Effectiveness of Fungal and Bacterial Isolates from Rhizosphere of Passion Fruits againts *Fusarium oxysporum* f. sp *passiflorae in Vitro*

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Abstract: Fusarium wilt caused by *Fusarium oxysporum* f.sp *passiflorae* is the most important disease on passion fruit that causes yield losses ranging from 50 to100%. The disease is difficult to control because the pathogen systematically infected plants and can survive up to five years in the soil in the absence of its hosts. The objective of this paper was to select potential antagonistic microbes in suppressing the growth of *Fusarium oxysporum* f.sp *passiflorae* (*Fop*) *in vitro*. Antagonists were isolated from the rhizosphere of purple passion fruit (*Passiflora edulis* form e*dulis*) and sweet passion fruit (*Passiflora* sp) from Gowa and Makassar. To obtain the best isolates, their ability to inhibit the growth of Fop and their production of cellulase, chitinase, pectinase as well as toxine compound were tested *in vitro*. The results showed that out of 22 fungal and bacterial isolates tested, four and three isolates respectively, gave an excellent growth inhibition to *Fop*. Highest percentage of growth inhibition was provided by fungal isolate U_1 (86.11%) and bacterial isolate Mb_2 (76.44%). The highest cellulase, chitinase and pectinase enzyme production were observed on fungal isolate U_1, followed by isolates U_7, M_1, M_2 and M_4**.** Only two bacterial isolates Ub_1 and Mb_3 showed highest cellulase, chitinase and pectinase enzyme production. The presence of toxin was detected by using a thin layer chromatography on fungal isolates U_1, M_4 and Mb_3. HCN compound from bacterial isolates were obtained from isolates Ub_1 and Mb_3.

Keywords: Fusarium wilt; antagonist microbes; biological control

1. Introduction

In Indonesia, passion fruit is widely grown by small-scale farmers for subsistence and commercialization. It has a potential to alleviate rural poverty. The major passion fruit growing areas in South Sulawesi are Gowa, Sinjai and Tana Toraja district, but Gowa serve as center area for purple passion fruit plantation and beverage industry. Based on our field observation, there are some problems causing reduction of passion fruit production. Beside the wilt disease that

attacked 5,915 plants from 9,820 plants in 2005, and increase up to 6,465 plants in 2006 (Dinas Pertanian, 2007).

Diseases are the major passion fruit production constraints worldwide. The low productivity in South Sulawesi is mainly due to diseases like Fusarium wilt (*Fusarium oxysporum* fsp.*passiflorae*) and brown spot (*Alternaria passiflorae*) (Gardener, 1989). The increase in disease incidence resulting from continuous cultivation in some areas can cause yield losses of 40-60 %. Between 2003-2006, production of passion fruits decreased from 18.780 to 7.519 ton (BPS, 2007).

The fungus is both seed and soil borne. The fungus produce resting spores (*chlamydospores*) that survives as in soil or in diseased plant tissues. Infected seeds harvested from wilted plants are a primary source of inoculums. The disease is characterized by sudden wilting of passion plants followed by drying and death of entire plants. Some control techniques have been used for suppressing the wilt disease such as sanitation, plant rotation and use of fungicide. However, none of the methode is able to reduce the intencity of wilt disease significantly. Biological control is an alternative control measures, which is effective in controlling plant pathogens and at the same time it is safer compared to synthetic fungicide. (Soesanto, 2008). Therefore, the specific objectives of this study was to evaluate the effectiveness of fungal and bacterial antagonist isolated from purple passion fruit and sweet passion fruit rhizospheres in inhibiting the growth of *Fusarium oxysporum* f. sp. *passiflorae in vitro* and the production of enzymes, toxine and volatile hydrogen cyanide (HCN).

