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Pseudomonas fluorescens as plant growth promoting Rhizo-Bacteria and biological control agents for white rust disease in chrysanthemum

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Key words: *P. flourescens*, PGPR, white rust *Puccinia horiana*, biological control, *in vitro* and *in vivo* testing.

Abstract

The use of plant growth promoting rhizobacteria (PGPR) to control disastrous diseases in many crops has been considered important recently. The research was conducted to evaluate several bacterial strains to control white rust in chrysanthemum. The research consisted of two chronological experiments, *in vitro* and *in vivo* testing of bacterial isolate against the disease. 16 bacteria isolates were collected, purified and applied on the rust-infected leaf. Three isolates showed more effective in suppressing white rust during *in vitro* testing and further identification confirmed these strains, Pf Kr 2, Pf Smd 2 and Pf Ktl were grouped into *P. flourescens*. *In vivo* testing of the Pf isolates also revealed consistent performances of these three Pf isolates in retarding the growth of fungal *Puccinia horiana* and even more effective than *Azotobacter* sp. and *Azospirilium* sp. The production of ethylene on the leaf was coincidence with the slower development and lower disease intensity on the treated plants. Among the three strains, Pf Kr 2 showed stronger suppression to the disease. Further investigations are needed to further elucidate the existence of specific interrelation between Pf strains and plant genotypes or cultivars. Prior to a selection of good bacterial inoculants, it is recommended to select cultivars that benefit from association with these bacteria.

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Introduction

White rust is the one of the most disastrous diseases at upper part of chrysanthemum plants in most world production centers. It is caused by a fungal pathogen by *Puccinia horiana* (Henn), an obligate parasite, which hosted in limitedly 12 species including chrysanthemum. The fungus attacks the plant by penetrating the leaf directly through the cuticle by enzymatic digestion and then colonizes the mesophyll tissue both inter- and intracellularly (Bonde *et al.*, 2005). The intensity of incidence fluctuates depending on susceptibility of cultivars, temperature and air humidity. Warm temperature and high air humidity are favorable to meet a rapid development and spreading of the disease in susceptible cultivars (Wojdyla, 2004).

The use of chemicals and screening of resistance varieties are the most common methods in controlling the disease in commercial nurseries. These costly practices were often applied regardless the presence of the symptoms and intensity of the diseases to ensure the marketable flower quality. In several countries like Indonesia, however, no active ingredient is found in commercial fungicide registered specifically for white rust control (Yusuf *et al.*, 2014). The application of chemical is also considered as the last effort in integrated pest management with the wise and precise considerations for type of the pest, method, dosage/concentration, interval and environmental friendly (Thornburn, 2015).

The use of biological agent has been reconsidered important in controlling diseases in many crops especially in the last few decades. The interest was encouraged as a part of responses to public concern about hazards associated with chemical pesticides. The recognition of plant growth promoting rhizobacteria (PGPR), a group of beneficial bacteria, as potentially useful for stimulating plant growth and increasing crop yield has been successfully applied in several crops, such as potato, radish, sugar beet and sweet potato (Farzana *et al.*, 2009). In chrysanthemum, however, only few antagonists with limited information have been known as biological control of the diseases, especially for white rust.

The mechanism of PGPR in protecting the plant from pathogen infections was through several ways involving the physiological and biochemical hypereactions as responses to the given bio-signals. One indicator on the plant mechanism is the production of ethylene associated with the plant response to wounding, pathogen attacks and other stressed (Bleecker and Kende, 2000). Ethylene appears to be able to promote either disease resistance or susceptibility depending on the particular plant-pathogen combination. These mechanism inferred that ethylene is produced after the signal is received and may be a stimulus for defense response by regulating a wide range of defense regulate genes, including those encoding pathogenesis-related (PR) protein (Kim *et al.*, 2015). Ethylene may also play a role in disease symptom development in correlation with the timing of increased ethylene production and development of disease symptoms on the plant (Goto *et al.*, 1980; Elad, 1990; Boller, 1991; Porcel *et al.*, 2014).

Several groups of bacteria, like *P. flourescens* (Pf), *Azotobacter* and *Azospirilium* have been observed to have potential use for controlling important diseases in several crops. Certain strains of Pf are able to reduce destructive attacks of *Verticilium dahlia* in eggplant (Soesanto, 2001), *Sclerotium rolfsii* in peanut (Soesanto, 2004) and *Fusarium oxysporum* in shallot (Santoso *et al.*, 2007), hot pepper (Maqqon *et al.*, 2006) and gladiol (Soesanto *et al.*, 2008). Considered as plant growth promoting rhizobacteria, these bacteria produced 2,4-diacetylflouroglucynol (Phl or DAPG) and siderophore (Raaijmakers and Weller, 1998; Soesanto, 2000) inducing root colony to protect the plant from soil borne pathogens (Soesanto and Rahayuniati, 2009).

Azotobacter spp. was also known as broad spectrum antifungal agents which was protecting the plants from fungal pathogens through HCN and sidephore productions. Synergistic interactions of these two

with other metabolites might function as stress factors including local and systemic host resistance that led for the suppressions against *Rhizoctonia solani* in cotton, guar and tomato (Chauhan *et al*., 2012), Cercospora in groundnut (Mali *et al*., 2011) and *F. oxysporum* (Boshale *et al*., 2013). These free living bacteria were able to fix N_2, produce vitamins and growth substances including IAA, gibberellins and cytokines which enhanced root growth and aided in nutrient absorption (Mali and Bodhankar, 2009).

