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PROTECTION OF HOT PEPPER AGAINST MULTIPLE INFECTION OF VIRUSES BY UTILIZING ROOT COLONIZING BACTERIA

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ABSTRACT

Mix virus infection is a common phenomena in nature. It results in severe of disease symptoms and yield loss. We utilized seven selected root colonizing bacteria (rhizobacteria) isolated from hot pepper rhizosphere to improve the effectiveness of virus management. The efficacy of those rhizobacteria in inducing plant growth and systemic resistance (ISR) on hot pepper against multiple infection of *Tobacco mosaic virus* (TMV), and *Chili veinal mottle virus* (ChiVMV) were evaluated in greenhouse trials. The rhizobacteria was applied as seed treatment and soil drench. All bacterial treated plants showed better growth character, milder symptom expressions than control and increased the peroxidase enzyme activities and ethylene but these depends on the species. It slightly affected the accumulation of TMV, however it suppressed the ChiVMV accumulation. Based on the morphological characters and full length nucleotide sequences analysis of 16S r-RNA, *Bacillus cereus* (I-35) and *Stenotrophomonas sp* (II-10) were the potential isolates as PGPR.

Key words : multiple viral infection, rhizobacteria, *Bacillus cereus*, *Stenotrophomonas* sp, ISR

INTRODUCTION

Hot pepper (*Capsicum annuum*) is one of important vegetables in Indonesia. However, infection by plant pathogens, including plant viruses become a serious constraint for hot pepper production. The main viral disease infecting hot-pepper are *Chili veinal mottle virus* (ChiVMV), *Pepper veinal mottle virus* (PVMV), *Pepper mottle virus* (PeMoV), *Pepper severe mosaic virus* (PeSMV) and *Cucumber mosaic virus* (CMV). In Indonesia ChiVMV, CMV, TMV and recently Geminivirus are important viruses infecting hot pepper (Duriat, 1996; Sulandari 2004). In nature, multiple infection by pathogens is a natural phenomena which causes damage more severe than a single infection by a pathogen.

Management strategies to control plant viruses in Indonesia is limited to the use of resistant cultivars, culture management and most farmers rely on chemical insecticides to control the insect vectors. To minimize the use of pesticides which pollute the environment and to improve the effectiveness of virus disease control, the utilization of beneficial root colonizing bacteria isolated from the plant rhizosphere referred to as Plant Growth Promoting Rhizobacteria (PGPR) might offer a promising viral diseases control method as previously reported to be effective in controlling fungi, bacterial and viruses (Maurhofer et al., 1994; De Meyer et al., 1999; Murphy et al., 2000; Murphy et al., 2003). Plants develop an enhanced defensive capacity against a broad spectrum of plant pathogens after colonization of the roots by selected strains of nonpathogenic bio-control bacteria (Pieterse et al., 2000).

In Indonesia, the availability of hot-pepper resistant cultivars against either pest or diseases are

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limited. Hence, this study sought to find and to evaluate the potential of rhizobacteria eliciting plant growth and induced systemic resistance (ISR) to protect hot pepper against multiple infection of TMV and ChiVMV.

MATERIALS AND METHODS

Rhizobacteria Isolates

Rhizobacteria was isolated from healthy rhizosphere of hot pepper cultivar in fields at Darmaga Bogor, West Java. Bacteria was isolated and was cultured on tryptic soya agar (TSA, Difco, USA). Bacterial isolation was performed as described previously (Lemanceau et al., 1995). Seven rhizobacteria isolates were used: I-2, I-16, I-25, I-30, I-35, II-7, II-10, and evaluated based on their ability to enhance plant growth and their ability to protect hot pepper against multiple virus infection.

Identification of Rhizobacteria

The potential isolate (s) as candidate PGPR was identified by sequencing the 16S r-RNA using a set of primer specific for prokaryotes 16S ribosomal RNA. The forward primer was 68f (5'-CAGGCCTAACACATGCAAGTC-3') and the reverse primer was 1387r (5'-GGGCGGWGTGTACAAGGC-3') as previously described (Marchesi et al., 1998). Further sequenced of the full length of 16S rRNA, additional primers designed according to the species. The homology and similarity of the nucleotide sequences were analyzed using WU-Blast2 software provided by EMBL-EBI (European Molecular Biology Laboratory – European Bio-Informatics Institute).