2. Materials and Methods

2.1 Isolation and Growth Condition

Six rhizosphere soil samples of purple and sweet passion fields were collected from different areas in Gowa district and Makassar to isolate fungal and bacterial antagonist. In order to screen them for the presence of potentially antagonistic strains of fungi and bacteria, one gram of soil sample was placed in a 250 mL conical flask containing 100 mL of sterile distilled water and mixed thoroughly. Different dilutions of working samples were prepared by serially diluting the stock solutions. Fifty microlitres of each of the dilutions was spread on Patato Dextrose Agar (PDA) and on King's mediaum B (KB) agar as described by Dhingra and Sinclair (1995). A large number of bacterial and fungal colonies were developed. Morphologically different colonies were selected and purified by sub-culturing

2.2 Dual Cultures Assay

A total of twenty two fungal and bacterial isolates (U_1, U_2, U_3, U_4, U_5, U_6, U_7, U_8, M_1, M_2, M_3, M_4, M_5, M_6 Ub_1, Ub_2, Ub_3, Mb_1, Mb_2, Mb_3, Mb_4 and Mb_5) were secrenened for their antagonistic activity against the wilt pathogen by employing the dual culture technique according to Singh *et al*.,(2002). The interaction was studied in 90-mm diameter petriplate containing PDA. One disc of each of isolates were placed on the solidified PDA medium at one side of plates and one of *F. oxysporum f sp. passiflorae* at opposite to test isolates. Plates were incubated at 25 ± 2°C. The radial growth of test pathogens in treated

and control plates were recorded after 2 days until two week of incubation and the percent inhibition of mycelial growth of the pathogens was calculated using following formula: I = (C-T/C) × 100 (Singh et al., 2002) where, I = Inhibition (%), C = Colony diameter in control plate and T = Colony diameter in treated plate.

All experiments were performed in triplicate. Duncan Multiple Range Test was used to evaluate the significant differences between treatments ($P \leq 0.05$). ANOVA analysis was done with the SPSS statistics software.

2.3 Extracellular Metabolites

Selection of extracellular metabolites was conducted by using of salt mineral medium or czapek dox agar using carboxy-methylcellulose, amylum, or chitin as sole carbon source (Gessner, 1980). After an incubation period, a Congo Red solution was used to reveal the hydrolytic zones.

3. Results and Discussion

In the present study, rhizosphere associated fungi and bacteria were evaluated for their antagonism against *Fop*, causative agent of wilt disease on passion fruit. The objective of the study was to select antagonistic fungi and bacteria active against *Fop in vitro*, and how their ability in producing secondary metabolites that correlated with antagonism. A total of 22 fungal and bacterial isolates were isolated from healthy rhizosphere of passion fruit plants collected from Gowa and Makassar. Antagonism of all the fungal and bacterial isolates was first evaluated against *Fop* in *vitro* test i.e., dual culture assay and extra-cellular metabolite efficacy test. On the basis of these tests, overall forty fungal and bacterial isolates were selected, which were found to control *Fop*. Dual culture assay can be used as standard test for the selection of biocontrol agent and shows cumulative effect of all mechanisms undergoing for bio-control i.e., diffusible and volatile antibiotic production, and lytic enzymes production.

3.1 Dual Culture Assay

Based on dual culture test indicated that the highest percentage inhibition of fungal isolates were showed by U_1 (86.11%), M_5 (82.82%) and M_6 (81.32%) isolates, whereas inhibition of bacterial isolates were observed by Mb2 (76.44%) and Ub2 (74.17%) and Mb1 (73.45%) isolates respectively.

Most of fungal and bacterial isolates tested had capacity to inhibit the growth of *Fop*. In some interactions there was no physical contact between any of the isolates and the pathogen (Figure 1). An inhibitory zone was observed suggesting the presence of fungistatic metabolites secreted by the bacterial or fungal isolates. Bacterial and fungal isolates significantly reduced pathogen growth in comparison to the control (Table 1).

The ability of all 22 isolates to produce secondary metabolites showed those fungal and bacterial isolates had different ability to produce extracellular enzymes, toxine and Hydrogen Cyanide (HCN). However, that ability of the isolates was not the only determinant of antagonist agents against *Fop*. Highest qualitative enzyme activity was observed by $U_{7,}$ $M_{1,}$ M_2 isolate, followed by M_3, Ub_1 and Mb_3 isolates respectively (Table 2 & Table 3).