The genus *Azospirilium* composed of free-living, nitrogen-fixing bacteria that are found to be associated with the roots and rhizosphere of several grasses including sugar-cane, maize, sorghum, and rice revealing a broad ecological distribution. They can colonize, by adhesion, the root surface or the intercellular spaces of the host plant roots. The potential role of these PGPR is to promote plant growth by several mechanisms including nitrogen fixation (Bashan *et al*., 2004) and phytohormone production, such as auxins, gibberellins, cytokinins, and nitric oxide as signals of plant growth promotion. Several studies revealed that *Azospirilium* is phylogenetically closer to Rhodospirillum and can grow in the presence of sucrose as sole carbon source and is also better adapted to soil acidity, which offers the bacterium additional advantages for colonization of plant root tissue in acid environments (Baldani and Baldani, 2005). Considering the potential use of PGPR as biological agents against fungal diseases, the research was conducted to evaluate the effectiveness of Pf, *Azotobacter* and *Azospirillum* in controlling white rust disease in chrysanthemum.

Materials and methods

The research was conducted at the laboratory of bacteriology and crop protection glass houses *of the,* Indonesian Ornamental Crops Research Institute (IOCRI) from January to December 2013. The research comprised of 2 experiments; (a) Isolation of Pf and *in vitro* screening of isolated Pf against *Puccinia horiana* and (b) *in vivo* testing of isolated PGPR to control white rust in chrysanthemum.

Isolation and in vitro screening of isolated Pf

Pf strains were collected from the soil at the rhizosphere of certain bamboo species (local name are 'Gombong' and 'Bitung') and healthy chrysanthemum leaves in several areas in West Java, Indonesia (Table 1). For about 10 g bamboo root and chrysanthemum leaves were separately collected and put into elenmeyer containing 100 ml sterile distilled water. The water containing the bamboo root and chrysanthemum leaves was put into rotary shaker with speed of 150 rpm for 30 min. The solution was then diluted (10^{-1}) by taking 1 ml solution into 9 ml distilled water. The diluted solution was shaken for 150 for another 30 min. The 10^{-1} suspension was diluted with the concentration of 10^{-2} up to 10^{-10}, then inoculated at 0.1% Triptic Soybroth Agar (TSA) medium. All the cultured bacteria were incubated for 48 h under temperature of 29-30C. The growing colonies were selected and grouped based on their morphological features. The selected single colony was isolated and then, reinoculated to get the pure colony for further testing.

The *in vitro* screening test of the bacteria isolates against *P. horiana* was carried out based on the modified method of Suhardi *et al.* (2011) with the bacteria isolates as the biocontrol agent. The source of white rust was the infected chrysanthemum leaves. The source of inoculums was the infected leaves. The leaves were selected for the early stage of infection, characterized by less than 5 yellow spots per leaf with no rust pustule (Fig. 1a). The leaves were soaked into the bacteria solution for about 10 min. The base of the leaf petiole was wrapped with cotton that was previously also dipped in bacteria solution. The leaf was then put into plastic jar containing wet cotton to preserve the humidity for the white rust and leaf during the screening test (Fig. 1b) based on the treatment design. The leaf was sprayed with bacteria solution within 3 days - intervals.

Table 1. Source and date of collection of Pf to be tested for *in vitro* screening against chrysanthemum white rust (*Puccinia horiana* Henn).

Isolate Code	Source of isolates	Location	Date of Collection
Pf Kr 1	Healthy Leaf (Chrysanthemum)	Ciherang, Pacet, Cianjur	12 June 2013
Pf Kr 2	Healthy Leaf (Chrysanthemum)	Ciherang, Pacet, Cianjur	12 June 2013
Pf Kr 3	Healthy Leaf (Chrysanthemum)	Ciherang, Pacet, Cianjur	12 June 2013
Pf Kr 4	Healthy Leaf (Chrysanthemum)	Ciherang, Pacet, Cianjur	12 June 2013
Pf Jl	Rhizosphere (Gombong bamboo)	Jambu Luwak, Ciawi, Bogor	14 May 2013
Pf Km	Rhizosphere (Gombong bamboo)	Kertamukti, Cipatat, Bandung Barat	14 May 2013
Pf Mm	Rhizosphere (Gombong bamboo)	Mandalawangi, Cipatat, Bandung Barat	14 May 2013
Pf Bd	Rhizosphere (Gombong bamboo)	Bedungan, Ciawi, Bogor	14 May 2013
Pf Mj	Rhizosphere (Gombong bamboo)	Babakan Jawa, Majalengka	14 May 2013
Pf Ktl	Rhizosphere (Bitung bamboo)	Katumlampa, Bogor Timur, Bogor	14 May 2013
Pf Tt	Rhizosphere (Gombong bamboo)	Titisan, Sukalarang, Sukabumi	14 May 2013
Pf Smd 1	Rhizosphere (Gombong bamboo)	Cadas Pangeran, Sumedang	14 May 2013
Pf Smd 2	Rhizosphere (Gombong bamboo)	Cadas Pangeran, Sumedang	14 May 2013
Pf Smd 3	Rhizosphere (Gombong bamboo)	Cadas Pangeran, Sumedang	14 May 2013
Pf Tp	Rhizosphere (Gombong bamboo)	Kampung Tipar, Ciawi, Bogor	14 May 2013
Pf Cmk	Rhizosphere (Gombong bamboo)	Cimangkok, Sukalarang, Sukabumi	14 May 2013

Fig. 1. (a) The selected chrysanthemum leaf with early stage of white rust infection used for the source of inoculums, (b) the leaf was then put into plastic jar containing bacteria isolates for *in vitro* screening test.

