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by Sulaeha Thamrin

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THE DEVELOPMENT OF FORMULA BASED ON TROPICAL ACTINOMYCETES TO PREVENT PEPPER STEM ROT DISEASES (A CASE OF ATTACK PEPPER (*PHYTOPHTHORA CAPSICI*) STEM ROT DISEASE)

RANTE HERLINA[1]*, ALI ALIMUDDIN[2] AND THAMRIN SULAEHA[3]

[1]Pharmacy Faculty, Hasanuddin University, Makassar, Indonesia
[2]Biology Department, Makassar Public University, Makassar, Indonesia
[3]Faculty of Agriculture, Hasanuddin University, Makassar, Indonesia

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Key words : *Phytophtora capsici, Streptomyces sp WGKP, pepper, biofungicide*

Abstract–The research to gain a biofungicide formula as a biological agent for suppressing and controlling pepper pathogen fungi has been conducted. This research was carried out by formulating active ingredient of the extract and biomass strain of *Streptomyces* sp WGKP. The research was using randomized block design (RBD) with 4 treatments and 6 replications. Application of formula was done by spraying of substances include (A) partial purified extract + 1Kg of carrier materials, (B) biomass (108 CFU/g carrier materials), (C) commercial fungicide, and (D) controls. The parameters were observed such as percentage of infection, the effectiveness of treatment, antifungal activity and viability of biomass to the shelf life. The results revealed that there were no differences in the percentage of infections among all of treatment, but significantly different from control on a field scale trial. The same thing is shown by the effectiveness of the treatment on the field scale trial. However, in tests *in planta* showed that the effectiveness of the treatment was significantly different between treatments. Therefore, our results suggest that the application of extracts and biomass from the strain WGKP as an active biofungicide material is very prospective to control and prevent the spread of fungi *Phytophthora capsici*.

INTRODUCTION

Commodities pepper, which is the mainstay of exports Indonesia, cannot be separated from production constraints which indicate that we are relatively lagging competitiveness with other countries such as Sri Lanka and India. This is caused by the agro-industrial system, especially in maintaining quality standards is still behind compared with the countries of the world pepper exporter (Lamour and Hausbeck, 2004; Sarma, 2003).

Particularly in Sulawesi pepper production continues to decline from year to year due to pests and diseases, especially pepper stem rot diseases caused by *Phytophthora capsici*. Widespread attacks *P. capsici* on pepper in 2005 reached 67% compared to other organisms. Meanwhile, the amount of loss caused by the disease at the beginning of 2006 amounted to 4.9 billion and the end of year 2007 increased to 19.5 billion dollars. The deadliest attack if the fungus attacks the base of the stem and roots of the plant. Pepper stem root disease can also attack any part of the leaf that causes spotting occur at the end, the middle or the edge of the leaf. Young plants and plants that have been outstanding for more than two years can be attacked by this disease. Various attempts have been made to control the disease, but have not given the expectations and showed satisfactory results. Biological control of soil-borne pathogens is an option that needs to be developed, because it is relatively cheap and easy to do, as well as environmental friendly (Sarma, 2003). The use of biological control agents derived from bacterial antagonists have been reported. *Pseudomonas fluorescens* and *Bacillus subtilis* are antagonistic bacteria that widely used as biological agents for several fungal and bacterial plant pathogens (Rajan

***Corresponding author's email**: h_rante@yahoo.co.id
Address : Rante Herlina, Pharmacy Faculty, Hasanuddin University, Makassar, 90-245, Indonesia, Tel: 22 +62-81241-31874

et al., 2002; Kim *et al*., 2006). However, the use of *Streptomyces spp*. groups has not been widely reported.