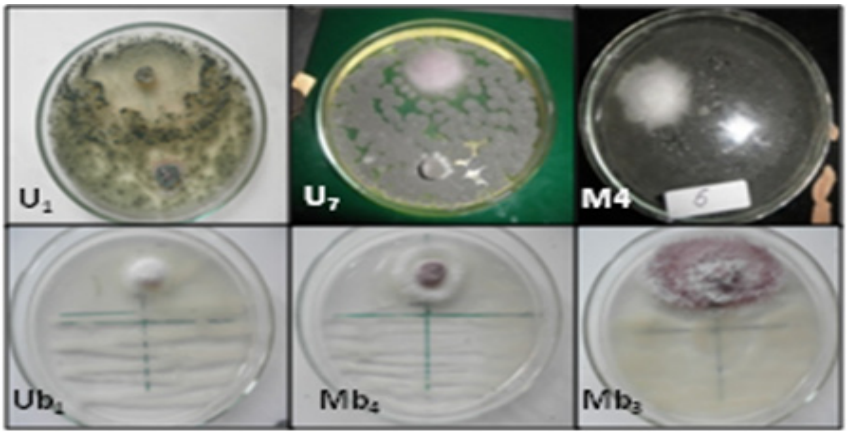

Figure 1. Dual culture assay between fungal and bacterial isolates to *Fusarium oxysporum* f.sp *passiflorae* (Fop), 7 days after inoculation (F = Fop colony, A = antagonist colony)

Table 1. Percentage of growth inhibition of *Fop* after treatment with different fungal isolates 2-14 days post inoculation

	Day Post Inoculation (dpi)			
Isolates	2	6	10	14
K	00.00^{a}	00.00^{a}	00.00^{a}	00.00^{a}
U_1	64.80^{d}	66.66^{f}	85.10^{f}	86.11^{g}
U_2	6.24^{ab}	10.69^{ab}	41.81^{b}	45.85^{bc}
U_3	10.50^{ab}	29.54^{bc}	40.31^{bc}	40.21^{bc}
U_4	25.27^{ab}	29.75^{bc}	40.16^{b}	42.90^{bc}
U_5	27.75^{ab}	53.37^{cdef}	55.25^{bcd}	58.95^{bcde}
U_6	28.72^{ab}	36.03^{bcd}	36.72^{b}	38.03^{b}
U_7	18.75^{ab}	38.75^{bcde}	64.54^{cdef}	72.43^{efg}
U_8	29.28^{bc}	40.50^{cde}	46.91^{bc}	47.62^{bcd}
M_1	40.12^{abc}	57.40^{cdef}	69.88^{bcde}	76.18^{cdef}
M_2	55.10^{cd}	73.73^{f}	75.16^{def}	78.54^{efg}
M_3	59.18^{d}	63.86^{f}	74.70^{def}	75.62^{efg}
M_4	55.63^{cd}	59.26^{def}	74.81^{def}	75.74^{efg}
M_5	62.09^{d}	66.37^{def}	82.82^{f}	82.82^{fg}
M_6	57.30^{d}	67.57^{ef}	79.63^{ef}	81.32^{efg}

Biological control of soil borne plant pathogens is a potential alternative to the use of chemical pesticides, which have already been proved to be harmful to the environment. There is a growing demand for sound, biologically-based pest management practices. Recent surveys of both conventional and organic growers indicated an interest in using biocontrol products (Rzewnicki, 2000; Van Arsdall and Frantz, 2001). Rhizosphere associated fungi and bacteria have drawn much attention, as they have the ability for root colonization and offensive mechanisms against the pathogen by the production of allelochemicals, including lytic enzymes, volatile and diffusible antibiotics and HCN (Cook and Baker, 1983). An important role of hydrolytic enzymes has been well

Table 2. Percentage of growth inhibition of Fop after treatment with different bacterial isolates 2-14 days post inoculation

