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Raw Secondary Metabolites of Chitosan-enriched *Pseudomonas fluorescens* P60 to Control Corn Sheath Blight

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Abstract. Corn is a strategic cereal with economic value and always face the corn sheath blight in the field. An alternative safely and environmentally friendly disease control is the use of biological agent *Pseudomonas fluorescens* P60 that produced raw secondary metabolites. This study aimed to test the effectiveness of chitosan enriched *P. fluorescens* P60 raw secondary metabolites against leaf blight and to determine the effect on the growth of maize. The research was conducted at the Laboratory of Plant Protection and Experimental Farm, Faculty of Agriculture, Jenderal Soedirman University. *In vitro* test used a completely randomized design and *in planta* test used a randomized block design consisted of four treatments and six replicates. The treatments consisted of control and the secondary metabolites of *P. fluorescens* P60 enriched with chitosan 1, 2, and 3%. Variables observed were inhibition ability, chitinase and protease analysis qualitatively, incubation period, disease intensity, infection rate, AUDPC, crop height, crop fresh weight, and root fresh weight. The results showed that the secondary metabolites of *P. fluorescens* P60 enriched with chitosan 3% was effective in inhibiting the growth of *Rhizoctonia solani* as 63.3%, produced chitinase and protease, and could control the disease by delaying the incubation period as 79.05%, lowering the disease intensity as 68.68%, lowering the infection rate as 100%, and lowering AUDPC as 83.32%. The secondary metabolites of *P. fluorescens* P60 enriched with chitosan 3% was effective in improving plant growth by increasing plant fresh weight as 33.9% and root fresh weight as 43.09% compared to control. Chitosan could be used for improving effectiveness of antagonistic bacteria raw secondary metabolites. The raw secondary metabolites of bacterial antagonists could be improved their effectiveness against plant diseases by enrichment of chitosan.

Key words: maize; *Rhizoctonia solani*; raw secondary metabolites; *Pseudomonas fluorescens* P60; chitosan

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INTRODUCTION

Maize is a strategic cereal with economic value and has the opportunity to be developed because of its position as the main source of carbohydrates and protein after rice as well as a source of feed (Langner et al., 2019). Until now, Indonesia still needs to import maize to meet the domestic needs (Bourgeois & Kusumaningrum, 2008). Therefore, efforts to increase maize production need greater attention. In fact, in the field, maize cultivation faces various plant diseases, such as those caused by the fungus *Rhizoctonia solani*, which can reduce the production and causes yield losses of up to 100% in susceptible varieties (Karima & Nadia, 2012).

The fungus *R. solani* is a soil-borne pathogen that damages crops, it has high adaptability and can survive in the soil for a long time in the form of sclerotia (Saveinai et al., 2017). Control of maize plant's disease that is often done by farmers is by using chemical fungicides. Controlling disease in this way has a negative impact (Keswani et al., 2019). Therefore, another alternative control is needed, namely the use of biological agents.

One alternative to disease control that is safe and environmentally friendly is to use the biological agent *Pseudomonas fluorescens* P60. These antagonistic bacteria produce raw secondary metabolites containing antibiotics and are PGPR (Plant Growth Promoting Rhizobacter), which actually stimulates plant growth and inhibits the growth of pathogens (Sahu et al., 2018; Alsohim, 2020). The use of these bacteria is reported to provide positive results on growth and production in agricultural crops (Jain & Das, 2016). The addition of chitosan has also proven to be potential in controlling pre- and post-harvest diseases of horticultural crops (Romanazzi et al., 2013).

Chitosan has many benefits in various fields; however, enrichment of *P. fluorescens* P60 raw secondary metabolites towards plant diseases is not understood yet. The purpose of this research was to examine the effectiveness of *P. fluorescens* P60 raw secondary metabolites enriched with chitosan against sheath blight and its effect on the growth of maize. The raw secondary metabolites of bacterial antagonists could be improved their effectiveness against plant diseases by enrichment of chitosan.

METHODS

The research was conducted at the Laboratory of Plant Protection and Experimental Farm, Faculty of Agriculture, Jenderal Soedirman University.

Pathogen exploration

Exploration of *R. solani* was obtained from maize showing symptoms of sheath blight. The samples were isolated on PDA until pure isolates were obtained (Gill et al., 2014).