The observation on the white rust development was conducted at 1, 3 and 7 days after applications. The conditions pustule was determined based on Suhardi *et al.* (2011) and the increase of number of developed pustules was counted. The percentage of white rust suppression by antagonist bacteria compared to the control was measured using the formula of :

$$PS = (T - C) \times 100\%$$

Where :

- PS = Percentage of Suppression
- T = Degree of infection on the treated plants
- C = Degree of infection of control plants (untreated)

The isolates that were able significantly to suppress the white rust development were selected and cultured on King's B medium. Pf isolate 18 taken from the laboratory of bacteriology was also included in this stage for a comparison of the selected isolates. After obtaining the pure culture of isolates, the morphological characterization and identification of isolates were conducted based on Schaad *et al.* (2001) and Price (1999) methods. The biochemical identification was performed based on Cowan and Steel (1974) method, the color of the colonies on King's and Na media and the growth rate at warmer temperature of 34-37C.

In vivo testing of isolated bacteria against white rust

Preparation of host plants

The tested chrysanthemum variety was 'Swarna Kencana' that was categorized as susceptible to white rust (Yusuf *et al.*, 2012). The rooted cuttings were collected from IOCRI seed production unit planted in polybags with the density of 5 cuttings/polybag. The media used was a mixture of top soil, bamboo humus and manure (70:15:15 v/v) with additional fertilizers of 200 kg/ha urea, 300 kg/ha SP-36 and 350 kg/ha KCl. The newly planted cuttings were then put inside

the glasshouse conditions and maintained under standard cultural practices. Long day condition was stimulated by providing additional light using 16 watt LED lamps during the night (10.00 pm to 02.00 am) every night for 30 days. The lamps were arranged 2 x 2 m for the distance among lamp points and 1.5 m high from the plants. Obamectin (Syngenta Co. Ltd) with the dosage of 18.4 g/l was sprayed once a week for prevention against insect attack and irrigated water was also given twice a week to maintain the optimum growth of the plants.

Application of bacteria isolates

The selected bacteria isolalates from in vitro testing and isolates of *Azotobacter* sp. and *Azospirillium* sp. that were previously reported effective in controlling fungal diseases by Indonesian Center for Agricultural Biotechnology and Genetic Resources (ICABGR) were used for *in vivo* testing againts white rust. Before planted, rooted cuttings were dipped for 15 min in the 10^{-10} cfu/ml bacterial solution. The bacterial solutions were also regularly sprayed into the plant every 7 days up to 60 days after planting. The sprayed solution was arranged in 0.5% in concentration with the density of 10^{-10} cfu/ml based on Taufiq *et al.* (2010). The solutions of 0.05% Methyl Jasmonate (Hersanti, 2004) and sterile water (control) were also sprayed with the same frequency and intervals as the bacterial treatments for comparison of the effectiveness. Observation of the white rust development was conducted from 10% plant samples at 3, 5, 7, 9 and 11 weeks after planting. The disease development was determined using Suhardi (2009) criteria as presented on Table 2. The disease intensity was calcuated using the following formula:

$$\text{Intensity (I)} = \frac{\Sigma\,(v \times n)}{(Z \times N)} \times 100\ \%$$

Table 2. Scale and damage criteria of white rust (*Puccinia horiana* Henn) infection on chrysanthemum (Suhardi, 2009).

Scale	Damage Criteria
0 :	Not infected (symptomless)
1 :	Very low, infection detected only on lower plant leaves and the intensity not exceed than 5% from total leaf area.
2 :	Low, infection detected on lower plant leaves and the intensity ranges 5-10% from total leaf area.
3 :	Medium damage, infection detected on middle and lower plant leaves and the intensity ranges 10-20% from total leaf area.
4 :	Heavy damage, infection detected on upper, middle and lower plant leaves and the intensity ranges 20-40% from total leaf area.
5 :	Very heavy damage, infection detected on upper, middle and lower plant leaves and the intensity was more 40% from total leaf area.

Where :

- I = Intensity of white rust infection (%)
- v = Scale of the observed damage
- n = number of infected plants categorized in the same damage scale
- Z = highest scale of the observed damage
- N = total number of observed plant samples

Concentration of ethylene production on the leaf was measured on 14 days after treatment application (DAA). The leaf samples were compositely collected from the plants from 3 replications. Ethylene was known to be directly connected with the activity of the antagonist bacteria inside the plants (Goodman *et al.*, 1986; Timmusk, 2003). A HPLC based analysis with the adopted method from from Association of Official Analytical Chemist (AOAC) Methods (1995) was carried out at ICABGR. The quantification of the ethylene concentration was calculated using Taufiq *et al.* (2010) based on the comparison of leaf area of leaf samples and standard of 100 ppm ethylene-producing area.

The comparative advantage analysis of each treatment was conducted in every steps of the respected treatment being comprehensively applied. These was scored based on the present and frequency of the treatment advantage after comparing to the

others. The higher the value of advantages was representing the more frequent of the treatment advantage presented. The advantageous treatment was scored as 1 and the less was given the score of 0 (Djatnika *et al.*, 2012). The scoring criteria for the basis of determination on presence and frequency of treatment advantages were:

a. Number of pustules on the treated plant at 7 DAA were low, the increase of number of pustule from 3 to 7 DAA were low and percentage of suppression of the treated bacteria on pustule development was higher than control.

b. Disease incubation was longer on the treated plants, intensity was low after 84 DAA and ethylene production on the leaf was higher.

Results and discussion

In vitro screening test of the bacteria isolates against white rust

Bacterial isolates performed differently in the suppression against *P. horiana* during *in vitro* testing. These could be seen from the development of pustule based on the increment of pustule numbers after 3 and 7 days and developmental stage of pustule after 7 days after bacterial application (Table 3). The number of pustule ranged from 25 to 87 per leaf after 7 days and the higher number of pustule was observed on leaf sprayed with sterile water (control). Most pustules on control treatment also reached more advance in developmental stages, that higher in the number of broken pustules (spore release) than the other treatments.