Isolates	Day After Inoculation			
	2	6	10	14
K	00.00^{a}	00.00^{a}	00.00^{a}	00.00^{a}
Ub_1	42.99^{bc}	58.26^{b}	62.20^{b}	71.32^{b}
Ub_2	37.06^{bc}	64.41^{b}	72.15^{b}	74.17^{b}
Ub_3	39.06^{bc}	55.47^{b}	67.65^{b}	71.71^{b}
Mb_1	55.03^{c}	66.26^{b}	71.21^{b}	73.45^{b}
Mb_2	43.11^{bc}	69.38^{b}	70.72^{b}	76.44^{b}
Mb_3	27.66^{abc}	59.27^{b}	65.45^{b}	68.25^{b}
Mb_4	53.97^{c}	55.15^{b}	59.65^{b}	67.01^{b}
Mb_5	21.80^{ab}	52.64^{ab}	70.27^{b}	71.64^{b}

Within each row means followed by the same superscript (a,b,c,d,e,f) are not significantly different. Duncan ($\alpha = 0.05$).

Table 3. Qualitatively measurement of fungal and bacterial isolates in producing secondary metabolites *in vitro*

Isolates	Enzyme			Toxin	Hydrogen Cyanide
	Cellulase	Chitin	Pectin		
U_1	++	+	+	+++	
U_2	+	+	+	-	
U_3	+	++	++	-	
U_4	-	-	-	-	
U_5	+	+	+	+	
U_6	+	+	+	+	
U_7	++	++	++	+	
U_8	-	-	-	-	
M_1	++	++	++	-	
M_2	++	++	++	-	
M_3	++	+	+	-	
M_4	+++	+	+	++++	
M_5	+	++	++	+	
M_6	-	++	++		
Ub_1	++	++	+	++	++
Ub_2	-	-	-	+	-
Ub_3	-	-	-	+	-
Mb_1	+	-	-	++	-
Mb_2	+	+	+	++	-
Mb_3	++	++	+	+++	++
Mb_4	+	+	+	+	-
Mb_5	-	+	-	++	-

- (no secondary metabolite);+ (weak); ++ (moderate); +++(strong); ++++(very strong)

documented as a variety of microorganisms also exhibit hyperparasitic activity, attacking pathogens by excreting these enzymes. The ability to control *Fop* might be through the secretion of diffusible and volatile metabolites. It may be concluded that they use these two latter mechanisms (volatile and diffusible antibiotic production) of biocontrol against fungus in this study (Singh, 2006; Woo *et al*., 2006).

It is well known that some fungal antagonist can parasitize fungal pathogens and produce antibiotics, besides the fungus have many positive effects on plants: increased growth and yield, increased nutrient uptake, increased fertilizer utilization efficiency, increased percentage and rate of seed germination and induced systemic resistance to plant diseases (Harman *et al*., 2004; Harman, 2006).

The present study demonstrated that both fungal and bacterial isolates have potential to be used as a biological control agent to protect passion fruit plants from *F. oxysporum f. sp. passiflorae*. However, antagonist fungi and bacteria with the highest level of bio-control *in vitro* may not perform as well *in vivo* since environmental conditions and competition with other microorganisms are much more restrictive. Therefore, the bio-control potential of these antagonist fungi may be further evaluated in field condition.

4. Conclusion

Dual culture test indicated that the highest percentage inhibition of fungal isolates were showed by U_1 (86.11%), M_5 (82.82%) and M_6 (81.32%) isolates, whereas inhibition of bacterial isolates were observed by Mb2 (76.44%) and Ub2 (74.17%) and Mb1 (73.45%) isolates respectively.Fungal and bacterial isolates had different ability to produce extracellular enzymes, toxine and Hydrogen Cyanide (HCN). However, those ability of the isolates were not the only determinant of antagonist agents against *Fop*. Highest qualitative enzyme activity was observed by $U_{7,}$ $M_{1,}$ M_2 isolate, followed by M_3, Ub_1 and Mb_3 isolates respectively.

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