THE EFFECTIVENESS OF RHIZOBACTERIA AS BIOPROTECTANTS TO MITIGATE FUSARIUM WILT DISEASE AND AS BIOSTIMULANTS TO IMPROVE THE GROWTH OF CHILI (*Capsicum annuum*)

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ABSTRACT

The aims of this study were to examine the potential of Rhizobacteria as bioprotectants and biostimulants on chili. The potential as bioprotectants was tested by the ability of these Rhizobacteria to inhibit the growth of the pathogenic fungus *Fusarium oxysporum* f.sp. *capsici* in vitro. Moreover, as biostimulants was tested by the ability of these rhizobacteria to produce IAA compounds and their ability to increase the plant growth parameter of chili. Four rhizobacteria were challenged for antagonistic activity against *F. oxysporum* f.sp. *capsici.* Chili seeds in this experiment were soaked in four different Rhizobacteria suspension for 15 minutes. For control, seeds were soaked with sterile water instead of Rhizobacteria suspension. Results of this experiment showed that four isolates of Rhizobacteria showed strong inhibitory activity against *F. oxysporum* f.sp. *capsici* on PDA medium. Percentage of inhibitory activity varied from 77,33% to 89,79%. The application of four isolates recorded significantly increased the plant growth parameters of chili. The plant height, number of leaves, leaf area, root length, shoot dry mass, root dry mass, and chlorophyll content on treated plants significantly higher than those of untreated control plants according to the Duncan's multiple range test (P<0.05%). These results indicate that the tested Rhizobacteria can be used as bioprotectants because they can inhibit the growth of *F. oxysporum* f.sp. *capsici* and simultaneously as a biostimulant for the ability to produce IAA compounds and stimulate the growth of chili.

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INTRODUCTION

Chili is an important and popular commodity in Indonesia which has a high economic value. This spice is one of the most widely used for household consumption, industry, and in food processing (Ismawati, 2016). However, the average productivity of chili in Indonesia is low. According to the Central Bureau of Statistics (2022), in 2021 national chili production in Indonesia decreased by 8.09% compared to the production in 2020. The national chili production decreased to 1.39 million tons in 2021 while in 2020 the national chili production was 1.5 million tons. One of the factors that lower chili production in Indonesia is because of fusarium wilt disease invasion.

Wilt disease on chili commonly caused by the fungal infection *Fusarium oxysporum* f. sp *capsici*. The use of synthetic fungicides to control *F. oxysporum* f.sp. *capsici* has been widely practiced and proven less effective in controlling this fungus. Besides, an excessive amount of applied synthetic fungicides lead to severe environmental pollution. One of the efforts that become a moral responsibility for us to reduce the use of synthetic fungicides is to utilize a more environmentally friendly approach like Rhizobacteria that potentially act as bioprotectants and biostimulants. As bioprotectants Rhizobacteria can produce substances that have biological activity as antimicrobials that act to protect plants from pathogents. While as biostimulants, Rhizobacteria can increase plant growth because they produce substances that have biological activity as growth promoters.

The purpose of this study was to examine the potential of Rhizobacteria as bioprotectants by challenging their ability to inhibit the growth of *F. oxysporum* f.sp. *capsici* in vitro and to examine the potential of rhizobacteria as biostimulants by testing their ability to produce IAA compounds and mediated the growth of chili.

MATERIALS AND METHOD

Time and Place of Research

This research was conducted at the Plant Protection Laboratory, Faculty of Agriculture, Udayana University from April to August 2022. The materials used in this study were 4 Rhizobacteria: *Pantoea agglomerans* TR78, *Klebsiella pneumoniae* KC66, *Klebsiella pneumoniae* SW04, *Stenotrophomonas maltophilia* KC29, PDA medium (4g Potato extract, 20 g sugar, 15g agar, Oxoid ltd., CM139) and chili seeds (Bara, East-West Seed). Chlorophyllmeter SPAD-502, Minolta Japan was used to measured the leaves chlorophill content.