Table 3. Number and developmental stage of pustules after 1, 3 and 7 days after isolated bacterial application under *in vitro* testing against white rust.

Isolate Code	Number and increment percentage of numbers of pustule (Days after isolates application/DAA)					Developmental stage of pustule after 7 days isolates application (DAA)			Degree of isolate suppression against white rust compared to control
	1*)	3*)	Increment percentage of number of pustule after 3 DAA (%)	7*)	Increment percentage of number of pustule after 7 DAA (%)	White spot *)	White spot with unbroken pustule*)	White spot with broken pustule*)	
Pf Kr 1	32 a	34 a	6.25	75 a	120.59	5 c	20 a	50 b	13.79
Pf Kr 2	27 a	27 a	0	30 b	11.11	0 d	23 a	7 c	65.51
Pf Kr 3	17 b	29 a	70.59	57 b	96.55	0 d	35 a	22 bc	34.48
Pf Kr 4	29 a	37 a	27.59	63 a	70.27	3 c	10 b	50 b	27.59
Pf Jl	27 a	28 a	3.70	47 b	67.86	7 c	10 b	30 b	45.98
Pf Km	16 b	17 b	6.25	72 a	323.53	12 b	30 a	30 b	17.24
Pf Mm	16 b	17 b	6.25	67 a	294.12	3 c	14 b	50 b	22.99
Pf Bd	27 a	32 a	18.52	69 a	115.63	35 a	15 b	19 c	20.69
Pf Mj	5 c	6 b	20	47 b	683.33	10 b	27 a	10 c	45.98
Pf Ktl	17 b	17 b	0	23 c	35.29	12 b	5 b	6 c	73.56
Pf Tt	12 b	15 b	25	73 a	386.67	15 b	23 b	35 b	16.09
Pf Smd 1	17 b	27 a	58.82	37 b	37.04	10 b	7 b	20 c	57.47
Pf Smd 2	15 b	15 b	0	25 c	66.67	2 c	11 b	12 c	71.26
Pf Smd 3	12 b	17 b	41.67	39 b	129.41	9 bc	10 b	20 c	55.17
Pf Tp	19 b	23 ab	21.05	49 b	113.04	12 b	17 ab	20 c	43.68
Pf Cmk	17 b	29 a	70.59	53 b	82.76	13 b	10 b	30 b	39.08
Control	15 b	35 a	133.33	87 a	148.57	0 d	7 b	80 a	-
CV (%)	7,2	7.9		6,9		7,5	10,1	9,2	

Remarks: values within the same column followed by the same letter were not significant under Duncan's Multiple Range Test (DMRT), α = 5%.

The suppression of white rust development by bacterial isolates was apparent at 3 days after application. The degree of suppressions was varied among the bacterial isolates. Three isolates namely, Pf Kr 2, Pf Ktl and Pf Smd 2 showed significant suppression on the number of white rust pustules. The increment of pustule on the leaves was absent (0) after 3 days application. The performances of these three bacteria isolates in suppressing the newly developed pustule were consistent up to 7 days application. The increase of pustule number was less than 10 pustule per leaf and these was much lower than the other treatments that might reached more than 20 newly developed pustules after 7 days.

The less number of newly developed pustules on the leaf treated by Pf Kr 2, Pf Ktl and Pf Smd 2 was seemed connected with the slower developmental stage of pustule. The development of white rust pustules under these three isolates were retarded as viewed from the lower number of white spot with broken pustule after 7 days isolates application (Table 3). The spore of fungal pathogen were released and spread from the body through the broken pustules. The broken pustule was also an indicator of the maturity of the spore. When the environment condition was conducive, the fungal spore might germinate and infect the leaf of the susceptible cultivar (Hanudin *et al.*, 2015).

Three isolates of Pf Kr 2, Pf Ktl and Pf Smd 2 showed better suppression against white rust compared to control. The degree of suppressions of Pf Kr 2, Pf Ktl and Pf Smd 2 were measured 66.51%, 73.56% and 71.26%, respectively, compared to the control. Based on these observations, three isolates Pf Kr 2, Pf Ktl and Pf Smd 2 were selected for further evaluation on their effectiveness against white rust *Puccinia horiana* under *in vivo* conditions.

Identification of selected bacteria isolates

All selected bacteria isolates showed rod with elevated with flat cell shape, ledge/wavy and included as gram negative based on KOH test (Table 4). Stojšin *et al.* (2015) stated that gram negative cell had a ccell wall consisted of three layers mucopeptide. The content of might as much as 3-12 % from the total dry weight. Mucoopeptide is a chemical compound composed of units of n-acetyl glucosamine (nag) and n-acetyl muramic acid (nam) bound in the composition β, 1-4 (Kaiser, 2014). Mucopeptide complex was often referred to by the name of murein (Acharya, 2013).

Table 4. Cell and colony features of Pf Kr 2, Pf Ktl and Pf Smd 2 based on morphology and biochemistry characteristics and predicted of isolate group.

