30. VIABILITAS DAN VIRULENSI TUJUH BELAS TAHUN PENYIMPANAN Fusarium oxysporum Schlecht. f.sp. zingiberi Trujillo DALAM TANAH STERIL

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Various pH Media Influence Production of *Pseudomonas fluorescens* P20 Raw Secondary Metabolites for Controlling Damping-off (*Pythium* sp.) in Cucumber Seedlings

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ABSTRACT

This research aims to determine the best pH media for the production of *Pseudomonas fluorescens* P20 raw secondary metabolites, its effect on controlling damping-off, and on cucumber seedling growth. *In vitro* test uses completely randomized design with four replicates and seven treatments consisted of pH 5.0; 5.5; 6.0; 6.5; 7.0; 7.5; and 8.0. *In planta* test uses a randomized block design with three replicates and ten treatments consisting of control, mancozeb 80%, and raw secondary metabolites with pH 7.0 and 7.5, and 4 concentration levels, i.e., 5, 10, 15, and 20%. Variables observe population density, inhibition ability, protease and chitinase qualitatively, germination ability, incubation period, disease incidence, the area under disease progress curve (AUDPC), crop height, number of leaves, root length, and crop fresh weight. The result shows that the best pH for the production of raw secondary metabolites is 7.0, indicated by population density as 5.68×10^{24} cfu/ml, inhibition ability as 50.8%, and the best protease and chitinase qualitatively. Application of the secondary metabolites on pH 7.0 could suppress disease intensity, incubation period, and AUDPC as 66.67, 77%, and 0%-day, respectively, and increase crop height, the number of leaves, root length, and crop fresh weight as 57.65, 37.19, 63, and 74%, respectively.

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the horticultural crops that belong to the Cucurbitaceae family which is widely used by the community. Cucumbers can be used as food, consumption of fresh fruit, cosmetics, industrial raw materials and as raw materials for medicine (Abdurrazak et al., 2013; Sahu and Sahu, 2015). Based on 2018 agricultural statistics, the production and productivity of cucumbers in Indonesia over the past 5 years have decreased (Ministry of Agriculture, 2018). Production and productivity of cucumbers in Indonesia are still relatively low compared to the potential production of hybrid cucumbers which reaches 20 t/ha.

The low productivity is thought to be caused by various factors, including poor seed quality, declining soil fertility, poor cultivation techniques, and problems with plant diseases (Singh et al., 2017). As a result of the presence of plant-pathogen attacks cause stunted plant growth, low crop productivity can even lead to crop failure (Soesanto et al., 2013a). Damping-off caused by *Pythium* sp. is one of the major diseases in cucumber seedlings, especially in the nursery. Seedlings can be damaged or die quickly after being moved to the field due to infection with sprouts (Sutton et al., 2006).

Control measures by farmers usually use pesticides; but the impact is not good for the environment or humans because it can cause soil, air, and water pollution, resistance, damage to the ecosystem balance, resurgence, and various human health problems (Aktar et al., 2009; Al-Zaidi et al., 2011). The use of environmentally friendly pesticides began to be developed through the use of soil microbes as biological agents and bio fungicides. One of the microbes used to control soil-borne pathogens is *Pseudomonas fluorescens* (Panth et al., 2020).

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The growth of biological agents is influenced by various factors, one of which is the degree of acidity (pH), which influences growth, production of secondary metabolites, and colonization of *P. fluorescens* bacteria in plant roots (Abeyratne & Deshappriya, 2018). Propagation media of *P. fluorescens* used must have an optimum pH so that it can produce growth and viability of good biological agents (Wickramasinghe et al., 2019). Based on this, it is necessary to research the best pH for the growth and production of *P. fluorescens* raw secondary metabolites, as well as the effectiveness of *P. fluorescens* raw secondary metabolites in cucumber plants infected with *Pythium* sp.

This study aims to determine the best pH of the media for the production of *P. fluorescens* P20 raw secondary metabolites, the effective pH media of *P. fluorescens* P20 raw secondary metabolites to control damping-off cucumber seedling, and the effect of the raw secondary metabolites application on the growth of cucumber plants.