The Potential of Rhizobacteria as Bioprotectant

The potential of Rhizobacteria as bioprotectants was examined by testing their ability to inhibit the growth of the pathogenic fungus *Fusarium oxysporum* f.sp. *capsici* in vitro. Inhibition testing was carried out based on the method of Mayadianti et al. 2020, namely first, preparation of growth media by pouring 10 ml of PDA media on a Petri dish. Furthermore, the fungus *F. oxysporum f.sp. capsici* was inoculated on PDA media, in the middle of the Petri dish, then each rhizobacteria, namely P. agglomerans TR78, K. pneumoniae KC66, K. pneumoniae SW04, and S. maltophilia KC29 were inoculated in 4 positions flanking the fungus, each 2 cm away from the fungal colony.

Each treatment was repeated 5 times. The design used in this study was a completely randomized design (CRD) with 5 treatments and 5 replicates. The treatments were the control fungus *F. oxysporum f.sp. capsici* (KTL0), rhizobacteria P. agglomerans TR78 + *F. oxysporum f.sp. capsici* (TR78), rhizobacteria K. pneumoniae KC66 + *F. oxysporum f.sp. capsici* (KC66), rhizobacteria K. pneumoniae SW04 + *F. oxysporum f.sp. capsici* (SW04), and rhizobacteria S. maltophilia KC29 + *F. oxysporum f.sp. capsici* (KC29). The growth rate of fungal colonies was determined using the equation (1). Determination of the percentage of rhizobacterial inhibition against fungal growth is determined by equation (2)

$$\text{Colony growth rate} = \frac{\text{Colony area at last observation}}{\text{Time interval}} \quad \text{.....(1)}$$

$$\text{Inhibition} = \frac{\text{Control colony area - Colony area of treatment}}{\text{Control colony area}} \times 100\% \text{.....(2)}$$

Test of Rizobacterial Potential as Biostimulant

The potential test of rhizobacteria as biostimulants was conducted by testing the ability of rhizobacteria to produce IAA compounds and testing their ability to increase the growth of chili plants. The test of the ability of rhizobacteria to produce IAA was conducted by colorimetric method based on Wahyudi et al. (2011). Rhizobacteria were grown for 48 hours in Nutrient Broth medium added with 1 mM L-tryptophan. The rhizobacterial culture was centrifuged at 1610 x g for 30 min, then the supernatant was separated from the bacterial sediment and filtered with Millipore filter paper (0.45 mm). Furthermore, IAA content in the filtrate was detected using Salkowski reagent (1 ml 0.5 M FeCl3 and 49 ml 35% HClO4). A total of 1 ml of rhizobacterial filtrate was put into a 2 ml eppendorf tube and then 1 ml of Salkowski reagent was added. The eppendorf tube containing the solution was incubated in a dark room at room temperature for 30 minutes. A change in the color of the solution in the eppendorf tube to pink indicates that the rhizobacteria produce IAA.

Test the ability of rhizobacteria in increasing the growth of chili plants was carried out by invigorating chili seeds with 2% suspension of each rhizobacteria. The 2% concentration was obtained from 2 ml of culture in PPDC media (5 g potato extract, 5 g peptone, 10 g dextrose, 1000 ml Coconut water) per 100 ml of water. The design used in this study was a randomized group design (RAK) with 5 treatments and 5 replicates. The treatments were control (KTRL); seed invigoration with P. agglomerans TR78 suspension (PAGS); seed invigoration with K. pneumoniae KC66 suspension (KLPE); seed invigoration with K. pneumoniae SW04 suspension (KLPN); seed invigoration with S. maltophilia KC29 suspension (STMA). Each replicate consisted of 10 plants. Seeds were then planted in pots that had been filled with planting media. Growth parameters observed were plant height, number of leaves, root dry weight, crown dry weight, root length, leaf area, and leaf chlorophyll content. Measurement of leaf chlorophyll

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content (SPAD unit) was determined with Chlorophyllmeter SPAD-502.