Cell feature	Characteristics of selected isolated bacteria		
	Pf Kr2	Pf Ktl	Pf Smd2
Morphology			
1. Cell shape	Rod	rod	rod
2. Doom elevation	Flat	flat	flat
3. Cell margin	circled	circled	circled
4. Gram (KOH 3%)	-	-	-
Biochemistry			
1. Color of colony when cultured at Na medium	cream	cream	cream
2. Color of colony when cultured at King's medium	florescent green	florescent green	florescent green
3. Growth at temperature of 34-37C	+	+	+
Predicted isolate group	P. fluorescens	P. fluorescens	P. fluorescens

The three isolates had flourescens green colony under King's B medium (Table 4). The flourescens green colony was caused by "pyoverdins" an iron chelating agent that was produced by a bacterium when grown under lacked-iron medium. Following the method of Godfrey and Marshall (2002), the three isolated bacteria (Pf Kr 2, Pf Ktl and Pf Smd 2) were grouped as *Psedomonas flourescens*, based on the morphological and biochemical features of their cells and colonies.

In vivo testing of isolated bacteria against white rust Puccinia horiana

In general, the symptom of disease arose when the interaction among three factors, the virulent pathogen, susceptible host and the environment were conducive for the pathogen to grow and developed (Francl, 2001). The early appearance of white rust in chrysanthemum was recognized as whitish-yellow spot on the upper leaf surface. The spot was then

developed into central spot and discoloration from white to dark brown. On the lower surface of the leaf, the color of early stage pustule was initially pink. The pustule was then enlarged, white turn into brown. Rust pustules were actually a collection teliospore that might germinate to form basidiospora and infected the plant (Suhardi, 2009).

The effectiveness of Pf Kr 2, Pf Ktl, Pf Smd 2, *Azotobacter* sp and *Azospirilium* sp. against white rust under in vivo conditions were varied. Disease intensity ranged from 0 to 37.78% with incubation period from 7.67 to 38.67 days after planting (DAP) (Fig. 2 & 3). White rust showed delay in incubation period when treated by the isolates. The longest postponement was detected at chrysanthemum plants treated by Pf Kr 2, that prolonged up to 38.67 DAP, followed by Pf Ktl and *Azospirilium* with the periods of 11.67 and 10.33 DAP, respectively.

Fig. 2. (Form left to right) Development of white rust Puccinia horiana attacks symptom on chrysanthemum leaves, from the early visible stage characterized by whitish spot and turn into dark brown with broken pustules.

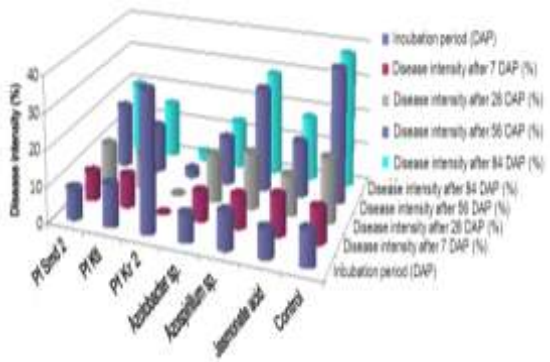

Fig. 3. Development of white rust intensity under different isolates application treatment and control on 7, 28, 56 and 84 days after planting (DAP).

The suppression of Pf Kr 2, Pf Ktl and *Azotobacter* sp. against white rust disease from 7 to 84 DAP were observed more effective compared to other isolates and control (Fig. 3). These viewed from the disease intensity less than 15.55%. The effectiveness of the isolates was also detected at the percentage of suppression that was averaged more than 58.84% from the control. The disease intensity on the plants treated by Pf Kr 2, Pf Ktl and *Azotobacter* sp. was recorded 2.22, 15.55 and 13.33% at 84 DAP with the percent suppression of 94.12, 58.84 and 64.72%, respectively.

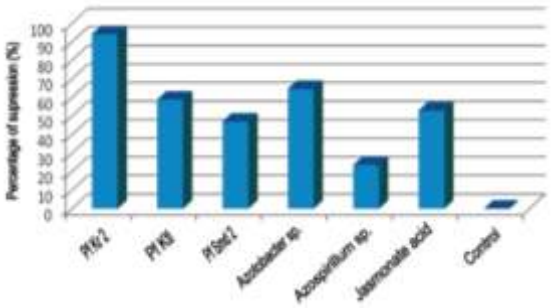

Fig. 4. Percentage of suppression of isolates treatment and control against white rust disease *Puccinia horiana* in chrysanthemum at 84 DAP.

The ethylene concentration on chrysanthemum leaves was seemed to be negatively related with the disease intensity. Higher ethylene production was observed when the disease intensity was high. These phenomenon was observed on the plants treated by Pf Kr 2, in that the disease intensity was the lowest (2.22 %) at 84 DAP (Fig. 3). The ethylene concentration on the plant leaf was the highest that was detected up to 723.935 ppm (Fig. 5).

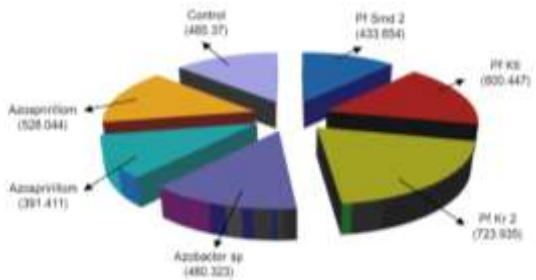

Fig. 5. Ethylene concentration on the leaf of chrysanthemum under different isolate treatment and control at 84 DAP (ppm).

Comparative advantages of each isolate

The comparative advantage of each isolate tretament was determined by the following facts :

a. Certain bacteria isolates were able to supress white rust diseases more effective under in vitro screening. The number of pustule at 7 DAP was categorized as few (23-30 spot/leaf) and the percentage of pustule increment from 1 and 3 to 7 DAA was low (11.11-66.67%). The percentage of supression of bacteria isolate againts white rust disease was measured high (> 65%) (Table 3).

b. Period of incubation of white rust plants was delayed (up to 38.67 DAP) on chrysanthemum plants treated by certain bacteria isolates under in vivo testing. The disease intensity was recorded lower (< 15.55%) at 84 DAP, thus the degree of supression of the isolates againts white rust Puccinia horiana was considered high (> 58%). Ethylene production, as predicted was correlated with the disease intensity. The lower disease intensity reflected the positive reaction of the isolates in systematically protecting the plant from white rust attacks. The plant produced higher ethylene as a response to the non-destructive infection of the bacteria inside the plant body. The higher ethylene concentration was observed up to 528.044–723.935 ppm on chrysanthemum leaf treated by certain bacteria isolates.