MATERIALS AND METHODS

Isolation of *P. capsici*

Borne fungi (*P. capsici*) isolated from the pepper plants affected by stem rot. Isolation is done by cutting the roots, stems and leaves to the size of 1cm^2. Pieces samples grown on Sabaorud agar and PDA media by plating the sample on the surface of agar media (Truong and Burgess, 2010). Plates were incubated at 30^{o}C for 4 days to grow visibly colonies/hypae around the sample pieces. Colony isolates that showed characteristic of *P.capsici* transferred to the same media repeatedly to obtain pure colonies. Pure fungal isolates that have been grown on PDA agar medium slant then stored in the refrigerator as stock.

Re-cultivation of Streptomyces WGKP strains

Strain WGKP that used as a source of active biofungicide material is isolates that have been known to inhibit fungal. Strain were re-cultivated on starch casein agar (SCA) media by using pour plate method and incubated at 35^{o}C for 7 days. Colonies that grew are replicated on the same media by streak culture method.

Production of antifungal compound

Production of antifungal compounds was done by batch fermentation system where strains were grown in media starter (inoculum) with composition (20g soluble starch, 0.5g NaCl, 1g KNO3, 0.5g K2HPO$_4$.3H$_2$O, 0.5g MgSO$_4$.7 H$_2$O, 0.01 g FeSO$_4$.7H$_2$O, 1000 mL of distilled water (pH 7.2 to 7.4). Sterilization media was done at a temperature of 121^{o}C at 1 atm pressure for 15 minutes. Production of antifungal compounds was done by using media such as media starter but added yeast extract and glucose 0.2 and 2% respectively. Production medium was carried out in a 500 mL Erlenmeyer flask, containing 150 mL. Furthermore, production medium was inoculated with 1%v/v WGKP strain at a concentration of cells reached 7.8×10^6 CFU/mL. Each fermentation flask was incubated at 30^{o}C for 8-9 days with shaking at speed of 150 rpm. After incubation was complete, the supernatant separated from the biomass by filtration. Liquid obtained was taken and used for the partial separation of antifungal compounds.

Production of biofungicide

The medium used for the production of biofungicide compound was ISP media consist of (10 g malt extract, 5 g peptone, 0.1 g NaCl; pH 7). Media was inoculated with 1%v/v strain at a concentration of cells reached 7.8 x106 CFU/ml. Each flask was incubated at room temperature for 7 days with shaking at the speed of 150 rpm. Media was separated by filtration to disengage biomass with supernatant. The resulting biomass was used as a biofungicide material, while the fermented liquid extracted for a partial separation of antifungal compounds (Rante *et al*. 2010).

Formulation of biofungicide

Biofungicide material in the form of partial purified extract was dissolved in 5% DMSO mixed with carrier material, which consists of : 1Kg talc powder (sterilized), 15g of calcium carbonate (to adjust pH) and 10 g of CMC (adhesive), mixing process was performed in aseptic conditions as described by Nandakumar *et al*. 2001. Biofungicide material thoroughly mixed and dried to below 20% moisture before use. The treatments used are labeled as (A) 1 gram of partially purified extract+1 Kg carrier, (B) viable cells (10^8 CFU/g carrier materials), (C) commercial fungicide and (D) untreated (used as control).

Application of biofungicide

Application was done by spraying method on the pepper plants in each treatment plot (field scale). Spray application was done 2 times a week for a month. Further observations were made at the third month after the application. At the end of the study, the calculation of the percentage of infection and the effectiveness of plant resistance were performed.

In the treatment plots *in planta* (polybag), infection with the fungal plant pathogens was done by spray (spore concentration was 1.5 x 10^6 CFU/ mL). Spray application of pathogenic fungi was applied 2 times a week. Furthermore, spray application with the treatment (extracts and biomass) was performed as treatment field scale limited to 2 times a week for a month. Observations were made on the third month after the spray application.