RESULTS AND DISCUSSION

Test of Rizobacterial Potential as Bioprotectant

The test results of the ability of rhizobacteria to inhibit the growth of *F. oxysporum f.sp. capsici* in vitro showed that rhizobacteria were able to inhibit the growth of *F. oxysporum f.sp. capsici* fungal colonies with the percentage of inhibition ranging from 77.33% to 89.79% (Table 1). The area of fungal colonies in the TR78 treatment amounted to 285.77 mm2 with a percentage inhibition of 77.33% when compared to the control. While the colony area in the SW04 treatment amounted to 267.82 mm^2 with a percentage inhibition of 78.76%, the colony area in the KC29 treatment amounted to 188.21 mm^2 with a percentage inhibition of 85.07%, and the colony area in the KC66 treatment amounted to 128.79 mm^2 with the highest percentage inhibition of 89.79% when compared to the control.

Table 1. The results of the rhizobacterial ability test in inhibiting the growth of the fungus *F. oxysporum f.sp. capsici* in vitro

Treatments	Fungal colony area (mm^2)	Colony growth rate (mm^2/day)	Inhibition (%)
KTL0	1261,01 a*	180,14 a	-
TR78	285,77 b	40,82 b	77,33
KC66	128,79 d	18,39 d	89,79
SW04	267,82 b	38,26 b	78,76
KC29	188,21 c	26,88 c	85,07

* Values in the same column followed by the same letter are not significantly different (p>0.05) according to Duncan's Multiple Range Test at the 5% level.

Fungal colonies of *F. oxysporum f.sp. capsici* in the control treatment grew normally with a colony growth rate of 180.14 mm2/day (Figure 1 and Table 1). While the fungal colonies in the rhizobacterial treatment grew abnormally or inhibited. This can be seen in the low value of fungal colony area which is a manifestation of fungal growth. The lower the area value of fungal colonies, the higher the inhibitory value of rhizobacteria against fungi. Fungal growth in the rhizobacterial treatment was abnormal due to the presence of antifungal compounds produced by rhizobacteria through the mechanism of antibiosis. Fungal growth in the rhizobacterial treatment was inhibited with colony growth rates ranging from 40.82 mm^2/day to 18.39 mm^2/day.

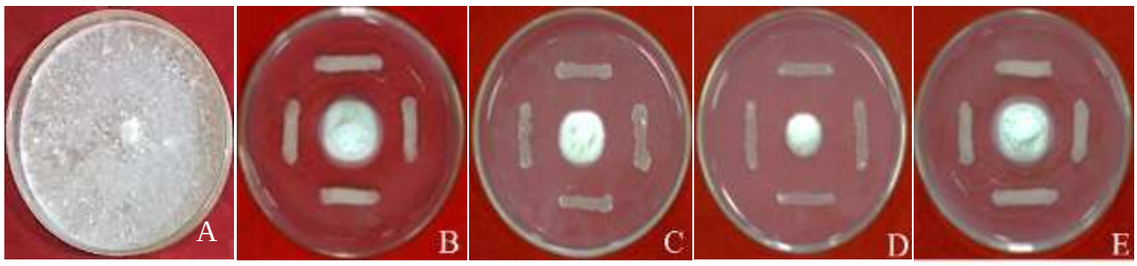

Figure 1. Test the ability of rhizobacteria in inhibiting the growth of fungus *F. oxysporum f.sp. capsici* in vitro A. KTL0 treatment; B. TR78 treatment; C. KC29 treatment; D. KC66 treatment; and E. SW04 treatment

Test of Rizobacterial Potential as Biostimulant

The results of colorimetric tests on rhizobacterial cultures showed that 4 tested rhizobacterial cultures experienced a color change from clear to pink (Figure 2). Meudt and Gaines (1967) reported that Salkowski reagent contains 35% HClO4 and 10 mM FeCl3 and when reacted with rhizobacterial cultures containing IAA there will be a color change from colorless to pink indicating that IAA is oxidized by HClO4. Rahman et al. (2010) reported that the color change to pink indicates that IAA is oxidized by HClO4 and then oxidized IAA reacts with FeCl3 to form tris-(indole-3-acetato) iron (III) complex. Kamnev et al. (2001) reported that the compound tris-(indole-3-acetato) iron (III) complex has the molecular formula FeC30H24N3O6 or Fe[(C8H6N)-CH2COO]3). Meudt and Gaines (1967) reported that IAA, 5-Hydroxyindole-3-acetic acid, and indole-3-acetamide compounds will be pink when reacted with Salkowski reagent. Colorimetric method can be used to determine the concentration of IAA, where the pinker the test solution, the higher the concentration of IAA in the solution. This is because one molecule of Fe will bind three molecules of IAA to form a tris-(indole-3-acetato) iron (III) complex.