The analysis of comparative advantages of each treatment was presented in Table 5. Treatment of isolate Pf Ktl, Pf Smd 2 and Pf Kr 2 had higher total number of comparative advantages form the rest of treatment. Pf Kr 2 showed the presence of all advantage parameters and had the highest, following Pf Ktl which did not affect the disease incubation period during in vivo testing. Based on these analysis of comparative advantages, three *P. flourescens* group isolates namely, Pf Kr 2, Pf Ktl, dan Pf Smd 2 were predicted to have capability to control white rust disease Puccinia horiana in chrysanthemum. The supressive mechanism of PGPR to the disease was thorugh several ways, incuding (a) systemic resistance induction to the plant, (b) production of siderophore, an iron chelat, made the ion unvailable for the pathogen, (c) secondary metabolite synthesis such as enzymes or cyanide that acted as antifungal agent, degrading the cell wall and supressing the growth of fungal pathogen, and (d) space and nutrition competitive abilities againts the pathogen (Beneduzi *et al*., 2012).

Table 5. Comparative analysis of the presence of advantage parameter of isolate treatment againts white rust disease on chrysasntemum under in vitro and in vivo testing.

Treatment	Frequemcy of advantage of each treatment on the parameters								
	Number of pustules at 7 DAP under *in vitro* testing	Percentage of increment on the number of pustule at 7 DAP under *in vitro* testing	Conditons of pustule on certain stage	Degree of supression of isolates againts white rust compared to control under *in vitro* testing	Incubation period under *in vivo* testing	Disease intensity after 84 DAP under *in vivo* testing	Degree of supression of the isolates compared to control under *in vivo* testing	Ethyene production	Total of comparative advantages
Pf. Smd 2	1	1	1	1	0	0	0	0	4
Pf. Ktl	1	1	1	1	0	1	1	1	7
Pf. Kr 2	1	1	1	1	1	1	1	1	8
Azotobacter sp.	0	0	0	0	0	1	1	0	2
Azospirilium sp.	0	0	0	0	0	0	0	0	0
Jasmonate acid	0	0	0	0	0	0	0	1	1
Control	0	0	0	0	0	0	0	0	0

Data on Table 7 indicated that Pf Ktl, Pf Smd 2 and Pf Kr 2 were able to supress white rust attacks in chrysanthemum based on in vitro and in vivo testing. Several reports informed that the action of Pf was related to the production of certain substances that was toxic to the fungal pathogen. Santiago *et al.* (2015) reported that Pseudomonads were the biggest bacterial group that had the capability in producing antibiotic and have been applied as biocontrol agents in many important crops. Pf stain A 506 was succesfully applied to control fire blight on apple (McManus and Jones, 1994), *Gaeumannomyces graminis* var. *tritici* pada gandum (Thomashow and Weller, 1988), *Ralstonia solanacearum* on tomato (Mulya, 1997), *Plasmodiophora brassicae* on chinese cabbage (Hanudin and Marwoto, 2003). *P. flourescens* was reported to be sucessful for controlling stem rot casued by *Phytium* spp. (Gurusidaiah *et al*., 1986) and the combination of Pf and *B. subtilis* was able to reduce destructive attacks of Fusarium wilt in carnation (Hanudin *et al*., 2004). Beside as a biocontrol agent, *P. fluorescens* can also be a solvent phosphate, produced IAA, ACC-deaminase enzyme, and siderophore (Alishahi *et al.* 2013).

Conclusion

In vivo screening of Pf bacteria revealed 3 bacteria isolates; Pf Kr2, KTL Pf and Pf Smd2 that performed more effective againts white rust. Idenification on morpholgical and biochemical features of the isolates had confirmed that the 3 isolates belonged to *P. flourescens* group.

The *in vivo* testing reconfirmed the effectiveness of these three isolates in controlling white rust better than to *Azotobacter* and *Azospirilium*. The ehtylene production on the chryanthemum plants was coincidence with the less disease intesity on the plants treated by these isolates. Based on the in vitro and in vivo testing, isolate of Pf Kr 2 was found to have more comparative advantages than Pf Smd 2 and Pf Ktl.

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References

Acharya T. 2013. Peptidoglycan (Murein/ Mucopeptide): Structure and medical significance. Filed under microbiology for beginners structure of bacterial cells. http://microbeonline.com/peptidoglycan-mureinmucopeptide-structure-and-medical-significance/. Accessed on 23 July 2015.

Alishahi F, Alikhani HA, Heidari A, Mohammad L. 2013. The study of inorganic insoluble phosphate solubilization and other plant growth promoting characteristics of indigenous *Pseudomonas fluorescens* bacteria of Kordan and Gonbad regions. International Journal of Agronomy and Agricultural Research **3 (12)**, 53-60.

Association of Official Analitycal Chemist. 1995. Salicylic acid 336-337.

Baldani JI, Baldani VLD. 2005. History on the biological nitrogen fixation research in graminaceous plants: Special emphasis on the Brazilian experience. Anais da Academia Brasileira de Ciencias **77 (3)**, 549-579.