Viral Inoculum

TMV was propagated in tobacco plants, while ChiVMV was propagated on hot pepper**.** TMV and ChiVMV were obtained from Laboratory of Virology collection, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University. The viral inoculum was tested serologically before use for inoculation.

Growth Condition and Rhizobacteria Treatment

The experiments were conducted in greenhouse trials to evaluate the rhizobacteria ability as PGPR to protect hot pepper against mixed infection of viruses. Rhizobacteria treatment (10^9 cfu/ml) and growing conditions were performed as described previously (Damayanti et al., 2007). Plants were grown in the greenhouse with humidity and temperature depending on the natural conditions. Six plants were used for each treatment unit.

Virus Inoculation

Plants per treatment were mechanically inoculated with mixed infected plant sap (1:10 w/v) in phosphate buffer (pH 7.0) (Merck, Germany) at 2 weeks post transplanting or 4 weeks after planting (WAP) into 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 viruses.

Evaluation of Plant Growth Characters and Disease Assessments

The plant height was measured from soil line to shoot apex taken at 1 day prior to inoculation (dpi) with viruses and at 4 and 8 weeks post-viral inoculation (wpi). The fresh weight of the above tissues, number of leaves, number of flowers/fruits (taken as single measure) were counted at 6-8 wpi.

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Disease Assessments

Disease severity rating was made at 2 wpi using rating scales developed using mock inoculated plants of treatment as a standard by using the following rating scales : 0 = no symptoms, 2 = mild mosaic symptoms on the leaves, 4 = severe mosaic symptoms on the leaves, 6 = mosaic and deformation of leaves, 8 = severe mosaic and severe deformation of leaves and stunted. Disease severity measured for every single plant used in the experiment.

ELISA test. Viral protein accumulation was detected by DAS-ELISA (double antibody sandwich Enzyme linked-immunosorbent Assay) method at 2 and 4 wpi with TMV, and ChiVMV antiserum. Samples were detected as composite leaf samples per treatment. The procedure was carried out according to manufacture's recommendation (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). Viral accumulation was quantitatively measured by using ELISA reader at 405 nm. The positive sample was considered for the presence of the virus when absorbance value was twice of healthy control.

Extraction and Quantification of Peroxidase (PO) Enzyme Activities. Peroxidase enzyme activities during 3 minutes was used as a parameter to determine whether ISR occurred or not. Extraction and quantification of peroxidase enzyme activities were done using a spectrophotometric method conducted at 1 wpi according to method described previously (Hammerschmidt et al., 1982; Damayanti et al., 2007) with minor modification. The reaction mixture was incubated at room temperature and the absorbance determined using a spectrophotometer at 420 nm with 30 second intervals for 3 minutes. The enzyme activity was expressed as a change in absorbance ($min^{-1}mg^{-1}$ protein). PO enzyme activity was measured from composite leaf samples of each bacterial treatment.

Quantification of Ethylene Production. Quantification of ethylene production was conducted by using gas chromatography (GC) method that was carried out at Balai Besar Pasca Panen, Cimanggu, Bogor, Indonesia. Leaves samples (2 g/each treatment) of bacterial-treated- and control plants were taken at 5 dpi and were measured for ethylene production, expressed as µmol/ g leaf. Ethylene production was measured from composite leaf samples of each bacterial treatment

Data Analysis

Data was analyzed by analysis of variance (ANOVA) and the treatment means were separated by Duncan's Multiple Range Test (DMRT)($\alpha = 0.05$) using SAS software version 6.12 (SAS Institute, Gary, NC, USA).