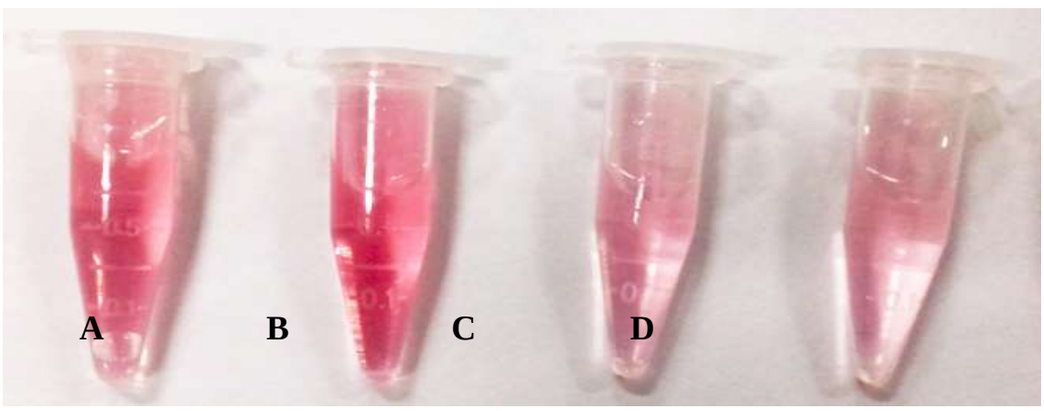

Figure 2. Colorimetric test results on rhizobacterial cultures. A. Filtrate of S. maltophilia KC29; B. Filtrate of P. agglomerans TR78; C. Filtrate of K. pneumoniae KC66; D. Filtrate of K. pneumoniae SW04.

The test results of the ability of rhizobacteria in increasing the growth of chili plants showed that the four tested rhizobacteria were able to increase the growth parameters of chili plants (Table 2). The rhizobacterial treatments were able to

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increase plant height ranging from 30.21% to 42.55% when compared to the control. PAGS treatment increased plant height by 42.55%, KLPE treatment by 34.04%, KLPN treatment by 36.59%, STMA treatment by 30.21% when compared to the control. Some researchers also reported similar results that rhizobacteria can increase plant height. Park et al. (2015) reported that the treatment of B. amyloliquefaciens IN937a, B. cereus BS107, B. pumilus INR7, B. subtilis GB03 on K. daigremontiana plants was able to increase plant height by 20.0% when compared to the control. Rathaur et al. (2012) reported that the treatment of rhizobacterial isolate PG9 increased the plant height of W. somnifera by 22.76% when compared to the control. Asghar et al. (2002) reported that the treatment of B. juncea plant seeds with rhizobacterial isolate S54 was able to increase plant height by 56.54% when compared to the control. Asghar et al. (2004) reported that treatment of Brassica napus plant seeds with rhizobacterial isolate S58 which produced 24.03 µg ml-1 IAA increased plant height by 39.86% when compared to the control.

Table 2. The results of the rhizobacterial ability test in increasing the growth parameters of chili plants

Treatments	Plant heaight (cm)	Leaves number (helai)	Leaves area (cm^2)	Root dry mass (g)	Shoot dry mass (g)	Chlorophyll content (SPAD)	Root length (cm)
KTRL	23,5 a	10,33 a	29,40 a	0,206 a	0,740 a	35,83 a	28,77 a
PAGS	33,5 b	13,67 a	33,85 b	0,547 c	1,693 b	43,91 b	23,67 b
KLPE	31,5 b	12,98 a	31,76 b	0,316 b	1,137 b	41,34 b	26,33 b
KLPN	32,1 b	12,24 a	33,38 b	0,322 b	1,150 b	42,67 b	29,50 a
STMA	30,6 b	12,54 a	31,47 b	0,351 b	1,173 b	42,87 b	25,23 b

Note: values followed by different letters in the same column indicate significantly different based on Duncan's test ($P < 0.05$).