Bashan Y, Holguin G, de-Bashan LE. 2004. *Azospirilium*-plant relationships: physiological, molecular, agricultural, and environmental advances

(1997–2003). Canadian Journal of Microbiology **50(8)**, 521-577.

Beneduzi A, Ambrosini A, Passaglia LMP. 2012. Plant growth-promoting rhizobacteria (PGPR) : Their potential as antagonists and biocontrol agents. Genetics and Molecular Biology **35 (4)**, 1044-1051.

Bleecker AB, Kende H. 2000. Ethylene : a gaseous signal molecule in plants. Annual Review of Cell and Developmental Biology **16**, 1-18.

Boller T. 1991. Ethylene in pathogenesis and disease resistance. In Mattoo AK and JC Suttle, Ed. The plant hormone ethylene. Boca Raton, FL: CRC Press 293-314.

Bonde MR, Murphy CA, Bauchan GR, Luster DG, Palmer CL, Nester SE, Revell JM, Berner DK. 2015. Evidence for systemic infection by Puccinia horiana, causal agent of chrysanthemum white rust, in chrysanthemum. Phytopathology **105 (1)**, 91-98.

Boshale HJ, Kadam TA, Bobade AR. 2013. Identification and production of *Azotobacter vinelandii* and its antifungal activity against *Fusarium oxysporum*. Journl of Environmental Biology **34**, 177-182.

Chauhan S, Wadhwa K, Vasudeva M, Narula N. 2012. Potential of *Azotobacter* spp. as biocontrol agents against *Rhizoctonia solani* and *Fusarium oxysporum* in cotton (*Gossypium hirsutum*), guar (*Cyamopsis tetragonoloba*) and tomato (*Lycopersicon esculentum*). Archives of Agronomy and Soil Science **58 (12)**, 1365-1385.

Cowan ST, Steel KJ. 1974. Identification of medial bacteria. 2nd Ed. Cambridge University Press, Cambridge 173.

Djatnika I. 2012. Seleksi bakteri antagonis untuk mengendalikan layu Fusarium pada tanaman Phalaenopsis. Jurnal Hortikultura **22 (3)**, 276-284.

Elad Y. 1990. Production of ethylene in tissue of tomato, pepper, french-bean and cucumber in response to infection by *Botrytis cinerea*. Physiological and Molecular Plant Pathology **36**, 277-287.

Farzana Y, Saad ROS, Kamaruzaman S. 2009. Growth and storage root development of sweet potato inoculated with rhizobacteria under glasshouse conditions. Australian Journal of Basic and Applied Sciences **3** (Supplement **2)**, 1461-1466.

Francl LJ. 2001. The Disease Triangle: A plant pathological paradigm revisited. The Plant Health Instructor. DOI: 10.1094/PHI-T-2001-0517-01. Reviewed on 2007. http://www.apsnet.org/edcenter/instcomm/teachingarticle/pages/disease-triangle.aspx. Accessed on 5 May 2015.

Godfrey SAC, Marshall JW. 2002. Identification of cold-tolerant *Pseudomonas viridiflava* and *P. marginalis* causing severe carrot post harvest bacterial soft rot during refrigerated export from New Zealand. Plant Pathology **51**, 155-162.

Goodman RN, Kiraly Z, Wood KR. 1986. The biochemistry and physiology of plant diseases. University of Missouri Press, Columbia 347-368.

Goto M, Yaguchi Y, Hyodo H. 1980. Ethylene production in citrus leaves infected with *Xanthomonas citri* and its relation to defoliation. Physiological Plant Pathology **16**, 343-350.

Gurusidaiah S, Weller DM, Sarkar A, Cook RJ. 1986. Characterization of antibiotic produced by strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces gramminis* var *tritici* and *Pythium* spp. Antimicrobial Agent and Chemoteraphy **29,** 488- 495.

Hanudin, Marwoto B, Djatnika I. 2015. Penyakit karat pada krisan dan pengendalian ramah

lingkungan dalam era masyarakat ekonomi ASEAN. Jurnal Pengembangan Inovasi Pertanian **8(1)**, 11-20.

Hanudin, Marwoto B, Saefullah A, Kardin K, Machmud M. 2004. Formula cair *P. fluorescens* untuk mengendalikan penyakit layu fusarium pada anyelir. Jurnal Hortikultura **14** (Eds. Khusus) 403-409.

Hanudin, Marwoto B. 2003. Pengendalian penyakit layu bakteri dan akar gada pada tomat dan caisim menggunakan *Pseudomonas fluorescens*. Jurnal Hortikultura **13**, 58-66.

Hersanti. 2004. Pengaruh ekstrak beberapa tumbuhan dalam menginduksi ketahanan sistemik tanaman cabai merah (*Capsicum annuum* L.) terhadap *Cucumber Mosaic Virus (*CMV). Disertasi, Sekolah Pascasarjana Universitas Padjadjaran 115.

Kaiser G. 2014. Peptidoglycan synthesis in a gram-positive bacterium. American Society for Microbiology. http://www. microbelibrary. org/library/bacteria/ 3661-peptidoglycan- synthesis-in-a-gram-positive-bacterium. Accessed on 23 July 2015.

Kim JS, Lee J, Lee Ch, Woo SY, Kang H, Seo SG, Kim SH. 2015. Activation of Pathogenesis-related genes by the *Rhizobacterium*, *Bacillus* sp. JS, which induces systemic resistance in tobacco plants**.** Plant Pathology Journal **31 (2)**, 195-201.

Mali GV, Bodhankar MG. 2009. Antifungal and phytohormone production potential of *Azotobacter* chroococcum isolates from groundnut (*Arachis hypogaea* L.) rhizosphere. Asian Journal of Experimental Science **23**, 293-297.

