

## SUPPRESSION ABILITY OF CRUDE EXTRACT DERIVED FROM MARINE BIOTA AGAINST *Fusarium oxysporum* f.sp. *vanillae*

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### ABSTRACT

The objective of this research was to investigate suppression ability of marine biota extracts against *Fusarium oxysporum* f.sp. *vanillae* of vanilla stem rot. Samples were collected at intertidal zones and in the depth of 1-7 m from seven beaches in Bali. Screening of active compounds of biota extracts were conducted using inhibition zone of well diffusion method on Potato Dextrose Agar (PDA). The extract was tested *in-vitro* in PDA medium using completely randomized design with three replicates. The methanolic extract of *Aglaophenia* sp. was able to suppress the growth of *F. oxysporum* f.sp. *vanillae* effectively, with minimum inhibition concentration (MIC) of 0.05 %. The extract inhibited colony growth diameter and total mycelial dry weight.

*Keywords* : *Fusarium oxysporum* f.sp. *vanillae*, *Aglaophenia* sp., well diffusion method.

### INTISARI

Penelitian ini bertujuan untuk mengungkapkan aktivitas antimikroba ekstrak biota laut terhadap *Fusarium oxysporum* f.sp. *vanillae* penyebab busuk batang pada tanaman vanili. Sampel biota diambil dari daerah intertidal dengan kedalaman 1-7 m pada 7 pantai di Bali. Skrining aktivitas antibiotik dilakukan menggunakan zone hambat dengan metode difusi sumur pada media PDA. Pengujian dilakukan secara *in vitro* dengan rancangan acak lengkap dengan tiga ulangan. Penelitian ini membuktikan bahwa ekstrak methanol *Aglaophenia* sp. mampu menekan pertumbuhan *F. oxysporum* f.sp. *vanillae* dengan konsentrasi hambat minimum (MIC) 0,05%. Ekstrak ini menekan diameter pertumbuhan koloni dan total berat kering miselium.

*Kata kunci*: *Fusarium oxysporum* f.sp. *vanillae*, *Aglaophenia* sp., metode difusi sumur

### INTRODUCTION

The increase of international competitive on trading triggers the effort of producers to produce vanilla beans in high quality without the presence of residual pesticides. Controlling *Fusarium oxysporum* f.sp. *vanillae*, (the pathogen of vanilla stem rot) using synthetic compounds would result in high pesticide residue on agricultural products. This method is no longer accepted in a sustainable agriculture (Hadisutrisno, 2004).

The use of natural product derived from marine organism is promising, because the bioactive compound is generally safe and biodegradable. More over, the secondary metabolites from marine organisms are unique and often better than those of terrestrial biota (Anggadiredja, 2004). Those facts trigger people to explore antimicrobes from marine biotas particularly from sedentary organisms. These organisms could not hinder from predator, therefore they develop defence mechanism by producing secondary metabolites including antifungal materials.

Some antibiotics derived from marine macro- and micro-algae have been developed, such as trichlorethane

and perchlorethane (Marasneh *et al.*, 1995). Some antifungi also have been extracted from marine biotas such as *Sargassum despiense*, *Turbinaria decurrense*, *Cystoseira tamariscifolia* and able to suppress *Fusarium*, *Penicillium*, and *Aspergillus* sp. (Mabrouk *et al.*, 1985; Souhaili *et al.*, 2004).

At the intertidal zones of Serangan and Canggus beach, Bali, 25 species of algae, 27 sponges, and 7 corals have been collected. The extract of these biotas has been found to have an inhibition activity against some fungi and bacteria (Suada *et al.*, 2008).

The purpose of this research was to investigate the suppression activities of the extract derived from marine biotas against *Fusarium oxysporum* f.sp. *vanillae* (*Fusarium*), the causal agent of vanilla stem rot.

### MATERIALS AND METHODS

#### Strain of Fungi and Marine Biota Samples

*Fusarium* (*Fusarium oxysporum* f.sp. *vanillae*) was isolated and identified in Agricultural Biotechnology Laboratory, Udayana University from infected vanilla plants collected from the field in Tabanan, Bali. Single

spore isolation technique was applied to obtained single strain of *Fusarium*. Samples of Marine biotas were collected at intertidal zones in the depth of 1-7 m at seven beaches (Serangan, Batu Bolong, Tanah Lot, Menjangan, Lovina, Tukad Abu, and Nusa Penida) of Bali Province.