The stability and viability test of the biofungicide active materials

The stability of the biofungicide active materials in a carrier was a benchmark of the biofungicide

expired time. The longer the period expired of biofungicide if the stability and viability of the active ingredient is not decreased. Method of testing the stability and viability of the active material are based on the modified method carried out by Badji *et al.* 2006. The stability test was performed by determining the antifungal activity against pathogenic fungi. The viability test was done based on shelf life. Biofungicide was kept in a room temperature of 25°C. The stability and viability were monitored every month for 4 months

Data Analysis Techniques

The data were calculated based on the percentage of infection, i.e. by the following formula (Suastika *et al*. 2005; Ponmurugan and Baby, 2006):

$$P = \frac{\Sigma I}{\Sigma L} \times \frac{100}{F}$$

Where, P is percentage of index infectious, ˜I is sum of all individual, ˜D is total number of leaves and F is value of maximum infection. Infections value is calculated based on the 6-point scale as follows: 0 is no infected leaves, 1 is 1% of leaf infected, 3 is 2-10% of leaf infected, 5 is 11-25% of leaf infected, 7 is 26-50 % of leaf infected and 9 is more than 50% of leaf infected.

The effectiveness of treatment is calculated using the formula (Djaya *et al*., 2003):

$$Et = \frac{(C - IAT)}{IAC} \times 100$$

Where, Et is Effectiveness of treatment, IAC is intensity of attacks on control (without spray) and IAT is intensity of the attacks on the treatment plant

RESULTS AND DISCUSSION

The fungi (*P. capsici*) isolated (**Fig. 1A**) from stem root desease has the same profile with standard fungi obtained from the Faculty of Agriculture, Hasanuddin University. Based on the morphology analysis, the isolates fungi were further used as the material for the fungal pathogens test. Biofungicide in the form biomass formula isolated from Streptomyces WGKP 150 strains were obtained by multiplying the biomass in semisolid media. The results indicate that strains WGKP grow with characteristic of bushy, white mycelium and covering all surfaces of semisolid media on the 8th day of the incubation period. Spore formation optimally looked after an incubation period of up to 12 days is marked by a color change of mycelium from white to gray. Nevertheless, the formation of partly mycelium color into black is an indication of exudate formation and maturation of spores. This is consistent with thecharacter shown by WGKP strains on the SCA media that appear to have gradations of color formation from white to gray to jet black after incubation up to 2 weeks.

The analysis number of cells revealed that at the end of the incubation period, the viable cell obtained at an average of 2.5×10^9 CFU/g growing media. This showed that the semi-solid media formulation used quite well as propagation medium for the spore of WGKP strain. As a material for biofungicide formula, the semi-solid media obtained then were extracted to release spores from growing medium. The result of extraction with physiological saline solution then analysed to determine the number of viable cells. Cell analysis showed that the extraction results obtained an average of 1.7×10^8 CFU/mL.

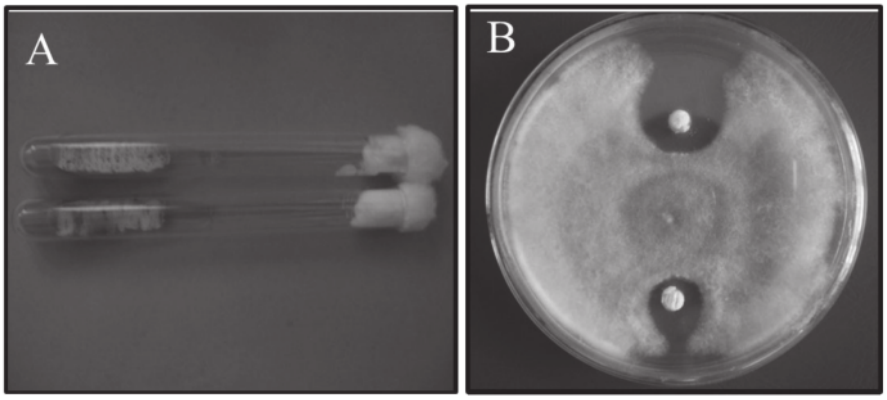

Fig. 1. (A). Isolates from WGKP strain and (B) antagonist test against fungi *Phytophthora capsici*

This indicates that the numbers of these cells are used to produce prospective biofungicide formula.