Preparation of isolate and chitosan

P. fluorescens P60 was prepared by transferring the bacteria using the line method, then growing it on solid King's B media (Lamichhane & Varvaro, 2013). Chitosan (Chitosanpharma, CV. Chimultiguna, Indramayu, Indonesia) solution was prepared for the concentration of 1% by adding 1 L of water mixed with 10 g of chitosan and so for the concentrations of 2 and 3%.

Making raw secondary metabolites

The raw secondary metabolites of *P. fluorescens* P60 were prepared in liquid Kings's B media and added with chitosan powder (according to treatment) then shaken (Daiki Orbital) for 3 days at a speed of 150 rpm at room temperature. Then the density was calculated for the application of the secondary metabolites in the field and the density used was 10^9 cfu mL^{-1} (Han et al., 2012).

Preparation of planting maize

The planting medium used was soil mixed with manure in a ratio of 2: 1, then it was put in a polybag sized 40 x 40 cm. Corn seeds of P20 (pioneer) variety were soaked for 2 hours according to treatment (sterile water and *P. fluorescens* P60 enriched with chitosan 1, 2, and 3%). The seeds were then planted in polybags according to the treatment.

In vitro antagonism test against *R. solani*

Testing for antagonistic activity was using PDA by placing pieces of *R. solani* using a cork borer (5 mm in diameter) 3 cm from the edge of the petri dish. Pieces of sterile filter paper (9 mm in diameter) were dipped in the secondary metabolites of *P. fluorescens* P60 enriched with chitosan according to treatment for ± 1 minute, then drained and placed at a distance of 3 cm from *R. solani* and incubated at room temperature (Donmez et al., 2015). A completely randomized design was used with 4 treatments, namely control and the secondary metabolites of *P. fluorescens* P60 enriched with 1, 2, and 3% chitosan.

Chitinase and protease test

The chitinase and protease tests were carried out by placing a piece of sterile filter paper (1 cm diameter) on 1% chitin-specific and Skim Milk Agar (SMA) media, respectively. A total of 4 pieces of sterile filter paper with a diameter of 1 cm were placed regularly on the surface of the 1% chitin media with details of 3 pieces of filter paper, each with a drop of 5 µl of the *P. fluorescens* P60 raw secondary metabolites enriched with chitosan (1, 2, and 3%) and 1 piece of filter paper dripped with 5 µl of sterile water as a control (Chu, 2012; Putri et al., 2016).

In planta test

In planta testing was carried out in polybags with a randomized block design of four treatments, namely control, the secondary metabolites of *P. fluorescens* P60 enriched with chitosan 1, 2, and 3%, repeated 6 times. *R. solani* were inoculated on the seeds in the soils by attaching 5 cork drills (1 cm in diameter). Inoculation of fungi was carried out simultaneously with planting seeds in polybags (Dutta & Deb, 2020). The infestation of *P. fluorescens* P60 raw secondary metabolites enriched with chitosan in a liquid formula was applied in two ways, namely by soaking the seeds for 2 hours at a dose of 20 ml and spraying directly onto plants with a density of 1.84 x 10^9, 1.96 x 10^9, and 2.18 x 10^9 cfu mL^{-1} for the concentration of 1, 2, and 3%, respectively, as much as 20 mL per crop after planting with 10-day intervals for further spraying up to 4 sprays.

Observed variables

The variables observed included the components of the pathosystem (inhibition, clear zone diameter, incubation period, disease intensity, infection rate, area under the disease progress curve (AUDPC), and growth components (plant height, fresh plant weight, and fresh root weight).

The percentage of inhibition is calculated using the following formula (Bekker et al., 2006): $P = \frac{(r2-r1)}{r2} x\ 100\%$,

where r1 = colony radius of R. solani facing the bacterial colony and r2 = radius facing the edge of the petri dish. Clear zone diameter in chitinase and protease test were measured. The incubation period was observed from the day after inoculation until the appearance of the first symptoms of disease in the test plants (days after inoculation).

