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Original Article

The effect of PGPR (Plant Growth Promoting Rhizobacteria) *Pseudomonas fluorescens* and *Bacillus subtilis* On Leaf Mustard Plant (*Brassica juncea* L.) Infected by TuMV (Turnip Mosaic Virus)

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Abstract

One problem that causing leaf mustard yield loss is the infection of Turnip Mosaic Virus (TuMV). The virus causes mild mosaic leaf with vein clearing, blister, malformation and stunting. The use of Plant Growth Promoting Rhizobacteria (PGPR) such as *Pseudomonas fluorescens* and *Bacillus subtilis* is one effort that could be used to solve the problem. Through the mechanism of induced resistance, these bacteria can elicit the defense signal in plant for the defence against pathogens. In this study the use of *Pseudomonas fluorescens* and *Bacillus subtilis* was performed to test their benefit on leaf mustard plant health against TuMV infection. This study was conducted with a randomized block design (RBD) by using 8 treatments and 4 replications. On the experiment of the effect of PGPR on the root length of leaf mustard plants, the design used was a completely randomized design (CRD) with 4 treatments and 4 replications. Leaf mustard plants inoculated with *Pseudomonas fluorescens* and *Bacillus subtilis* had longer roots than those without the inoculation of *Pseudomonas fluorescens* and *Bacillus subtilis*. In addition, *Pseudomonas fluorescens* and *Bacillus subtilis* was also able to reduce the incubation period and disease intensity of TuMV on the leaf mustard plant. The activity of catalase enzyme and phenol content was elevated in the leaf of leaf mustard plant inoculated with PGPR. The results suggested that catalase and phenol production probably play a role in plant defense of leaf mustard against the infection of TuMV.

Key word : Mustard, *Turnip Mosaic Virus* (TuMV), PGPR

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INTRODUCTION

Leaf mustard (*Brassica juncea* L.) is one of important vegetable in Indonesia. According to BPS (Badan Pusat Statistik) (2012) the leaf mustard plant production in 2011 was 580.969 tons. Over the last 15 years the biggest production in 1999 is 1.447.910 tons. According to DEPKES RI (1979), nutrient content of 100g mustard is calories 22 cal; protein 2,3 g; fat 0,3 g; carbohydrates 4 g; fiber 1,2 g; calcium 220,5 mg; phosphorus 38,4 mg; iron 2,9 mg; vitamin A 969 SI; vitamin B1 0,09 mg; vitamin B2 0,1 mg; vitamin B3 0,7 mg; and vitamin C 102 mg.

TuMV (Turnip Mosaic Virus) is the most dangerous virus that attacks the leaf mustard. Symptoms of this disease are mild mosaic with vein clearing, blister, malformations and stunting (Firdaus, 2009).

Jetiyanon and Kloeper (2002) in Ashrafuzzaman *et al*., (2009) showed that PGPR (Plant Growth Promoting Rhizobacteria) could be used as a biological control agent by inducing resistance in plants. PGPR also serves to increase nitrogen capture, synthesis of phytohormones,

dissolving minerals such as phosphorus and siderophores to availability of iron in plant roots (Lalande *et al.*,1989; Glick, 1995; Bowen and Rovira, 1999 in Ashrafuzzaman *et al*., 2009). Luttge *et al.* (1979) stated that phytochrome and phytohormon are signal system defense of plants. This can be seen with the increasing of resistance compound such as peroxide that occurs in the plant tissues.

Several types of PGPR such as *Pseudomonas fluorescens* and *Bacillus subtilis* can reduce the development of plant virus through the induction of plant resistance mechanisms. *P. fluorescens* is able to produce the antibiotic phenazine derivatives are used for biological control of the pathogen. *B. subtilis* can increase plant growth by producing various growth hormones such as IAA (*indole - 3 - acetic acid*), cytokinins and gibberelin (Fernando, 2005).

The aims of this study is determine the effect of PGPR to the performance of leaf mustard plant which was infected by virus TuMV.

MATERIALS AND METHODS

The experiments were performed in the greenhouse, of Faculty of Agriculture, Brawijaya University, Malang from May to July 2013.

Materials used in this study are mustard seeds (*Brassica juncea* L.) cultivar Tosakan. PGPR *P. fluorescens* and *B. subtilis*.

The effect of the application of *P. fluorescens* and *B. subtilis* of plant roots growth.

Experiment was performed in completely randomized design with 4 treatments i.e. the application of *P. fluorescens* (P1), *B. subtilis* (P2), combination of *P. fluorescens* and, *B. subtilis* (P3) and without application of PGPR (P4). Each treatment was repeated four times.