Biofungicide total volume of material production reaches 50 liters. Production was carried out during the 10-day incubation period and seen that the formation of mycelium / biomass at the end of the incubation appearing as clumps biomass. Furthermore, the production of active compounds was performed by extracting production media using ethyl acetate. The active compound used in the experiments is the result of the combined fractions. The fraction that is used is characterized by the formation of a brownish yellow color which recognized as antifungal active spot. Spot that showed a brownish yellow color as an indicator of the active compound tested to ensure the target antifungal activity. *In vitro* test results showed that the extracted compounds are active against the fungi test marked by the formation of a clear zone around the paper disks. Retrieved 2 fractions (A2 and A3) inhibit fungal growth in *in vitro* test. The test results are used as the basis of the use of the active fraction biofungicide material formula.

Formula obtained in part is not completely soluble in water. Hence, the process of homogenization (blender) is needed. However, there were particles that settle to the bottom of the water if left a few minutes. Therefore we need a stirring process periodically if it will be used for the spray application. The application process of biofungicide in field scale is limited focusing on the healing process or controlling pepper plants which are begin being attacked by fungal pathogens. During the first month of treatment, both the biomass and the active compounds showed signs of recovery from the attack of pathogenic fungal. This is evident from the reduced diameter of the spot or colonies of fungus on the leaves of pepper slowly with increasing time of application. Observations on the third month showed that the percentage of infections result no significant observations from the 3 treatment which are (A) the extract, (B) biomass and (C) a fungicide, while the controls were significantly different (Table 1). This is evident in the analysis of the effectiveness of treatment showed that the extract and biomass is quite effective in suppressing the development of pathogens.

The results of effectiveness analysis are yet to be declared correlated with the percentage of infections suppression on field scale because pathogenic inoculation process was not done. Nevertheless the result may be references to extrapolate the percentage of infection, such as biomass and extract treatment were able to suppress the development of infection significantly. As reported by Ali *et al*. 2011 that WGKP strain has the ability to inhibit all groups of fungi (board spectrum), which showed a large zone of inhibition on the test antagonist with unicellular and multicellular fungi test.

Statistical analysis of the percentage of infection shows that there is difference among the treatments, but differed very significantly with controls (Table 1). This suggests that the use of biofungicide WGKP strain was able to suppress the growth of pathogenic fungal. To determine percent effectiveness of biofungicide in application on fungi attack case, then it can be summarized that biofungicide quite effective in suppressing the growth of pathogens. This can be seen from the tests done on the effectiveness of the treatment *in planta* test was different significantly with control in all treatments. But there were no significantly different between extracts and biomass. Therefore, it was concluded that biofungicide WGKP strain can be used either extract or biomass.

When compared with the standard fungicide, the data showed that the use of fungicides significantly different with all treatments. Contrary to the limited field-scale trials which showed that there were not different among all treatments. This might be due to the treatment on a scale *in planta* are more controlled compared to the field scale. Another factor that is

Table 1. The effect of treatment against fungal pathogenicity components in pepper plants

Treatment	The average effectiveness of field-scale treatment (%)	The percentage of infection (%)	The average effectiveness of treatment *in planta* (%)
Extract (A)	15,67[a]	31,62[a]	10,90[a]
Biomass (B)	18,33[a]	34,81[a]	10,72[a]
Fungicide (C)	21,7[a]	21,21[a]	21,83[b]
Control (D)	0,12[b]	47,10[c]	0,31[c]

Note: Data were followed by the same letter in the same column indicate no real different $p>0.05$.

believed to influence was the rain. Because rain can wash the treated material so the concentration is less than the test *in planta*. To get information about the effect of shelf life against antifungal activity of biofungicide formula, the viability test was performed. The results are shown in the form of diameter inhibition zone against fungal test. It showed that the formula which uses partially purified compounds (treatment A) were decreasing in activity after a month storage period (Fig. 2).