MATERIALS AND METHODS

Research conducted at the Laboratory of Plant Protection and screen house, Faculty of Agriculture Universitas Jenderal Soedirman in October 2018 to March 2019.

Preparation of P. fluorescens P20

The bacteria of *P. fluorescens* P20 (Soesanto et al., 2013a) were grown on King's B agar. Furthermore, the density of the bacterial population was calculated using the TPC (Total Plate Count) method, i.e., the bacteria were harvested, the bacterial suspension was diluted to a certain level, taken as much as 100 µl and flattened with the L glass and incubated for 24 hours (Sukmawati & Hardianti, 2018).

Preparation of *Pythium* sp.

The pathogen *Pythium* sp. was isolated from cow dung by growing cucumber seeds in cow dung, then the plants affected by fallen seedlings were taken and grown on a PDA medium. Isolate of *Pythium* sp. obtained was later propagated by growing on a PDA medium and incubated for 3 days (Sutton et al., 2006).

Producing Raw Secondary Metabolites

King's B broth was prepared as much as 100 ml for each pH treatment. The media was measured by its pH. The pH treatment was done by adding HCl and NaOH, so that the King's B had 7 pH ranges, 5; 5.5; 6; 6.5; 7; 7.5; and 8 (Shi et al., 2017). In the next step, *P. fluorescens* P20 isolate with a density of 10^9 cfu/ml was put into King's B broth as much as 1 ml. The media was shaken using the Daiki Orbital Shaker at 150 rpm for 2 days (Soesanto et al., 2013a).

Cultivating

The planting media consisted of a mixture of soil and manure (5:1) was put into a polybag. The growth media was inoculated with *Pythium* sp. by placing a 1-cm plug from the leading edge of a colony grown on PDA (Conway, 1985). The seeds were soaked in each treatment for 30 minutes. Furthermore, the seeds were immersed in soil with a depth of 1 cm.

Maintenance

Plant maintenance includes the application of raw secondary metabolites which were carried out once every 3 days as much as 10 ml, drainage was done 7 days after planting (Zuyasna et al., 2009) , watering was done 1-2 times a day done in the morning or evening, weed control was done 1- 2 times every week at the age of 2 and 3 weeks after planting mechanically.

Experimental Design

In vitro test used a completely randomized design with 7 treatments with 4 replicates. The treatments were pH media 7.5 (control), and pH media 5.0; 5.5; 6.0; 6.5; 7.0; and 8. *In planta,* experiment used a randomized block design with 10 treatments and 3 replicates. The treatments were sterile water; fungicide (mancozeb 80%); concentrations of 5, 10, 15, and 20% pH media 7; and concentrations of 5, 10, 15, and 20% pH media 7.5.

Observed Variables

The parameters observed in the *in vitro* study were the population density of *P. fluorescens* P20 (Brugger et al., 2012) as formula 1.

$$\sum \text{Population} = \frac{1000}{V} \times NC \times DL \quad \text{..... 1)}$$

Where: ∑ Population = number of bacterial population (cfu/ml), NC = number of bacterial colonies, V = Volume of distributed suspension, and DL = dilution level. Qualitatively analysis of chitinase and protease was according to (Niranjana & Bavithara, 2020; Saima et al., 2013), respectively; the growth inhibition was calculated by the formula 2 (Ngegba et al., 2018).

$$P = \frac{(r1-r2)}{r1} x100\% \quad \text{..... 2)}$$

Where: P = percentage of inhibition, r1 = the radius of the pathogenic colony growing in the opposite direction to the isolate antagonist, r2 = the radius of the pathogen that grown close to the isolate antagonist). The rate of germination was estimated using the formula (Tian et al., 2014): ∑G/t, where G is the percentage of seed germination at one-day intervals and t is the total germination period.