The effect of *P. fluorescens* and *B. subtilis* application on the defense response of leaf mustard plant against TuMV

The study was performed in a randomized block design (RBD) consisted of 8 treatments i.e. plant applied with *P. fluorescens* (P1 and P4) , *B. subtilis* (P2 and P5), combination of *P. fluorescens* and *B. subtilis* (P3 and P6), All the treatments of P1 to P3 were not inoculated with TuMV, in contrast the treatment of P4, P5 and P6 were inoculated with TuMV. As controls the leaf mustard plant was only inoculated with TUMV (P7), and leaf mustard plant without inoculation of TUMV as well as the application of PGPR (P8). Each treatment was repeated four times.

Assay of Catalase (CAT)

Catalase activity was assayed according to the method of Luck (1974). A 20% homogenate of leaves was prepared in 0.067 M phosphate buffer (pH 7.0). The homogenate was centrifuged and the supernatant was used for the enzyme assay.H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer (Merck Spectroquant Pharo 300, EU). The enzyme solution containing H_2O_2-free phosphate buffer served as a control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

Assay of Peroxidase (POD)

The method proposed by Reddy *et al*. (1995) was adopted for assaying the activity of peroxidase. 20% homogenate was prepared in 0.1M phosphate buffer (pH 6.5) from the leaves samples, clarified by centrifugation and the supernatant was used for the assay. To 3.0ml of pyrogallol solution (0.05 M in 0.1M phosphate), 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5ml of H_2O_2 was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer (Merck Spectroquant Pharo 300, EU). One unit of peroxidase is defined as the change in absorbance/minute at 430 nm.

Assay of Total Phenol

Determination of total phenolic content was performed according to Singleton and Rossi, (1965) with minor modification. Respectively of 0.1 mL of leaf extract was added in 0.1 mL Folin-Ciocalteu reagent solution then vortexed for 1 minute. The solution was added by 2 mL solution of sodium carbonate 2% (Na_2CO_3). This mixture was kept in a dark room for 30 minutes. Extract solution absorbance was read at a wavelength of 750 nm with a UV-Vis spectrophotometer (Merck Spectroquant Pharo 300, EU). The results are expressed as mg gallic acid / kg extract.

Measurements of Plant Roots Length

Root length was observed after 3-5 fully expanded leaves were appeared . Plants were removed and observed the root length differences between the plants that applied by PGPR and without PGPR .

Incubation Period and Symptoms

The incubation period is the period of time from inoculation to the appearance of symptoms of the mustard plant. The observation of incubation was started one day after inoculation until the appearance of the first symptoms.

Disease Intensity

Disease intensity was measured according to the method proposed by Horsfall and Barrat (1945) in Bock (2009) :

$$P = \frac{\sum (n \times v)}{N \times Z} \times 100\%$$

Scoring of the disease intensity is as follows:
0 = healthy leaf
1 = mosaic symptom on leaves ≤ 25 %
2 = extensive mosaic on leaves ≥ 25 % - ≤ 50 % with blister
3 = extensive mosaic on leaves ≥ 50 % blistering and malformation
4 = malformation, leaf scald and dwarf