Rhizobacteria treatment was able to increase the number of leaves ranging from 18.48% to 32.33% when compared to the control. PAGS treatment increased plant height by 32.33%, KLPE treatment by 25.65%, KLPN treatment by 18.48%, STMA treatment by 21.39% when compared to the control. Some researchers also reported similar results that rhizobacteria can increase the number of leaves. Rathaur et al. (2012) reported that the treatment of rhizobacterial isolate PG10 increased the number of leaves of Withania somnifera plants by 21.62% when compared to the control. Rajan and Radhakrishna (2013) reported that the treatment of Klebsiella sp. LEE19 which produced IAA 212.3 µg ml-1 increased the number of leaves of Hibiscus rosasinensis plants by 334.28% when compared to the control. Yaqoob et al. (2013) reported that the treatment of Azospirillum sp. G1 producing IAA in Triticum aestivum plants increased the number of leaves by 75% when compared to the control. Gusmaini et al. (2013) reported that the treatment of bacterial isolate 5 MD on Andrographis

paniculata plants increased the number of leaves by 116.67% when compared to the control. Sudharani et al. (2014) reported that the treatment of Azotobacter chroococcum Az on cabbage plants increased the number of leaves by 25.68% when compared to the control.

Rhizobacterial treatments were able to increase leaf area ranging from 7.04% to 15.13% when compared to the control. PAGS treatment increased leaf area by 15.13%, KLPE treatment by 8.02%, KLPN treatment by 13.53%, STMA treatment by 7.04% when compared to the control. Some researchers also reported similar results that rhizobacteria can increase leaf area. Sivasankari et al. (2014) reported that the application of 5 ml suspension of B. cereus suspension with the addition of 2 mg ml-1 tryptophan in the media increased the leaf area index of cowpea plants aged 15 HST by 127.45% when compared to the treatment of 5 ml of B. cereus suspension without the addition of tryptophan. cereus suspension without the addition of tryptophan. Gholami et al. (2009) reported that seed coating treatment of corn seeds with a suspension of P. putida DSM291 with a population density of 108 cfu ml-1 increased leaf area by 190.56% when compared to the control. Karakurt and Aslantas (2010) reported that spraying a suspension of B. subtilis OSU-142 with a population density of 109 cfu ml-1 at first bloom and full bloom of apple plants increased leaf area by 13.71% when compared to the control. Turan et al. (2014) reported that treatment of cabbage seeds with Pantoea agglomerans RK-92 increased leaf area by 27.25% when compared to the control.

Rhizobacterial treatments were able to increase root dry weight ranging from 53.39% to 165.53% when compared to the control. PAGS treatment increased root dry weight by 165.53%, KLPE treatment by 53.39%, KLPN treatment by 56.31%, STMA treatment by 70.38% when compared to the control. Some researchers also reported similar results that rhizobacteria can increase root dry weight. Qi et al. (2016) reported that six strains of B. thuringiensis increased the dry weight of tomato plants ranging from 35.3% to 201.9% when compared to the control. Reetha et al. (2014) reported that B. subtilis which produces IAA 12.67 µg/ml on Nutrient Broth media increased the dry weight of onion plant roots by 15.47% when compared to the control. While Zahid et al. (2015) reported that the treatment of bacterial isolate HJR3 which produces IAA increased the dry weight of roots by 211.11% when compared to the control. Swain et al. (2007) reported that B. subtilis CM4 which can produce IAA 2.1 mg/l with the addition of 0.1 g/l tryptophan in Nutrient broth increased the dry weight of roots and crowns of Dioscorea rotundata plants by 254.54% and 291.67% when compared to the control. Khalid et al. (2004) reported that the treatment of IAA-producing rhizobacteria isolate Ha21 on wheat cultivar Pasban-90 plants can increase root dry weight by 11.43% when compared to the control.