The parameters observed in the *in planta* study were incubation period, disease incidence, calculation of the magnitude of disease incidence using the formula 3 (Noordzij et al., 2010):

$$DI = \frac{n}{N} x\ 100\ \% \quad \text{..... 3)}$$

Where: DI = disease incidence, n = number of symptomatic plants, N = total plants observed. AUDPC is calculated using Simko & Piepho (2012) our analysis shows that this approach severely underestimates the effect of the first and last observation. To get a better estimate of disease progress, we have developed a new formula termed the area under the disease progress curve (AUDPC) formula 4.

$$AUDPC = \sum_{i=1}^{n-1} \left|\frac{Y_i+Y_{i+1}}{2}\right| (t_{i+1} - t_i) \quad \text{..... 4)}$$

Where: Y_i is an assessment of disease (percentage, proportion, ordinal score, etc.) at the i^{th} observation, t_i is time (in days, hours, etc.) at the i^{th} observation, and n is the total number of observations. Plant growth parameters included root length, plant height, number of leaves, and plant fresh weight.

Data Analysis

Data were analyzed by variance test (F test) at an error level of 5%. If there are real and very real differences, proceed with the DMRT (Duncan's Multiple Range Test) at an error rate of 5%.

RESULTS AND DISCUSSION

The Effect of the Treatments on *P. fluorescens* P20 Density

The population of *P. fluorescens* P20 bacteria on the King's B broth with various pH treatments after 48 hours of shaking show different population densities (Fig. 1). Based on the results (Fig. 1) the highest bacterial population is at treatment P5 (pH 7). The growth of bacteria on the media increases with the increasing pH of the media. However, at certain pH bacterial growth decrease. This is because bacteria require optimum pH for growth (Kim et al., 2018). pH may affect microbial metabolisms that catalyze redox reactions to synthesize ATPs and hence microbial community structures by modulating the thermodynamics and kinetics of redox reactions (Jin & Kirk, 2018). The effect of pH on bacterial growth is also related to the activity of enzymes that catalyze reactions related to bacterial growth. No optimal growth pH can disrupt bacterial growth (Respati et al., 2017). According to Slama et al. (2019), antagonistic bacteria can grow in a wide pH range at pH 5-10, while *P. fluorescens* has the optimum pH for growth that is 7-8. The bacterial population can be seen from the turbidity of the bacterial growth medium after cornering. Bacteria failed to grow at pH below pH 5 and above pH 10.

The results of the statistical analysis indicated significantly different results of the inter-treatments. The best treatment was at pH 7.5, 6.5, 7.0, and 8.0. *P. fluorescens* P20 was able to inhibit the growth of *Pythium* sp. up to 69.25%. These results were in line with the counting bacterial colonies. Based on the calculation of bacterial colonies (Fig. 1), the four pH levels were mediums with a high bacterial population. The presence of clear zones in the antagonist test shows that the hyphal growth inhibition of *Pythium* sp. is caused by *P. fluorescens* P20 with antibiosis mechanisms. This is following (Deveau et al., 2016; Neidig et al., 2011) that *Pseudomonas* sp. can inhibit the growth of fungi because it produces antibiotic compounds (antifungal), siderophore, and other secondary metabolites.

The Effect of the Treatments on Inhibition Ability The Effect of the Treatments on Chitinase and Protease Production Qualitatively

P. fluorescens is an antagonistic bacterium that is known to produce various enzymes, including chitinase and protease (Alhasawi & D. Appanna, 2017; Fajingbesi et al., 2018) are useful in food industries, medically, pharmaceutical industries and even agriculturally. The results of the raw secondary protease enzyme test of *P. fluorescens* P20 are qualitatively seen from the clear zone formed. Based on Table 1, the best results with the largest clear zone are found in pH 7.5 and 7.0 media. This is presumed because at that pH was the optimum growth pH for *P. fluorescens* P20. These results are supported by observations of colony calculations that show that pH 7.0 and pH 7.5 have the highest level of colony density. According to Rodarte et al. (2011), the clear zone in the protease enzyme test