Disease intensity observations were carried out every week, since the first symptoms appeared, using the following formula DI = [$\sum$ (v x n)/(Z x N)] x 100%, where: DI = disease intensity (%), v = attack category score, n = number of plants attacked in each

category, Z = the highest attack category score, and N = number of plants observed, with attack category 0 = No symptoms on sheath blight, 1 = sheath blight symptom of 0-20%, 2 = sheath blight symptom > 20-40%, 3 = sheat blight symptom > 40-60%, 4 = sheath blight symptom of > 60% (Ravat et al., 2019). The infection rate was calculated based on the van der Plank formula (1963), namely r = 2.3/t (log 1/(1-Xt) – log 1/(1-Xo)), where r = infection rate, Xo = proportion of initial disease, Xt = proportion of disease at time t, and t = time of observation.The total area under the disease progress curve (AUDPC) is calculated using a formula (Ling et al., 2017).):

$$AUDPC = \sum_{i=1}^{n-1}\left(\frac{X_i + X_{i+1}}{2}\right)(t_{i+1} - t_i)$$

where AUDPC = disease progression curve (%-days), X_i = the incidence of certain disease in the i^{th} measurement, t_1 = time (in days) of measurement i, and n = the total number of measurements.

Data analysis

Data diversity was analyzed using the F test with an error rate of 5%. When significantly different found, continued by HSD (Honest Significantly Difference) at an error level of 5%.

RESULT AND DISCUSSION

Rhizoctonia solani

The isolation results indicated *R. solani* with hyphal characteristics such as hypha that can grow spread on Petri dishes. The young hyphae were white and the old hyphae were brown, the hyphae branching formed an angle of 90°, and formed a sclerotium that spread randomly in the colony. Sclerotia were white when young, then turned brown to black. Sclerotia were initially round in shape then became irregular. Based on the observation of pathogen characteristics, the fungi resulted from the exploration was *R. solani* in accordance with Gill et al. (2014).

The growth of *R. solani* suppressed by the raw secondary metabolites *in vitro*

Based on statistical tests (Table 1), the *P. fluorescens* P60 enriched with 1, 2, and 3% chitosan resulted significantly different in inhibition level compared to control (Figure 1). It is assumed that the secondary metabolites of *P. fluorescens* P60 enriched with 1, 2, and 3% chitosan can inhibit the growth of *R. solani.*

This is according to Neidig et al. (2011) and Raaijmakers and Mazzola (2012) that the antagonist bacteria *P. fluorescens* is reported to be able to produce secondary metabolites, including siderophore, pterin, pyrrole, and phenazine. Siderophores can act as fungistasis and bacteriostasis. Chitosan itself has the ability as an antifungal so that it can inhibit fungal growth and is able to damage the fungal cell walls. This is in line with Palma-Guerrero et al. (2008) and de Oliveira Junior et al. (2015) that chitosan treatment can damage fungal pathogen to experience cellular disorganization ranging from the breakdown of the cell wall to cytoplasmic disintegration.

Table 1. Inhibition (%) of *P. fluorescens* P60 raw secondary metabolites enriched with chitosan against *R. solani*

Treatment	Inhibition level (%)	
control	0	a
P. fluorescens P60, 1% chitosan	56.6	b
P. fluorescens P60, 2% chitosan	58.9	b
P. fluorescens P60, 3% chitosan	63.3	c

Note: Numbers followed by different letters in the same column show a significant difference based on HSD with an error level of 5%.