Plants Growth Observation

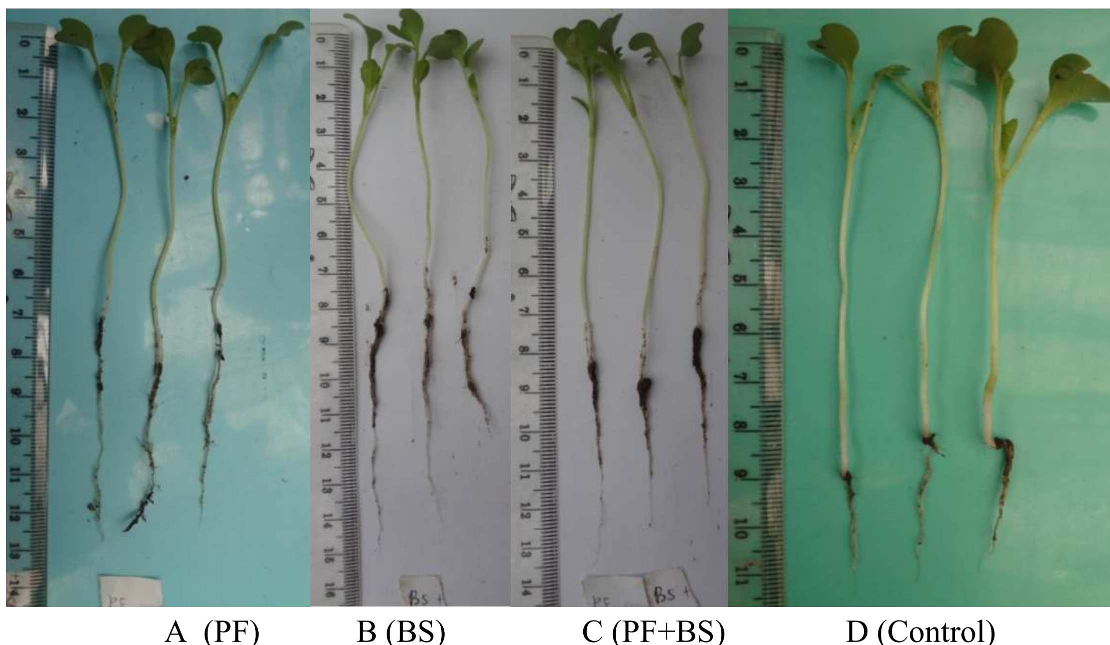
The observation of plant growth were performed including the number of leaves, chlorophyll content, wet and dry weight and leaf area. The number of leaves was observed after inoculation of leaf mustard plants with virus.

The measurement of chlorophyll content of leaves was performed using a SPAD chlorophyll meter (SPAD 502, USA). Wet and dry weight of plants was performed when plants were harvested. Plant dry weight was observed after drying in the oven for 24 hours at a temperature of 80°C. Leaf surface areawere calculated using the Leaf Area Meter (LI-3100C area meter, USA).

RESULTS AND DISCUSSION

PGPR (*Plant Growth Promoting Rhizobacteria*) affected The Growth of Plant Roots.

Based on observations at 6 DAI (Day After Planting), there was a difference in root length of plants in each treatment. Figure 1 showed the average of root length of each treatment i.e. PF (*P. fluorescens*) 6.625 cm, BS (*B. subtilis*) 7.1 cm, PF+BS (*P. fluorescens* and *B. subtilis*) 5.735 cm, and a control (no treatment) 2,95 cm. Figure 1 showed that the PGPR treated with can stimulate the growth of roots of leaf mustard plants. According to Minorsky (2008) PGPR inoculation can increase the growth, germination, and harvest of cultivated plants.



A (PF) B (BS) C (PF+BS) D (Control)

Figure 1. The effect of PGPR on root length of leaf mustard plants at 6 days after planting (DAP) on leaf mustard plant applied by A. PF (*P. fluorescens*); B. BS (*B. subtilis*); C.PF + BS (*P. fluorescens* and *B. subtilis*) , and D. control without the addition of PGPR

It has been known that PGPR is able to colonize the root surface area and associated with plant roots. According Khalid *et al.,* (2004), many reports showed *B. subtilis* and *P. fluorescens* have the ability to efficiently colonize the roots and improve the plant yield by increasing plant metabolism. *B. subtilis* as well as *P. fluorescens* were reported also able to produce indole-acetic-acid (IAA), which serves as a plant growth stimulant (Idris *et al.,* 2004).

Incubation Period and Symptoms of TUMV

Based on Table 1, PGPR treatment affected the incubation period of TUMV on leaf mustard plants. On Table 1 it can be seen that the addition of PGPR showed longer incubation period than control. This inhibition could be due to the induction of plant resistance against TUMV infection. Walters (2007) stated that the plant immune system can recognize and respond to pathogen invasion, hence plants can activate other pathways to induce the resistance. *P. fluorescens* and *B. subtilis* is capable to trigger induced plant resistance to produce compounds of salicylic acid, jasmonic acid and ethylene that serves as a signal of plant defense in plants (Koornneef, 2008). These compounds stimulate the plant to produce an enzyme used defense against the pathogen infection.

Table 1. Incubation period of TuMV symptom on leaf mustard

Treatment	Incubation period (days)
PF + TuMV	20,25
BS+ TuMV	20
BS + PF + TuMV	19,5
TuMV only (control)	14,75

Table 2. Disease intensity of TuMV on leaf mustard

Treatment	Disease intensity (%)
PF	0,70711 a
BS	0,70711 a
PF + BS	0,70711 a
PF + TuMV	5,13964 b
BS+ TuMV	7,19578 c
BS + PF + TuMV	5,34455 b
TuMV (only)	7,16921 c
No PGPR and TUMV	0,70711 a

Description: numbers followed by the same letter in the same column, showed no significant different based on DMRT (5%). The average number above have been transformed in $\sqrt{x + 0,5}$

According to Avdiushko *et al*., (1993), several enzymes produced by plants to fight against pathogens that infect plants are superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and Polyphenol Oxidase (PPO).