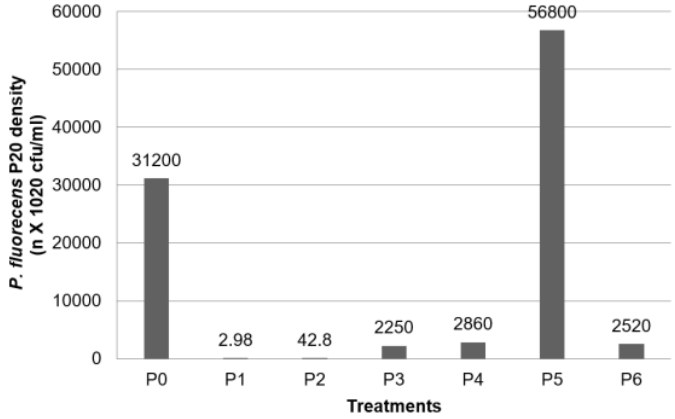
is a sign that bacteria has proteolysis activity, with the loss of casein from the agar proteolysis media. Based on the chitinase enzyme test (Table 1), the best pH was at pH 7.5 and 7.0. In those pH, a wide clear zone is performed compared to other pH's. This is in line with high population densities at pH 7.0 and 7.5. The wider clear zone shows *P. fluorescens* P20 in the treatment has a higher ability to degrade chitin compared to other pH's. According to Alhasawi & D. Appanna (2017), chitinase is an enzyme owned by *P. fluorescens* which functions to degrade chitin.

The Effect of the Treatments on Germination

Germination is calculated based on the percentage of seed that germinates normally. The analysis shows that all treatments are not significantly different from germination (Table 1). The germination test in all treatments of *P. fluorescens* P20 raw secondary metabolites was not significantly different because it could be caused by the growth hormone possessed by *P. fluorescens* P20. *P. fluorescens* produces the hormone Indole Acetic Acid (IAA) (Marathe et al., 2017), and gibberellins (Jain & Das, 2016). Both of these hormones support the germination process. IAA has a role for cell division and the formation of plant tissue (Tank et al., 2015), while the gibberellins hormone which are well-known phytohormones that are involved in regulating seed germination to promote seeds germination (Li et al., 2016), so the seeds will germinate more easily.

The Effect of the Treatments on Pathosystem Components

All treatments tested influence incubation period and disease incidence based on the statistical result (Table 2). The treatments can prolong the incubation period and decrease the disease incidence.



Remarks: P0: pH 7.5 (Control), P1: pH 5.0, P2: pH 5.5, P3: pH 6.0, P4: pH 6.5, P5: pH 7.0, and P6: pH 8.0.

Fig. 1. Population density of *P. fluorescens* P20 bacteria at various pH treatments.

Table 1. The results of the *P. fluorescens* P20 inhibition test against *Pythium sp.*

pH	Inhibition ability (%)	Protease	Chitinase	Germination (%)
7.5	69.25 b	++	++	100 a
5.0	35.41 a	+	+	95 a
5.5	34.07 a	+	+	95 a
6.0	39.50 a	+	+	95 a
6.5	47,91 ab	+	+	95 a
7.0	63.42 b	++	++	100 a
8.0	60.00 b	+	+	100 a

Remarks: Numbers followed by different letters in the same column show a marked difference in DMRT of a 5% error rate; ++: broad clear zone, +: narrow clear zone, -: no clear zone is formed

Incubation Period

The incubation period is calculated from the pathogen inoculation until the onset of initial symptoms. Symptoms of lodging shoot caused by *Pythium* sp., i.e., the lower part of the plant is pale white, shrinks, and the plant falls (Lamichhane et al., 2017). Based on data analysis results between treatment and control (Table 2) show very significant different results on the incubation period of the disease. This was due to the influence of the *P. fluorescens* P20 raw secondary metabolites application and also by the application of fungicides that can inhibit the development of pathogenic *Pythium* sp. Supported by Khabbaz & Abbasi (2014) root rot, and other soil-borne diseases of various crops. In this study, antagonistic bacteria were isolated from a commercial potato field and screened for their growth inhibition of fungal and oomycete pathogens in laboratory tests. The biocontrol potential of the 3 most effective antagonistic bacteria from the in vitro tests was evaluated against seedling damping-off and root rot of cucumber caused by Pythium ultimum. Based on phenotypic characteristics, biochemical tests, and sequence analysis of 16S\ u201323S rDNA gene, the 3 antagonistic bacteria were identified as Pseudomonas fluorescens isolate 9A-14, *Pseudomonas fluorescens* (isolate 9A-14), *Pseudomonas* sp. (isolate 8B-45), and *Bacillus subtilis* (isolate 8B-1) promote plant growth and suppressed Pythium damping-off and root rot of cucumber seedlings in growth-room assays. The short incubation period in sterile water treatment is due to the absence of raw secondary metabolite applications so that pathogens more easily infect the roots of cucumber plants. The other treatments delayed the incubation period by 77% compared to sterile water. Plants show no symptoms of attack by *Pythium* sp. This is presumably because the application of the *P. fluorescens* P20 raw secondary metabolite can suppress the development of pathogens. *P. fluorescens* has several mechanisms that can inhibit the growth of pathogens by producing antibiotics including Phenazine-1-carbocyclic acid (PIC), HCN, and 2,4 diacethylephloroglucinol (Almario et al., 2017; Deveau et al., 2016).