Rhizobacteria treatment was able to increase the crown dry weight ranging from 53.64% to 128.78% when compared to the control. PAGS treatment increased the crown dry weight by 128.78%, KLPE treatment by 53.64%, KLPN treatment by 55.40%, STMA treatment by 58.51% when

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compared to the control. Some researchers also reported similar results that rhizobacteria can increase crown dry weight. Cakmakci et al. (2007) reported that barley seed application treatment with Paenibacillus polymyxa RC05 which produces IAA 32.8 µg/ml in Tryptic Soy Broth (TSB) media with the addition of 25 mg tryptophan increased the crown weight by 54.23% when compared to the control. Ekinci et al. (2014) reported that watering treatment with Bacillus megaterium KBA-10 suspension which produces IAA 6.7 ng/µl in Tryptic Soy Broth increased the dry weight of cauliflower plant crowns (Brassica oleracea L.) by 45.38% when compared to the control.

Rhizobacterial treatment was able to increase leaf chlorophyll levels ranging from 15.37% to 22.55% when compared to the control. PAGS treatment increased leaf chlorophyll content by 22.55%, KLPE treatment by 15.37%, KLPN treatment by 19.09%, STMA treatment by 19.64% when compared to the control. Some researchers also reported similar results that rhizobacteria can increase leaf chlorophyll levels. Mahmood et al. (2016) reported that seed coating treatment of Mung Bean seeds with Bacillus drentensis P16 which produces IAA 3.86 mg/l in Luria Bertani Broth media without the addition of tryptophan increased chlorophyll content by 25.19% when compared to the control. Igbal et al. (2016) reported that seed coating treatment on corn seeds with rhizobacteria isolate MA-11 which produced IAA 12.08 µg/ml on GPM broth media without the addition of tryptophan increased the total chlorophyll content by 13.56% when compared to the control. Yildirim et al. (2015) reported that PGPR treatment in cabbage plants increased leaf chlorophyll by 3.43% when compared to the control. While Turan et al. (2014) reported that treatment of cabbage plant seeds with Bacillus megaterium TV-91C increased leaf chlorophyll by 5.74% when compared to the control. Erdogan et al. (2016) reported that the treatment of Rhodococcus erythropolis RC9 which produced 22.6 µg ml-1 IAA increased leaf chlorophyll of strowberry plants by 28.82% when compared to the control.

The rhizobacterial treatments of P. agglomerans TR78, S. maltophilia KC29 and K. pneumoniae KC66 were able to reduce the root length while K. pneumoniae SW04 was able to increase the root length of chili plants (Figure 3). The rhizobacterial treatments of P. agglomerans TR78, S. maltophilia KC29 and K. pneumoniae KC66 reduced root length by 17.72%, 12.30%, and 8.48% respectively when compared to the control while K. pneumoniae SW04 increased root length by 2.53% when compared to the control. The rhizobacterial treatment was able to reduce and increase the root length presumably due to differences in the concentration of IAA compounds produced by the rhizobacteria. Loper and Schroth (1986) reported the same research results that rhizobacteria are able to reduce root length and increase root length depending on the level of concentration of IAA compounds produced by rhizobacteria, the higher the IAA produced by rhizobacteria, the higher the reduction power of primary root length, where the percentage of reduction power of primary root length of sugar beet plants in the rhizobacterial treatment of P. fluorescens E8 which is high in concentration. fluorescens E8 which

produces IAA 0.57 µg ml-1, rhizobacterial treatment of enterobacteriaceae group isolate 111 which produces IAA 0.79 µg ml-1, rhizobacterial treatment of P. fluorescens B10 which produces IAA 1.22 µg ml-1 respectively by 0.84%, 9.25%, 10.46% while the treatment of P. putida A1 which produces IAA 0.44 µg ml-1 is able to increase root length by 2.88%.

