating rhizobacteria treatment delayed the incubation time and symptom expressions.

Furthermore, all bacterized-plants showed lower severity than control, especially plants treated with I-6, I-16, and I-35 (Table 1). In addition, some of plants treated with I-6, I-16, and I-35 treatment remained symptompless until the end of the experiment leading to lower incidence than non-bacterized control.

However, the symptom severity did not parallel with the TMV accumulation on the basis of ELISA absorbant value. The mean ELISA absorbance values for the plants infected with TMV were high at 2 wpi and decreased at 4 wpi. At 2 wpi, all ELISA absorbance of bacterized-plants except for plants treated with I-1, I-8, II-5, and II-10 was different and the lowest absorbance value showed by plants treated with I-6 isolates. At 4 wpi showed the TMV accumulation decreased than non-bacterized control, even not different significantly (p = 0.4638), except absorbance value of plants treated with I-6 (Table 1).
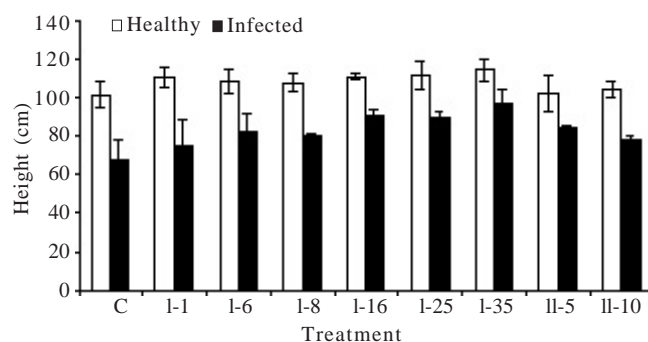

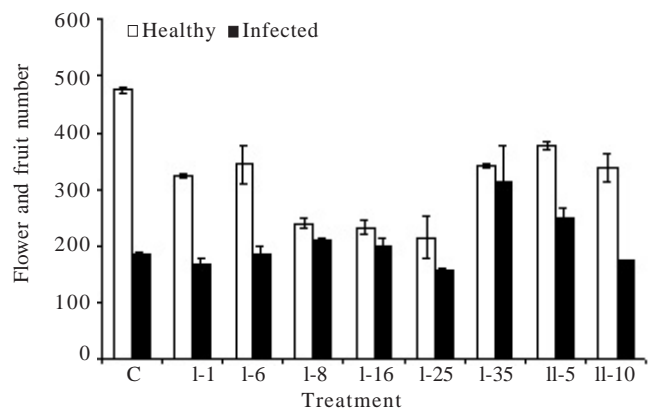Figure 1. Plant height of healthy and TMV infected plants.

Figure 2. Number of flower and fruits of healthy and TMV infected plants.
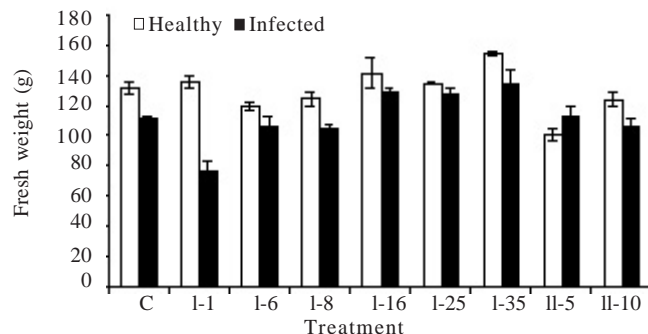

Figure 3. Total fresh weight of healthy and TMV infected plants.

Table 1. Enzyme-linked immunosorbent assay (ELISA) values, and severity of hot pepper treated with rhizobacteria and challenged with TMV

Treatment	ELISA Values*		Severity**
	2 wpi	4 wpi	
Control	2.283 ± 0.004[a]	2.235 ± 0.088[a]	8.0 ± 0.0[a]
I-1	2.133 ± 0.005[a]	1.878 ± 0.361[ab]	6.0 ± 0.0[ab]
I-6	0.680 ± 0.014[e]	0.958 ± 0.495[b]	2.7 ± 3.1[b]
I-8	2.202 ± 0.005[ab]	1.589 ± 0.867[ab]	4.7 ± 1.2[ab]
I-16	2.005 ± 0.027[d]	1.550 ± 0.644[ab]	3.3 ± 3.1[b]
I-25	2.106 ± 0.057[c]	1.448 ± 0.931[ab]	4.7 ± 1.2[ab]
I-35	2.116 ± 0.035[bc]	1.592 ± 0.741[ab]	2.7 ± 2.3[b]
II-5	2.282 ± 0.010[a]	1.821 ± 0.653[ab]	5.3 ± 1.2[ab]
II-10	2.235 ± 0.088[a]	1.590 ± 0.908[ab]	4.7 ± 1.2[ab]

*Absorbance value of ELISA at wavelength 405 nm, Positive result of ELISA test = absorbance value is twice of the healthy absorbance. The means of healthy absorbance at 2 wpi = 0.309, and at 4 wpi = 0.285; **Means followed by different letters within a column represent a significantly different (á = 0.05) by DMRT
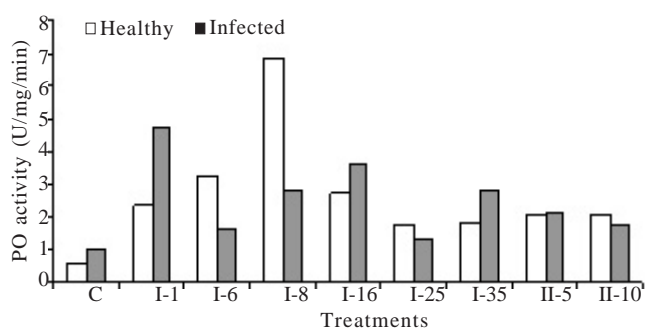

Figure 4. Peroxidase enzyme activity of bacterized-and non-bacterized plants either healthy (white boxes) or challenge inoculated with TMV (black boxes).