Disease Incidence

The analysis showed that sterile water and other treatments were highly significantly different, but among the treatments are not significantly different (Table 2). The application of raw secondary metabolites and fungicides reduce the incidence of disease by 33.33% compared to sterile water treatment. This is in line with research conducted by Soesanto et al. (2013a) in chilli. Raw secondary metabolites of *P. fluorescens* P60 reduced disease incidence by 60-85%. The application of *P. fluorescens* secondary metabolites reduces the incidence of disease might be related to the mechanism they have. P. *fluorescens* is known to be able to produce secondary metabolites, including siderophore. Siderophore functions as a fungistatic that inhibits the development of pathogenic fungi (Deveau et al., 2016), as well as affects the resistance of plants (Marathe et al., 2017).

Area Under the Disease Progress Curve (AUDPC)

The highest AUDPC value occurred in sterile water treatment. The low AUDPC value is proportional to the low disease progression. The lower the AUDPC value, the lower the ability of pathogens to develop and cause disease in plants (Roylawar et al., 2021). These results are under Soesanto et al. (2013a) study which states that the incidence of disease in control plants is generally higher. It is added by Smolińska & Kowalska (2018), that the high incidence of plant diseases in control is caused by the activity of pathogens which more quickly infects plant tissue and the lack of plant resistance mechanisms to pathogenic infections. Masi et al. (2018) report that plants that are not treated with secondary metabolites of biological control agents caused higher disease events compared to other treatments.

The application of *P. fluorescens* raw secondary metabolites was able to suppress the development of the disease well because of the lower AUDPC value compared to sterile water treatment. The treatment could reduce the incidence of disease by 100%. The high emphasis on the occurrence of disease which is suspected due to the application of the raw secondary metabolites was able to make plants more resistant to seedling disease. *P. fluorescens* antagonist can control plant pathogens by producing antibiotics and siderophores, being able to colonize plant roots and induce plant deficiencies (Soesanto et al., 2013b; Deveau et al., 2016).

The Effect of the Treatments on Growth Components

Based on a statistical result, the treatments given affected plant height, number of leaves,

root length, and weight of fresh plant compared to control (Table 3). The treatments could increase plant growth components.

Crop Height

The results of the analysis show that the observed variables are significantly different in all treatments compared to control (sterile water). The best plant height is at a concentration of 5% pH 7.0 as 57.65% while the lowest plant height is at control (Table 3). Plant height increase is thought to be due to the hormone produced by the *P. fluorescens* P20. According to Marathe et al. (2017) *P. fluorescens* can produce IAA hormones that can support plant growth.

Number of Leaves

The number of plant leaves (Table 3) show significantly different results compared with the sterile water treatment (control). The highest number of leaves is in the treatment of fungicide, a concentration of 5% pH 7.0, and concentration of 10% pH 7.0 as 37.19%, respectively, compared to control. This is thought to be caused by the treatment of *P. fluorescens* P20 raw secondary metabolites that can act as plant protection from the fungal pathogen and as a PGPR, which stimulates plant growth. According to Sivasakthi et al. (2014) and Marathe et al. (2017), the direct mechanisms of PGPR involve nitrogen fixation, phosphorus solubilization, HCN production, production of phytohormones such as auxins, cytokinins and gibberellins and lowering of ethylene concentration.