Mali GV, Patil RC, Bodhankar MG. 2011. Antifungal and phytohormone production potential of *Azotobacter* chroococcum isolates from groundnut (*Arachis hypogaea* L.) rhizosphere and their effect on nodulation and dry mass, along with native rhizobia in pot culture experiment. Research Journal of Chemistry and Environment **15 (2)**, 1-7.

Maqqon M, Kustantinah, Soesanto L. 2006. Penekanan hayati penyakit layu Fusarium pada tanaman cabai merah. Agrosains **8 (1)**, 50-56.

McManus PS, Jones AL. 1994. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. Phytopathology **84**, 627-633.

Mulya K. 1997. Penekanan perkembangan penyakit layu bakteri tomat oleh *Pseudomonas flourescens* PFG32. Jurnal Hortikultura **7 (2)**, 685-691.

Porcel R, Zamarreno AM, Jose Maria GM, Aroca R. 2014. Involvement of plant endogenous ABA in *Bacillus megaterium* PGPR activity in tomato plants. BMC Plant Biology **14 (36)**, 1-12.

Price NP. 1999. Carbohydrate determinants of Rhizobium-legume symbioses. Carbohydrate Research **317 (14)**, 1-9.

Raaijmakers JM, Weller DM. 1998. Natural plant protection by 2,4-diacetylphloroglucinol producing Pseudomonas spp. in take-all decline soils. Molecular Plant-Microbe Interactions **11**, 144-152.

Santiago MC, Udaondo Z, Ramos JL. 2015. Draft whole-genome sequence of the antibiotic-producing soil isolate *Pseudomonas* sp. strain 250J. Abstract. Environmental Microbiology Report **7 (2)**, 288-292. doi: 10.1111/1758-2229.12245. Accessed on 23 July 2015.

Santoso SE, Soesanto L, Haryanto TAD. 2007. Penekanan hayati penyakit moler pada bawang merah dengan *Trichoderma harzianum*, *Trichoderma koningii*, dan *Pseudomonas fluorescens* P60. Jurnal Hama dan Penyakit Tumbuhan Tropika **7 (1)**, 53-61.

Schaad NW, Jones JB, Chun W. 2001. Laboratory guide for identification of plant pathogenic bacteria. St. Paul, Minnesota, APS Press 373.

Soesanto L, Rahayunati RF. 2009. Pengimbasan ketahanan bibit pisang Ambon Kuning terhadap penyakit layu Fusarium dengan beberapa jamur antagonis. Jurnal Hama dan Penyakit TumbuhanTropika **9 (2)**, 130-140.

Soesanto L, Rokhlani, Prihatiningsih N. 2008. Penekanan beberapa mikroorganisme antagonis terhadap penyakit layu Fusarium gladiol. Agrivita **30 (1)**, 75-83.

Soesanto L. 2000. Ecological and biological control of Verticillium dahliae. Ph.D. Thesis, Wageningen University Wageningen.

Soesanto L. 2001. *Pseudomonas fluorescens* P60 sebagai Agensia Hayati Jamur *Verticillium dahlia* Kleb. Jurnal Penelitian Pertanian Agrin **5 (10)**, 33-40.

Soesanto L. 2004. Kemampuan *Pseudomonas fluorescens* P60 sebagai agensia pengendali hayati penyakit busuk batang kacang tanah in vivo. Eugenia **10 (1)**, 8-17.

Stojšin V, Balaž J, Budakov D, Stanković S, Nikolić I, Ivanović Ž, Popović T. 2015. First Report of *Pseudomonas syringae* pv. *aptata* Causing Bacterial Leaf Spot on Sugar Beet in Serbia. Plant Disease **99 (2)**, 281-282.

Suhardi, Silvia E, Saefuloh A. 2011. Pengendalian penyakit karat putih (*Puccinia horiana* Henn) pada krisan dengan mikoparasit: Koleksi dan karakterisasi. IOCRI yearly report TA. 2011, 22. (unpublished).

Suhardi. 2009. Sumber inokulum, respons varietas dan efektivitas fungisida terhadap penyakit karat putih pada tanaman krisan. Jurnal Hortikultura **19 (2)**, 207- 209.

Taufiq M, Rahman A, Wahab A, Hidayat SH. 2010. Mekanisme ketahanan terinduksi oleh Plant Growth Promotting Rhizobacteria (PGPR) pada tanaman cabai terinfeksi *Cucumber Mozaic Virus* (CMV). Jurnal Hortikultura **20 (3**), 274-283.

Thomashow LS, Weller DM. 1988. Role of a phenazine antibiotic from *Pseudomonas florescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. Jornal of Bacteriology **170**, 3499-3508.

Thornburn C. 2015. The rise of demise of integrated pest management in rice in Indonesia. Insects **6**, 381-408.

Timmusk S. 2003. Mechanism of action of the plant growth promoting bacterium Paenibacillus polymyxa. Acta Universitas Upsaliensis (UPPSALA). Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 908, 40.

Wojdyla AT. 2004. Development of *Puccinia horiana* on chrysanthemum leaves in relation to chemical compounds and time of their application. Journal of Plant Protection Research **44 (2)**, 91-102.

Yusuf ES, Djatnika I, Suhardi. 2014. Collection and characterization of mycoparasite from rust disease on chrysanthemum. Jurnal Hortikultura **24 (1**), 56-64.

Yusuf ES, Nuryani W, Djatnika I, Suhardi, Winarto B. 2012. Potensi beberapa fungisida nabati dalam mengendalikan karat putih (*Puccinia horiana* Henn) dan perbaikan mutu krisan. Jurnal Hortikultura **22 (4)**, 385-391.