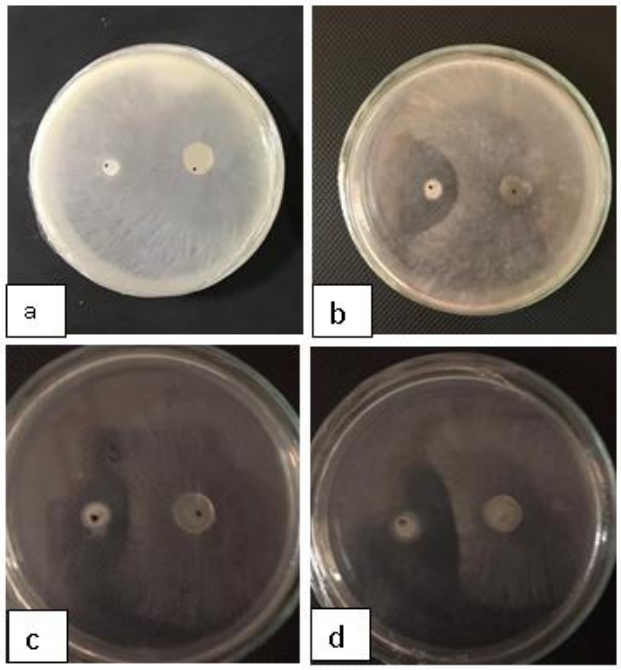

Figure 1. Test of inhibition. Notes: control (a) and the secondary metabolites of *P. fluorescens* P60 enriched with 1% (b), 2% (c), and 3% (d) chitosan.

The secondary metabolites of *P. fluorescens* P60 enriched with 3% chitosan showed the highest inhibition level compared to other treatments. The difference in inhibitory power is caused by differences in the content of active substances. The higher the chitosan concentration, the more content of

active substances so that the inhibitory ability formed is also greater. Pawar et al. (2019) stated that the concentration of antifungal compounds is one of the factors that influence the efficiency and effectiveness of these antifungal agents. Chitosan is also reported to inhibit the proliferation of *Botrytis cinerea*, cause fungal damage and stimulate changes in *Rhizopus stolonifer* and *B. cinerea* cells (Meng et al., 2010).

The secondary metabolites enriched with chitosan on chitinase production

The results of statistical analysis of *P. fluorescens* P60 secondary metabolites enriched with chitosan 1, 2, and 3% were significantly different from the control in producing chitinase and protease (Table 2). The ability of *P. fluorescens* P60 secondary metabolites enriched with chitosan to produce chitinase enzymes was indicated by a clear zone around the colony resulted in chitinase activity. Each treatment has a different ability to produce chitinase. This is in accordance with Al Ahmadi et al. (2008), that the presence or absence of chitin can be seen from the presence or absence of clear zones formed around bacterial colonies when grown on chitin agar medium. This indicates that the isolate secretes the chitinolysis enzyme outside. The different diameters of each isolate indicates the different activity of each chitinolytic enzyme secreted.

Chitosan as a source of carbon and nitrogen for bacterial growth can stimulate chitinase production (Berger et al., 2012). As explained by Zhang et al. (2018), the presence of a substrate can stimulate a microorganism to secrete its cell metabolites. Enzymes will react when there is a substrate.

Table 2. Result of *P. fluorescens* P60 raw sedondary metabolites with different concentration of chitosan on chitinase and protease

Treatment	Clear zone diameter	
	chitinase (mm)	protease (mm)
control	0 a	0 a
P. fluorescens P60, 1% chitosan	6.3 b	14.5 b
P. fluorescens P60, 2% chitosan	7.5 b	16.16 b
P. fluorescens P60, 3% chitosan	9.83 b	20.16 b

Note: Numbers followed by different letters in the same column show a significant difference based on HSD with an error level of 5%.

The secondary metabolites enriched with chitosan on protease production

Positive results are indicated by the presence of a clear zone around the bacterial colony in SMA media. The qualitative test results showed that the secondary metabolites of *P. fluorescens* P60 enriched with chitosan was proteolytic bacteria because it was able to produce protease. SMA media contains peptone and skim milk as the main carbon sources for the metabolic needs of bacteria (Maitig et al., 2018). The results of statistical tests for the enzyme activity of *P. fluorescens* P60 secondary metabolites enriched with chitosan 1, 2, and 3% were significantly different from the control (Table 2). Enzyme activity can be influenced by activators (substances that increase enzyme activity) and inhibitors (substances that decrease enzyme activity). In accordance with the opinion of Ethica et al. (2018), the reaction speed catalyzed by enzymes is strongly influenced by the material and the substrate concentration.

Effect of *P. fluorescens* P60 secondary metabolites enriched with chitosan on the components of the pathosystem

Symptoms of sheath blight were found on leaf blades in the form of blight that slightly reddish and turn gray (Figure 2). The infection started from the lowest leaf midrib and continued upward. The statistical test results of the incubation period of *P. fluorescens* P60 secondary metabolites enriched with 1, 2, and 3% chitosan showed a significant effect compared to control (Table 3).

Figure 2. Symptoms of sheath blight. (A) treatment of the secondary metabolites, and (B) control (Source: Personal Documentation).