Disease intensity of TuMV On Leaf Mustard Plant

The observations of disease intensity of TuMV on leaf mustard plant showed that the application of *P. fluorescens* and *B. subtilis* as well as their combination could reduce the disease intensity of TuMV on leaf mustard (Table 2). Kloepper (1992) stated that PGPR can trigger plant defense mechanisms against pathogens or soil borne diseases, through the mechanism of induced resistance. Some studies showed that the seeds inoculated with PGPR showed the disease intensity lower than a mustard seed that was not inoculated with PGPR when both plants were infected with the plant viruses (Taufiq *et al*., 2005).

Analysis of Catalase, Peroxidase Activities and Total Phenol Content of Leaf Mustard

Analysis of the catalase enzyme in leaves of leaf mustard plants showed significantly different activity of catalase among the treatment at 1 WAI (week after inoculation of TUMV). However, at 2 and 3 WAI did not show any difference among the treatments (Table 3). This result indicated that the duration of induction of catalase enzyme activity by PGPR was limited only around 1 week after inoculation, then the induction was decreased. Table 3 also showed that the addition of *P. fluorescense* showed the highest activity of catalase compared with that of other treatments.

Increased catalase enzyme allegedly as a result of the accumulation of compounds such as peroxidase. Catalase enzyme plays a role in the decomposition of peroxide (H_2O_2) into water and oxygen which is not toxic to the cells (Agrios, 2005).

Table 3. Catalase activity on leaf mustard

Treatment	Catalase (Unit/mL)		
	1 st Week	2nd Week	3rd Week
PF	19,5463 b	4,9743	3,4709
BS	4,4062 a	1,6610	8,0658
PF + BS	5,8290 ab	1,9919	2,6040
PF + TuMV	9,3507 ab	0,9838	3,7165
BS + TuMV	5,1761 ab	6,5702	2,8400
BS + PF + TuMV	11,3294 ab	3,3772	6,3286
TuMV only	5,1915 ab	3,0022	1,4216
No PGPR and TuMV	5,1672 ab	1,8044	4,0257

Description: numbers followed by the same letter in the same column showed not significantly different based on Duncan's test (5%)

Table 4. Peroxide activity on leaf mustard

Treatment	Peroxide (unit/mg)		
	1 st Week	2nd Week	3rd Week
PF	0,0187	0,0571	0,0466
BS	0,0279	0,0382	0,0460
PF + BS	0,0178	0,0297	0,0538
PF + TuMV	0,1418	0,0654	0,0481
BS+ TuMV	0,0487	0,0746	0,0674
BS + PF + TuMV	0,0282	0,0514	0,0519
TuMV	0,0215	0,0580	0,0464
No PGPR and TuMV	0,0349	0,0416	0,0538

Table 4 showed no different of peroxidase activities between the application of PGPR and control. This result indicates that the application of PGPR did not affect the production of peroxidase. The result of Table 3 and 4 indicates that the application of PGPR could induce only specific peroxidase enzyme i.e catalase but not all types of peroxidase. Catalase is a member of peroxidase enzymes that ussually elevated during stress condition caused by abiotic as well as biotic factor such as pathogen infection or induction by rhizobacteria. These result also indicate that during infection of TUMV the all peroxidase enzymes activity also elevated due to TUMV infection. Riedle-Bauer (1997) reported that mosaic virus infection on plants will led to the accumulation of antioxidant enzymes such as peroxidase enzymes.

Total phenol levels in each treatment showed differences (Figure 2). The addition of *P. fluorescense* showed the highest levels of total phenol compared to that of other treatments. According to Campos-Vargas (2005) phenol accumulation in plant defense response is correlated with accumulation of phenyl Alanin Lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD). Therefore accumulation of total phenol in planta was elevated even in the leaf mustard plant inoculated with TuMV only, due to the induction of defense response by TuMV infection.