RESULTS AND DISCUSSION

Evaluation of rhizobacteria treatment on plant growth characters

Plant height measured at 1 day before viral inoculation (4 WAP) was significantly higher on bacterial treated plants than control (data not shown). In addition, bacterial treated plants visually exhibited greater vigor, fitness and leaf size than control plants. However, at 8 WAP, plants treated with I-16, and I-30 at 12 WAP did not show any difference with control plants respectively (Table 1, Healthy). Similar results were obtained when plants were challenge inoculated by mixed viruses at 4 weeks post inoculation (wpi) (8 WAP) and 8 wpi (12 WAP) (Table 1, Infected). It showed that even plants were infected severely by the viruses, these were able to growth well, indicating the protective effect of rhizobacteria to plants against viral infection. Further, most bacterial isolates showed the ability to enhance plant growth until 3 months after seedling, suggesting the long term persistence of rhizobacteria in soil. Enhancement of plant health might be by stimulating the plant host or mutualistic

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symbionts and/or the plant may be affected by hormones which mediate processes of plant cell enlargement, division, and extension in symbiotic as well as non-symbiotic roots as previously described (Gardener, 2004).

Table 1. Effect of rhizobacteria treatment on plant height (cm)

Treatment	8 WAP*	4 wpi	12 WAP	8 wpi
	Healthy	Infected**	Healthy	Infected**
Control	38.25 ± 3.74 e	25.45 ± 3.56 c	94.17 ± 10.28 b	53.50 ± 18.38 c
I-2	56.25 ± 3.90 c	34.67 ± 2.23 b	107.80 ± 6.99 a	80.48 ± 3.34 ab
I-16	36.17 ± 4.54 e	42.15 ± 2.38 a	115.83 ± 2.64 a	73.83 ± 1.94 b
I-25	61.83 ± 2.73 ab	36.42 ± 4.01 b	111.08 ± 7.23 a	77.17 ± 5.21 ab
I-30	45.83 ± 2.56 d	34.50 ± 4.37 b	93.57 ± 6.80 b	71.32 ± 8.54 b
I-35	64.20 ± 2.54 a	44.10 ± 2.75 a	114.83 ± 8.57 a	87.50 ± 12.88 a
II-7	59.42 ± 5.64 bc	44.00 ± 4.75 a	113.63 ± 6.13 a	83.50 ± 5.60 ab
II-10	62.67 ± 2.88 ab	42.42 ± 4.78 a	111.02 ± 3.17 a	86.20 ± 7.50 a

Number in columns followed by the same letter are not significantly different (α= 0.05) by DMRT
*WAP- week after planting (WAP).
**TMV and ChiVMV were inoculated at 4 WAP. Non-bacterial treated plants were used as control

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The bacterial treated plants generally produced more leaf flowers than control plants (Table 2). Similar results were obtained in the fresh weight of healthy plants. However, the number of flower of I-16-treated plants decreased similar to control plants. Furthermore, plants treated with I-16, I-30, II-7 and infected with mixed viruses did not show any difference in fresh weight compared to control. Other treatments (I-2, I-25, II-10) showed slight difference while I-35 treated plants were significantly different.

Table 2. Effect of rhizobacteria treatment on plant leaf, flower numbers and fresh weight.

Treat-ment	Leaf Numbers		Flower Numbers		Fresh Weight (g)	
	Healthy	Infected*	Healthy	Infected*	Healthy	Infected*
Control	93.17 ± 29.08 e	35.67 ± 18.04 e	75.83 ± 13.88 b	52.50 ± 22.85d	72.02 ± 16.81 d	58.73 ± 4.75 b
I-2	176.00 ± 16.25 ab	90.00 ± 11.49 bc	191.50 ± 39.93 a	226.33 ± 52.11ab	99.10 ± 7.01 bc	65.61 ± 5.82ab
I-16	140.50 ± 29.51 dc	105.17 ± 20.23 b	202.00 ± 49.39 a	64.00 ± 6.81 d	98.87 ± 4.45 bc	55.47 ± 16.33 b
I-25	156.17 ± 24.77 bc	71.67 ± 16.97 cd	174.83 ± 50.76 a	232.00 ± 56.95 a	97.63 ± 6.86 bc	66.47 ± 14.98 ab
I-30	118.33 ± 9.33de	58.50 ± 16.31 d	185.50 ± 42.40 a	164.17 ± 64.99 c	88.37 ± 9.56 c	48.88 ± 11.12 b
I-35	172.17 ± 38.58 abc	162.50 ± 32.49 a	183.67 ± 34.78 a	137.33 ± 20.49 c	130.37 ± 27.40 a	78.87 ± 26.06 a
II-7	150.17 ± 11.69 bc	107.67 ± 11.69 b	182.17 ± 40.54 a	178.67 ± 21.79bc	90.98 ± 14.01 c	58.90 ± 7.08 b
II-10	199.17 ± 24.56 a	104.33 ± 18.93 b	176.17 ± 39.29 a	221.67 ± 18.93ab	108.76 ± 6.94 b	65.60 ± 6.03 ab