Table 3. The effect of treatments on testing the incubation period, disease intensity, infection rate, and AUDPC

Treatment	Incubation period (dai)	Disease intensity (%)	Infection rate (per unit per day)	AUDPC (%-days)
control	6.99 a	26.6 b	0.0067 b	103.33 c
P. fluorescens P60, 1% chitosan	15.65 b	8.76 a	0.0007 a	63.77 b
P. fluorescens P60, 2% chitosan	25.53 c	9.22 a	0.00035 a	61.86 b
P. fluorescens P60, 3% chitosan	33.36 d	8.33 a	0 a	11.45 a

Note: Numbers followed by different letters in the same column show a significant difference based on HSD with an error level of 5%. dai = days after inoculation.

The rapid appearance of sheath blight symptoms in the controls was due to the aggressiveness of the pathogen. The secondary metabolites of *P. fluorescens* P60 enriched with 3% chitosan had the longest incubation period by 79.05% compared to control. Giving different chitosan concentrations will have a different effect on the incubation period. In line with what Hadrami et al. (2010), that the difference in the concentration of chitosan used affects the parameters of disease progression. The secondary metabolites of *P. fluorescens* P60 was also able to inhibit the growth of *R. solani*. This is in accordance with the statement of Soesanto et al. (2019), *P. fluorescens* P60 was able to delay the incubation period of disease, reduce disease incidence, and reduce the number of end pathogens.

The disease intensity of the secondary metabolites enriched with chitosan 1, 2, and 3% was significantly different (67.07, 65.34, and 68.68%, respectively), compared to control (Tabel 3). The effectiveness of the *P. fluorescens* P60 secondary metabolites is because of the secondary metabolites content, including siderophores, pterine, pyrrole, and phenazine. In addition, chitosan has an active group that binds to microbes so that chitosan can inhibit microbial growth (de Souza et al., 2015).

The infection rate of *P. fluorescens* P60 secondary metabolites enriched with chitosan 1, 2, and 3% had a significant effect compared to control (Table 3). The secondary metabolites were able to reduce the rate of infection. Chitosan molecules have toxicity and can inhibit the development and growth of fungi. These results are consistent with Berger et al. (2012) and Zhang et al. (2018) that chitosan can stimulate chitinase production and can stimulate a microorganism to secrete its cell metabolites.

The secondary metabolites of *P. fluorescens* P60 enriched with chitosan 1, 2, and 3% had a significant effect in AUDPC compared to the control treatment (Figure 3). The secondary metabolites can suppress development of the pathogen so that it can reduce the rate of infection. In line with Hamadayanty et al. (2012) and de Souza et al. (2015), that chitosan can damage fungal cell walls. In addition, *P. fluorescens* P60 produced a number of activities that can inhibit pathogen development, resulting in low AUDPC values.

The secondary metabolites of *P. fluorescens* P60 which was enriched with 3% chitosan resulted in the lowest AUDPC value of 88.92% compared to the control (Table 3). This may be due to the provision of the 3% chitosan concentration used that is more precisely when compared to other treatments. It is in line with what was said by Wang et al., (2012) that the difference in the concentration of chitosan used had an effect on the parameters of disease progression and inhibit fungal growth.

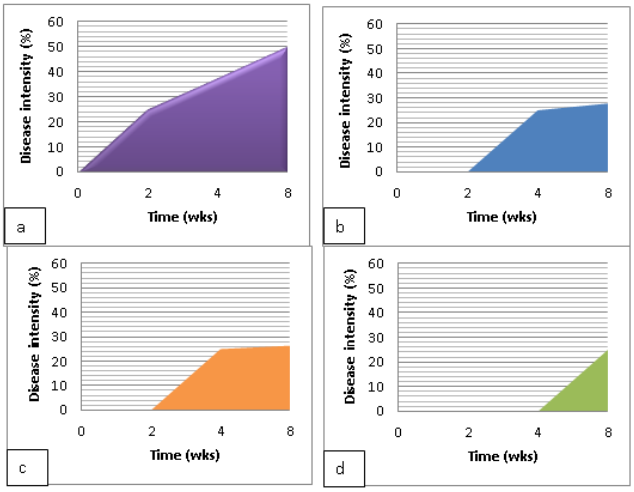

Figure 3. AUDPC value for leaf blight. Information: control (a) and the secondary metabolites of *P. fluorescens* P60 enriched with 1% (b), 2% (c), and 3% (d) chitosan.