Table 2. The effect of the treatments on pathosystem components

Treatments	Incubation period (dai)	Disease incidence (%)
Sterile water	6.83 a	66.67 a
Mancozeb 80%	30 b	0 b
Conc. 5% pH 7.0	30 b	0 b
Conc. 10% pH 7.0	30 b	0 b
Conc. 15% pH 7.0	30 b	0 b
Conc. 20% pH 7.0	30 b	0 b
Conc. 5% pH 7.5	30 b	0 b
Conc. 10% pH 7.5	30 b	0 b
Conc. 15% pH 7.5	30 b	0 b
Conc. 20% pH 7.5	30 b	0 b

Remarks: Numbers followed by the same letters in the same column show no significant difference in the 5% DMRT test; dai=days after inoculation.

Table 3. The effect of the treatments growth components

Treatments	PH (cm)	NL	RL (cm)	FP (g)
Control	33.83 a	7.33 a	9.91 a	7.58 a
Fungicide (mancozeb 80%)	77.22 b	11.67 c	20.84 b	26.52 b
Conc. 5% pH 7.0	79.89 b	11.67 c	24.67 b	29.41 b
Conc. 10% pH 7.0	79.56 b	11.67 c	19.78 b	28.31 b
Conc. 15% pH 7.0	69.11 b	10.33 bc	23.06 b	22.99 b
Conc. 20% pH 7.0	62.66 b	9.67 b	26.89 b	20.11 b
Conc. 5% pH 7.5	68.78 b	10.67 bc	18.99 b	22.71 b
Conc. 10% pH 7.5	66.67 b	11.00 c	18.44 b	21.21 b
Conc. 15% pH 7.5	61.78 b	10.67 bc	20.06 b	18.87 b
Conc. 20% pH 7.5	62.33 b	10.67 bc	21.61 b	19.13 b

Remarks: Numbers followed by the same letters in the same column show no significant difference in the 5% DMRT test. PH = Plant Height, NL = Number of Leaves, RL = Root Length, FP = Weight of Fresh Plants.

Root Length

The analysis of plant root lengths shows significantly different results compared to controls. The longest root is shown by the concentration of 20% at pH 7.0 and did not differ from the other concentrations, while the shortest root was in the control treatment. The application of *P. fluorescens* P20 raw secondary metabolites can increase root length by 63% compared to controls. This is presumably because the treatments are able to protect roots from attack by pathogens, so that plant roots can grow well. Soesanto et al. (2013b) state that the increase in root length in the treatment of raw secondary metabolites due to the mechanism of *P. fluorescens* which can suppress the growth of pathogens is also related to its ability to produce growth hormones that can stimulate plant root growth and act as PGPR (Sivasakthi et al., 2014).

Crop Fresh Weight

The analysis of fresh plant weights (Table 3) showed very significant different results. The highest fresh plant weight was at a concentration of 5% pH 7.0 as 74% compared to control, while the lowest one was at sterile water treatment. This proves that the treatment of *P. fluorescens* P20 raw secondary metabolites application stimulate plant growth. This is consistent with the opinion of Sivasakthi et al. (2014) and Soesanto et al. (2013b), that *P. fluorescens* in addition to being able to suppress the growth of pathogens, can also produce growth hormones that can stimulate plant growth.

CONCLUSION AND SUGGESTION

The best pH media for growth and production of *P. fluorescens* P20 raw secondary metabolites is pH 7.0 which has the highest population density of 5.68 x 10^{24} cfu/ml. The pH media is effective to control cucumber seedlings damping off. The raw secondary metabolites of *P. fluorescens* P20 increase plant growth variables including plant height by 57.65%, number of leaves by 37.19%, root length by 63%, and plant fresh weight by 74%. Based on the results of the study it could be suggested that for the growth of *P. fluorescens* and the production of the raw secondary metabolites the King's B broth at pH 7.0 can be used.

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30. VIABILITAS DAN VIRULENSI TUJUH BELAS TAHUN PENYIMPANAN Fusarium oxysporum Schlecht. f.sp. zingiberi Trujillo DALAM TANAH STERIL

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