Number in columns followed by the same letter are not significantly different (α = 0.05) by DMRT
* TMV and ChiVMV were inoculated at 4 WAP. Non-bacterial treated plants were used as control

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Disease Assessments

All bacterial treated plants inoculated with mixed viruses exhibited phenotype symptoms milder than control plants (Fig. 1). The mildest symptoms were displayed by plants treated with I-16 and I-35. The protective effect afforded by isolates in suppressing the symptoms varied in severity, suggesting that the variety of the bacteria species might lead to different effects on plants after the challenge inoculation of viruses. In addition, the effect of I-16 on disease severity was more prominent than its ability to promote plant growth (Table 1 and Fig. 1).

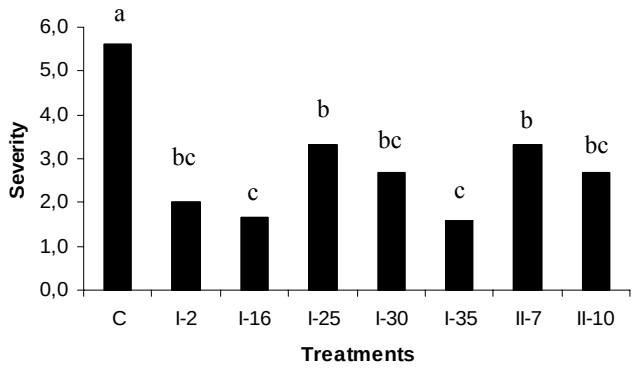

Figure 1. Disease severity of control plants (C) and bacterial treated plants. inoculated by mix viruses

ELISA test. Based on ELISA absorbance value, the rhizobacteria treatment affected slightly on TMV either at 2 or 4 wpi, while ChiVMV accumulated lower levels of the virus than control plants (Table 3). It suggested that bacterial treatment able to suppressed the ChiVMV better than TMV.

Table 3. ELISA test of control and inoculated plants infected by virus

Treatment	ELISA Absorbance value*			
	TMV		ChiVMV	
	2 wpi	4 wpi	2 wpi	4 wpi
C**	1.624	1.895	0.326	0.472
I.2	1.889	1.942	0.187	0.386
I.16	2.198	1.765	0.080	0.074
I.25	2.100	1.769	0.221	0.410
I.30	1.810	1.712	0.234	0.360
I.35	2.039	1.751	0.089	0.065
II.7	1.955	1.660	0.195	0.358
II.10	1.925	1.770	0.187	0.341

*Means of ELISA absorbance value obtained from duplex measurement of composite samples per treatment. Positive results of ELISA = twice of healthy Absorbance value Healthy absorbance value of TMV= 0.095, and that of ChiVMV = 0.031

** C = control plants inoculated by mixed viruses

The accumulation of TMV was more prominent than that of ChiVMV, indicating the stability of TMV to compete with other viruses during multiple infection. Alternatively, rhizobacteria treatment did not decrease the TMV accumulation, but it suppressed the severity. Even the rhizobacteria treatment slightly affected TMV, however all rhizobacteria treated plants exhibited milder symptom expression compared with control plants (Figure 1). The protection afforded to rhizobacteria treated