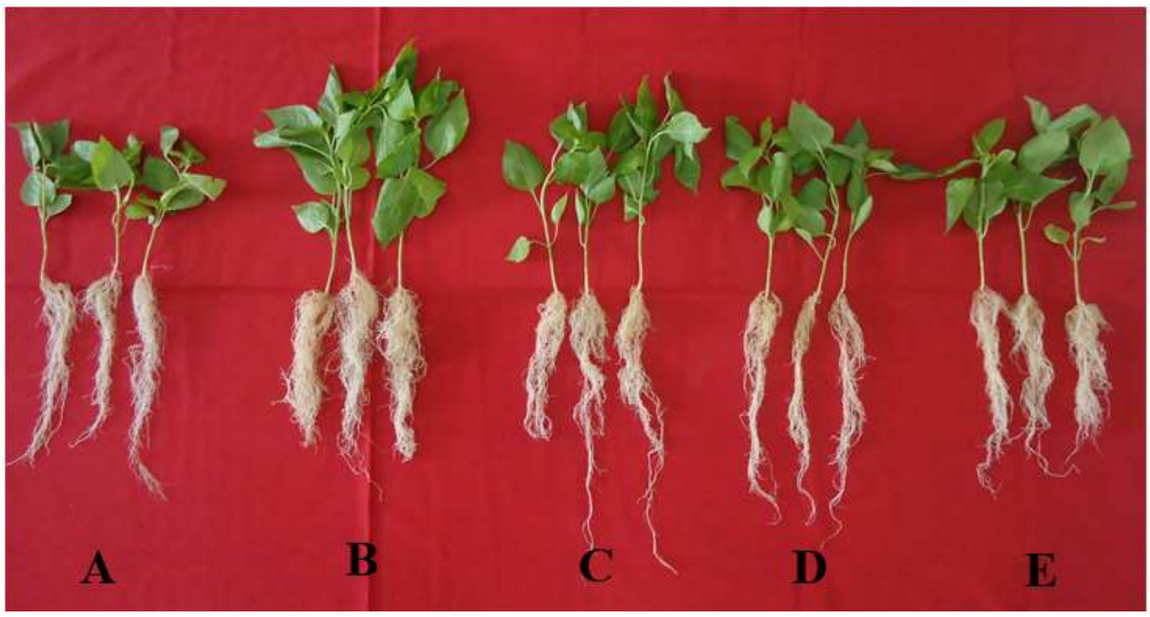

Figure 3. The results of the rhizobacterial ability test in increasing the growth of chili plants A. KLPE treatment; B. PAGS treatment; C. KLPN treatment; D. KTRL treatment; and E. STMA treatment.

Shahzad et al. (2010) reported that the treatment of rhizobacterial isolate JI22 can reduce root length with a reduction power of 8.06% when compared to the control. Kim and Mulkey (1997) reported that IAA in high concentrations will reduce the rate of root elongation, this is because IAA in high concentrations can induce the formation of ethylene and it is known that one of the ways ethylene works is to inhibit the process of elongation of plant cells. Four species of rhizobacteria tested, namely P. agglomerans TR78, K. pneumoniae KC66, K. pneumoniae SW04, and S. maltophilia KC29, have the potential as bioprotectants because they produce substances that are antifungal against the fungus *F. oxysporum f.sp. capsici* with an antibiosis mechanism and have the potential as biostimulants because they are able to produce IAA compounds in colorimetric tests using the Salkowski reagent and are able to increase the growth of chili plants.

CONCLUSION

The results of this study concluded the four tested rhizobacterial species have potential as bioprotectants because they are able to inhibit the growth of the fungus *F. oxysporum f.sp. capsici* with inhibition ranging from 77.33% to 89.79% when compared to the control. Moreover, the four tested rhizobacterial species have potential as biostimulants because they are able to produce IAA compounds in colorimetric tests using Salkowski reagent and are able to increase chili plant growth parameters such as plant height, number of leaves, leaf area,

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root dry weight, crown dry weight, leaf chlorophyll content, and root length.

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