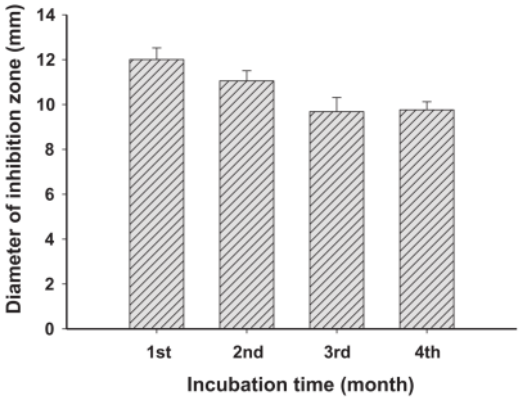

Fig. 2. The relationship between diameter inhibition zone and shelf life of biofungicide materials

The average diameter of inhibition zone began to decrease in the first month of shelf life decreased from 12.00 mm to 11.05 mm. The activity even decreased to 9.76 mm at 4 months of storage. Storage period on the 4th month already approached expiration, meaning that there are only about 1.76 mm inhibition from 8 mm paper disks used. This indicates that the extracts of antifungal biofungicide could not survive stored for more than 4 months, so the use of purified extract can only be used a maximum of 3 months during storage.

In this study, the formula used for application is before 1 week old, so the prediction that the use of biofungicide materials in the 3rd month still needs to be done further testing. This is to determine the effectiveness of biofungicide despite the decreasing in activity after 3-month shelf life.

The shelf life determination of the biofungicide materials is useful to determine the ability of biofungicide material after the storage period. The test was conducted using a pour plate method which showed the viability of WGKP strains was respectable. This is evident from viability cell remains high despite stored for 4 months. Based on the data in Fig. 3 shows that in the first month of storage, the average of biomass material viability cell reached 4,,e108 CFU/gram. This number are maintain even after 4 month which showed the number of viable cells reached 4.25,,e108 CFU/gram.

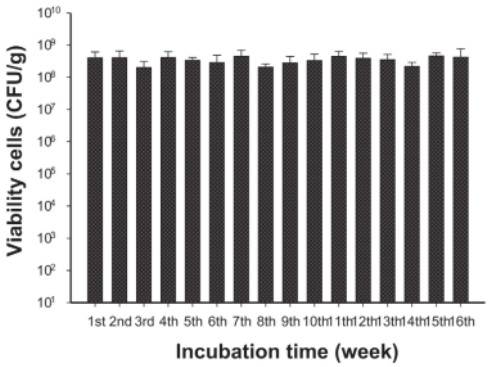

Fig. 3. The relationship between viability cell and shelf-life of biomass biofungicide.

This result is much better when compared to the shelf life of the purified extract that reached expiration after 3 month. The ability and activity of WGKP strain were caused by the high durability of spore in isolates used. One characteristic of Streptomyces is the formation of spores that can survive in extreme environmental conditions.

Long shelf life capability of these strains showed that the fungicide made from biomass is quite good to use as a material for biofungicide formula. Although it is not certain that the biomass storage durability, but it can be presumed that this biomass can last more than 1 year. This is indicated by the number of surviving cells is equal to the first month of storage even though it is saved after 4 month. Statistical analysis showed that no difference in the viability of the strain after a storage period of 16 weeks.

CONCLUSION

Based on the research results, it can be summarized that the use of biofungicide active material from Streptomyces WGKP strains and active antifungal extract are able to suppress the growth of pathogenic fungal that attack pepper plants (*P.capsici*). The biofungicide active materials (from biomass) show no decreasing in ability to grow after

4 months of storage. Therefore, biofungicide from active WGKP strain can be used as a candidate of biofungicide formula in controlling fungal of pepper plants.

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