The bacterial treatments increased the peroxidase (PO) enzyme activity in comparison to non-bacterized control (Figure 4). After challenge inoculation with TMV some of bacterial treatments increased the PO activity higher than healthy plants (Figure 4).

Identification of Rhizobacteria. Based on the plant growth characters and disease assessments, the potential candidates as PGPR were the isolate I-6, I-16, and I-35. The I-6 and I-35 were gram-positive, whitish colony, produces spores in the center of the cell, and rod shape. The I-16 was gram-negative, whitish colony with rod shape. The nucleotide sequencing of the 16S r-RNA showed the I-6 has 99% nucleotide homology to *Bacillus* sp., the I-35 has 100% homology to *Bacillus cereus*, and I-16 has 99% homology to *Brevibacterium sanguinis*. The I-16 and I-35 were deposited in DDBJ (DNA Database of Japan) with accession no.AB288106 and AB288105.

DISCUSSION

Some of the rhizobacteria isolates used in this study could enhance growth of hot pepper TM-999 resulting of plants vigor and fitness greater than control treatment to some extent. However, the role of rhizobacteria either as growth promoter or as a plant systemic resistance inducer seemed affected by greenhouse environment conditions. Since the humidity and temperature being uncontrolled and mostly extremely higher than compared to that of in nature. It affects the biological

activity of the rhizobacteria. The high temperature and humidity caused specific abiotic stress for either plants or rhizobacteria as seen on the blossom flowers. The optimum temperature range for hot pepper growth is 24-28 ºC, while higher temperature affected to the blossom and fruit production (Widodo 2002). In these trials the average of daily temperature was above 32 ºC. Hence, all blossom flowers could not develop into fruits, due to flowers fallen off soon after the blossom especially for the non-bacterized control plants. However, many flowers from bacterized-plants produced more fruits than control plants even the flower numbers lower than control (Figure 2).

The effectiveness of biological control using microorganism such rhizobacteria depends on crucial factors such environment condition and soil type. However, some of isolates showed their ability to enhance plant growth subsequent to virus inoculation resulted in milder symptom and some of plants remained symptomless. The protection afforded rhizobacteria-treated plants resulted from the enhancement growth of hot pepper, thereby allowing them to respond to inoculation with TMV. This suggested that rhizobacteria treatment for some extend able to induced plant systemic resistance to overcome TMV infection on hot pepper TM-999.

Zehnder *et al.* (2000) previously evaluated the application of *B. subtilis* IN937b, *B. pumilus* SE34, and *B. amyloliquefaciens* IN937a against CMV on tomato. The treatment with those *Bacillus* strains resulted in reduction of severity even the virus titer in the plants was not affected by bacterial treatment; ELISA values as indication of viral titer within the plant was not changed by bacterial treatment. Similar results was shown on TMV in these experiments. It was indicated that rhizobacteria treatment might not prevent TMV replication. Bacterial treatment might affect the movement of virus and/or the symptom expressions. Alternatively nutritional factors especially nitrogen levels might serve to offset or mask the symptom. This masking symptom may play role during early stage of systemic infection of rhizobacteria treated plants by TMV when symptoms were delay or not apparent, even though virus accumulation was similar to that of control plants as previously reported by Murphy *et al.* (2003) against CMV on tomato.

Some of bacterized-plants increased the PO activity after TMV inoculation, while others were not. It suggested that some of rhizobacteria might able to enhanced plant's defense response through elevated PO activity (I-1, I-16, I-35, II-5), while others might PO-independent. The role of polyphenol oxidase enzyme and peroxidase oxidizes phenolics to quinones and generates hydrogen peroxide (H_2O_2). H_2O_2 is an antimicrobial, also releases highly reactive free radicals and further increases the rate of polymerization of phenolic compound into lignin-like substances. These substances are then deposited in cell walls and papillae and interfere with the further growth and development of pathogen (Hammond-Kosack & Jones 1996; Agrios 2005). The result was suggested that some of rhizobacteria isolates (I-16 and I-35) are able to activate the plant's defense response of virus leads to the greater degree of resistance might be by increasing the PO

activities, while others might be by PO-independent. However, the increasing of PO activities did not prevent the TMV accumulation, suggested the PO elicit plant's defense response at the early of infection stage rather than viral suppression. Alternatively, the disease suppression afforded by rhizobacteria treatment might be caused by enhancement of plant growth which made plants could increase plant resistance to overcome the virus infection by ISR with PO-independent mechanism which was not covered from these experiments.

Bacillus spp. can promote crop health and some strains expressed activities that suppress pests and pathogens (Gardener 2004). In most cases, *Bacillus* spp. that elicit ISR typically elicit plant growth promotion (Kloepper *et al*. 2004) and our results also supported the previously reports (reviewed in Kloepper *et al*. 2004).

Bacillus cereus was previously reported had activities to suppress pests and pathogens or promote plant growth, while *Brevibacterium* genera had not been reported yet as PGPR. This finding extended the role of *Brevibacterium* in plant disease suppression. Treatment hot pepper seeds and plants with these rhizobacteria might improved the hot pepper health and its productivity through the promotion of host nutrition and growth and stimulation of plant host defenses rather than antagonism (Figure 1-4). The *B. cereus* treatment was able to protect hot pepper and maintained plant growth and production even plants being infected by TMV. Among the three species, the *B. cereus* was the best potential candidate as PGPR for protecting hot pepper against TMV.

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