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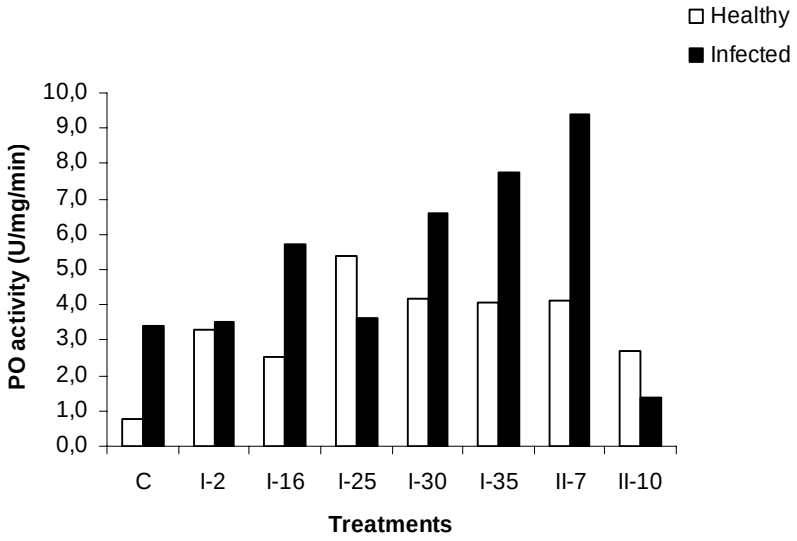
plants appeared to have been a result of the enhanced growth of hot pepper, thereby allowing them to respond to inoculation with viruses.

Peroxidase Enzyme Activities. Generally, all rhizobacteria treated plants increased the PO activity comparing with control (healthy), and the PO activities increased to some extent after challenge inoculated with viruses, except for plants treated with I-25, and II-10 (Figure 2). This suggests that some rhizobacteria treatments might induce plant's systemic resistance through increasing peroxidase enzyme activity (PO dependent) but not for I-25 and II-10. It showed that some rhizobacteria might able to enhanced plant's defense response through elevated PO activity (I-2, I-16, I-30, I-35, II-7) while others might be PO-independent (I-25, I-10) and it might be depends on the species. The polyphenol oxidase enzyme and peroxidase oxidizes phenolics to quinones and generates hydrogen peroxide (H_2O_2). It well known that H_2O_2 is an antimicrobial compound, which releases highly reactive free radicals and further increases the rate of polymerization of phenolic compounds into lignin-like substances. These substances are then deposited in cell walls and papillae and interfere with the further growth and development of pathogens (Agrios, 2005; Hammond-Kosack and Jones, 1996). Certain PGPR do not induce pathogenesis related (PR) proteins but rather increase accumulation of peroxidase, phenylalanine ammonia lyase, phytoalexines, polyphenol oxidase, and/or chalcone synthase (reviewed by Compant et al., 2005). These experiments also showed that some of isolates except I-25 and II-10 were able to induce the peroxidase after challenge with virus infection.



Ethylene Production. Overall, rhizobacteria treatment did not show increased ethylene production (healthy) (Figure 3). However, the ethylene production increased when plants were challenge inoculated with viruses, except for I-2 and I-30 treated plants. In this case, induction of ISR either through increased ethylene production or the ACC (1-aminocyclopropane-1carboxylate) converting capacity to ethylene is still unclear and needs further investigation. However, rhizobacteria treatments (I-16, I-25 I-35, II-7, II-10) and challenge inoculation with viruses increased the ethylene production compared with their healthy plants. A greater production of ethylene in the initial phase of infection might contribute to enhanced resistance against pathogens. In other words, ethylene might act as the primary signal in enhancing the defense capacity or may activate some defense genes. However, even plants treated with I-2 and I-30 did not increase ethylene production, but treated plants

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showed milder disease severity than control.

Improvement in plant health and productivity by PGPR mediated by three different ecological mechanisms; (1) antagonism of pests and pathogens, (2) promotion of host nutrition and growth and (3) stimulation of plant host defenses (Gardener, 2004).