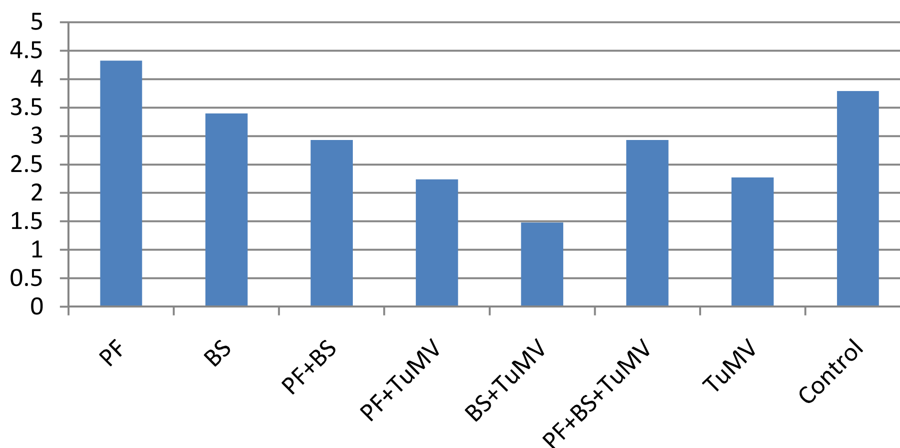


Figure 2. Analysis of Total Phenol (mg/L)

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The Growth of Leaf Mustard Plant

Wet weight and dry weight of leaf mustard plants showed differences between plants inoculated with TuMV and without inoculation of TuMV. Overall, TuMV infection reduced the wet weight and dry weight of leaf mustard. No significant differences showed among treatments with inoculation of TuMV. Also, no significant differences showed among treatment without inoculation of TuMV. These results probably due to the high variability of the measurement data. However, there was indication of the increase of wet weight as well as dry weight of leaf mustard by application of PGPR in the condition of infection or no infection of TuMV (Table 5).

Similar indication were shown on the data of leaf area (Table 6). Based on Table 6, it can be seen that the inoculation of TuMV decrease the leaf area of leaf mustard plants. Similar to wet and dry weight, no significant differences of leaf area showed among treatments with inoculation of TuMV. Also, no significant differences showed among treatment without inoculation of TuMV, probably due to the high variability of the measurement data. Data of leaf number showed no significant difference among the treatment.

Significant effect of PGPR was shown on the chlorophyll content. The application of all PGPRs increase the chlorophyll content in TuMV infected leaf mustard plant, indicated that the addiion of PGPR could support leaf mustard plant to defense against TuMV infection (Table 7). TuMV infection on leaf mustard plant typically showed the reduction of chlorophyll content since TuMV could produce chlorosis or loss of chlorophyll in leaves. Thus the addition of PGPR could inhibit the loss of chlorophyll caused by TuMV infection.

Overall, mosaic virus such as TuMV could affect plants by reduction in plant growth and the production of plant biomass (Power, 1992; Zhang *et al.,*2000; in Escriu, 2003). In this study, beside its function in increasing plant growth, the application of PGPR at least could reduce the severity of the disease and inhibit the symptom development of the TuMV showed by the reduction in incubation period and disease intensity of TUMV. The PGPR affected the performance of leaf mustard plants against TUMV probably by increasing the defese response i.e. the catalase activity and accumulation of total phenol in the leaves, resulted in the inhibition of chlorosis caused by TUMV infection.

Table 5. Wet and Dry Weight of Leaf Mustard

Treatment	Wet Weight (Gram)		Dry Weight (Gram)	
PF	32,15	cd	2,15	bc
BS	36,47	d	2,6	c
PF + BS	34,1	cd	2,175	bc
PF + TuMV	18,15	ab	1,1	ab
BS+ TuMV	13,67	a	0,925	a
BS + PF + TuMV	23,35	abc	1,65	abc
TuMV	12,75	a	0,7	a
No PGPR and TuMV	26,8	bcd	1,775	abc

Table 6. The Amount Of Leafs and Leaf Area Surface

Treatment	Amount of Leafs		Leaf Area Surface (Cm^2)
PF	6,8125	b	548,821
BS	6,875	b	505,564
PF + BF	7,25	b	440,787
PF + TuMV	6,625	b	198,885
BS+ TuMV	6,625	b	258,065
BS + PF + TuMV	7	b	435,881
TuMV	5,625	a	213,154
No PGPR and TuMV	7,3125	b	500,598

Description: numbers followed by the same letter in the same column, showed not significantly different based on Duncan's test (5%)

Table 7. The chlorophyll content.

Treatment	Amount of Chlorophyll (Unit)
PF	25,4438
BS	25,8438
PF + BF	26,2375
PF + TuMV	24,7063
BS+ TuMV	25,0063
BS + PF + TuMV	24,9875
TuMV	23,3625
No PGPR and TuMV	35,4438

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