Effect of the secondary metabolites enriched with chitosan on the growth components

The results of statistical tests showed that plant growth did not show any differences between treatments (Table 4). The use of *P. fluorescens* P60 secondary metabolites enriched with chitosan did not have a high effect on plant height. This is presumably because the auxin hormone produced by *P.*

fluorescens P60 does not work optimally in environments with high light intensity so that the auxin hormone becomes inactive and inhibits plant elongation. In line with the opinion of Nurnasari & Djumali (2012), auxin work is strongly influenced by the presence or absence of sunlight. The auxin hormone decomposes when exposed to sunlight.

The fresh weight of plants treated with *P. fluorescens* P60 secondary metabolites enriched with chitosan 1, 2, and 3% showed a significant difference with an increase of 29.65-33.9% compared to control treatment (Table 4). The high yield weight was in line with the low disease intensity which proved that the secondary metabolites was able to suppress development of the pathogens. As stated by Soesanto et al. (2019), antagonists can stimulate plant growth and can also increase plant-induced resistance. The use of *P. fluorescens* P60 is very effective in accelerating seed germination and seed vigor so that it will affect the level of plant development and further to plant fresh weight.

Table 4. Effect of treatment on growth components

Treatment	Crop height (cm)	Crop fresh weight (g)	Root fresh weight (g)
control	55.27 a	196.65 a	81.62 a
P. fluorescens P60, 1% chitsan	60.14 a	279.52 b	116.74 b
P. fluorescens P60, 2% chitsan	63.04 a	295.70 b	126.94 bc
P. fluorescens P60, 3% chitsan	63.66 a	297.49 b	143.43 c

Note: Numbers followed by different letters in the same column show a significant difference based on HSD with an error level of 5%.

In addition, chitosan is also able to increase plant growth because it plays a role in improving plant metabolism. According to Kumar (2000), chitosan has reactive N groups and is able to increase the performance of the N element in the soil and the hydrophilic properties of chitosan to help absorb water in the soil. Water and N elements in the soil are external factors that can accelerate plant growth effectively if they are met (Ohta et al., 2004).

The statistical test results of fresh root weight of plants treated with the secondary metabolites enriched with chitosan 1, 2, and 3% were significantly affected compared to the control (Tabel 4). This is thought to be due to the mechanism of the secondary metabolites, to produce growth hormones that can stimulate plant root growth and act as PGPR in addition to suppressing pathogens. In line with Viti et al. (2010), *P. fluorescens* P60 produces IAA which can be used as the main criterion in selecting PGPR, because IAA affects root length, root surface area and the number of root tips. In addition, chitosan can also increase plant growth and development by stimulating auxin biosynthesis (IAA) from tryptophan. Chitosan molecules can function as plant growth promoters and come from environmentally friendly polysaccharides (Chakraborty et al., 2020).

The highest root fresh weight was found in the secondary metabolites of *P. fluorescens* P60 enriched with 3% chitosan by 43.09% compared to the control (Tabel 4). As stated by Fu et al. (2015), IAA hormone at certain concentrations in plants can stimulate plant root growth and increase apical dominance so that plants can grow taller. The addition of chitosan to the secondary metabolites of *P. fluorescens* P60 can improve the effectiveness and ability of the secondary metabolites. The raw secondary metabolites of bacterial antagonists could be improved their effectiveness against plant diseases by enrichment of chitosan.

CONCLUSION

The secondary metabolites of *P. fluorescens* P60 enriched with chitosan 3% was effective in inhibiting the growth of *Rhizoctonia solani* as 63.3%, produced chitinase and protease, and could control the disease by delaying the incubation period as 79.05%, lowering the disease intensity as 68.68%, lowering the infection rate as 100%, and lowering AUDPC as 83.32%. The secondary metabolites of *P. fluorescens* P60 enriched with chitosan 3% was effective in improving plant growth by increasing plant fresh weight as 33.9% and root fresh weight as 43.09% compared to control.

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