The majority of plant growth promoting bacteria that activate ISR appear to do so via an SA-independent pathway involving jasmonate and ethylene signals (reviewed in Compant et al., 2005). It seemed that ISR afforded by isolates might increased the jasmonic acid (JA) production and leads increasing of sensitivities to ethylene as resemble of the case of PGPR WCS417r in *Arabidopsis* (Knoester et al., 1999). ISR is associated with an increase in sensitivity to JA and ethylene rather than an increase in their production, which might lead to the activation of partially set of defense genes (Hase et al., 2003; Pieterse and van Loon, 1999). Unfortunately, in these experiments, JA production was not measured for further elucidation of the role of JA in ISR on hot pepper plants.

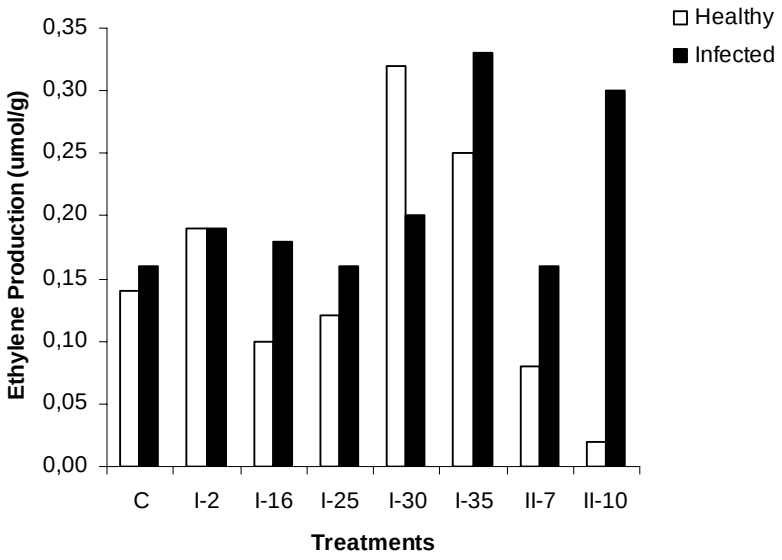

Figure 3. Ethylene production of healthy and virus infected plants

Identification of Rhizobacteria Species

Isolates I-35 and II-10 have the potential as PGPR as these are able to protect hot pepper plants against multiple virus infection. Previously, I-35 identified as *Bacillus cereus* (DDBJ Accession No.AB288105) was reported to enhance growth and protect hot pepper plants against TMV infection (Damayanti et al., 2007). In most cases, *Bacillus spp* that elicit ISR typically promote plant growth. *B. cereus* has been previously reported to have activities that suppress pests and pathogens or promote plant growth (reviewed in Kloepper et al., 2004). These results also support the previous reports for *B. cereus* (I-35) that consistently exhibited its ability as PGPR and/or enhanced plant systemic resistance event in plants infected by mixed viruses.

Rhizobacteria species were identified by testing the morphological characters of the bacteria (gram type, colony form, cell type) and nucleotide sequences of 16S r-RNA. The II-10 isolate is a

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gram-negative, whitish colony on TSA, and rod shaped. Based on these characters and 16S r-RNA sequences, the isolate II-10 was identified as *Stenotrophomonas sp* (DDBJ Accession No. AB288107). *Stenotrophomonas* genera is still few reported as PGPR. However, *Stenotrophomonas maltophilia* was the most frequent species recovered from weeds rhizosphere in Canada and reportedly could promote potato growth (Sturz et al., 2001). Our results here extend the role of *Stenotrophomonas* genera as PGPR. These results might be the first evidence of *Stenotrophomonas sp* as inducer of plant systemic resistance against plant viruses in hot pepper.

CONCLUSION

Based on the evaluation of plant growth characters and disease assessments, the potential candidates of root colonizing bacteria as PGPR which could protect hot pepper against multiple infection of viruses are *Bacillus cereus* (I-35) and *Stenotrophomonas* sp (II-10).

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