**Medium and Culture Conditions**

The medium used for isolation of *Fusarium* was Matuo medium (Matuo, 1972). The medium consisted of 1.00 g K<sub>2</sub>PO<sub>4</sub>, 0.50 g KCl, 0.50 g MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.01 g Fe-Na-EDTA, 2.00 g L-Asparagine, 20.00 g D-Galaktose, and 1.00 L aquadest. The medium was sterilized at 121°C for 20 min. Antimicrobe substances were added by 1.00 g PCNB 75% WP, 0.50 g Oxgall, 1.00 g Na<sub>2</sub>B<sub>2</sub>O<sub>7</sub>, and 0.30 g Streptomycin sulfate. *Fusarium* were then cultured in potato dextrose agar medium (PDA, *Nissui*) and potato dextrose broth (PDB, *DIFCO*) pH 4.5.

**Well Diffusion Method**

Two millilitres of spore suspension of *Fusarium* (2x10<sup>5</sup> conidia/ml) were placed into petri dishes, then 20 ml PDA were added, shaken gently until the conidia well mixed. Over the solid medium of PDA wells were made by cork borer Ø 5 mm. The wells were filled with 40 µl conidium extracts in various concentrations, then incubated at room temperature for 48 h (Berghe and Vlietinck, 1991; Rios *et al.*, 1988). The inhibition zone appeared on the medium was examined. Through this experiment the minimum inhibition concentration (MIC) was determined.

**Fresh Biomass Extraction**

Two grams of marine biotas (Figure 1, Table 1) collected from 7 beaches were chopped, ground, then 5 ml solvents were added for maceration. Solvents used in this experiment were n-hexan, chloroform, acetone, ethanol, methanol, and water. The filtrate was put in eppendorf tubes, centrifuged at 5000 rpm and the supernatant was transferred into new eppendorf tubes. The filtrate aerated and bioassayed using well diffusion method (as described above) against *Fusarium* with the concentration of 50% (w/v) on PDA medium.

**Dry Biomass Extraction**

The extract *Aglaophenia* sp. was found as the most active extract and showed the widest inhibition zones toward the growth of *Fusarium* sp. Its dry material was then tested. The biota biomass was air dried (up to 12% water content, indirect sun light), then ground into powdered. Maceration of the powdered biotas was diluted in methanol at the ratio of 1:2 (w/v) at room temperature for 24 h. The filtrate was evaporated in vacuum rotary evaporator at 40°C. The crude extract obtained was freeze dried.

**Inhibition on Colony Growth Diameter**

*Fusarium* colony plug, 5 mm in diameter, of 3 days culture was placed on the centre of PDA medium in Petri dish in various extract concentrations (0, 0.05, 0.10, 0.15, and 0.20%). The cultures were incubated for 7 days at room temperature (fungi on controlled culture were growing and covered all surface of the medium). The diameter of growing colonies was measured daily.

**Inhibition of Mycelial Dry Weight**

The mycelia of the treatments above were harvested by adding 1% HCl, then oven dried at 80°C, up to constant weight. Mycelia harvested from every treatments above were weighed.

This experiment was also run in PDB liquid medium. The cultures were incubated for 7 days at room temperature and the mycelia were filtered using Whatmann paper no 2. The mycelia were oven dried at 80°C up to constant weight. All data were analysed with Complete Random Design and the treatments were analysed using DMRT 5% differentiation.

**RESULTS**

**Diversity of Marine Biotas Collected from Bali Beaches**

Fifty three types of marine biotas belong to algae, sponges, and hydrozoa were found and collected from seven beaches in Bali. Twelve biotas exhibit antifungal activity against *Fusarium* e.g.: algae: *Gracilaria arcuata*, *Euचेuma cottonii*; sponges: *Haliclona* sp., *Tedaria ignis*, and *Spongilla* sp.; corals: *Aglaophenia* sp. (Figure 1; Table 1).

MIC is the smallest concentration an extract that would inhibit the growth of microbes visually after two days incubation (Andrews, 2006). MIC refers to susceptibility of microbe against ones extract. According to Hoffmann *et al.* (1993) and Andrews (2006), the MIC extract less than 0.1% is reliable to be tested. Foreexample, if the extract of *Aglaophenia* sp. in methanol exhibited inhibition zone of 58 mm, then its MIC less than 0.1% was tested for the next steps.

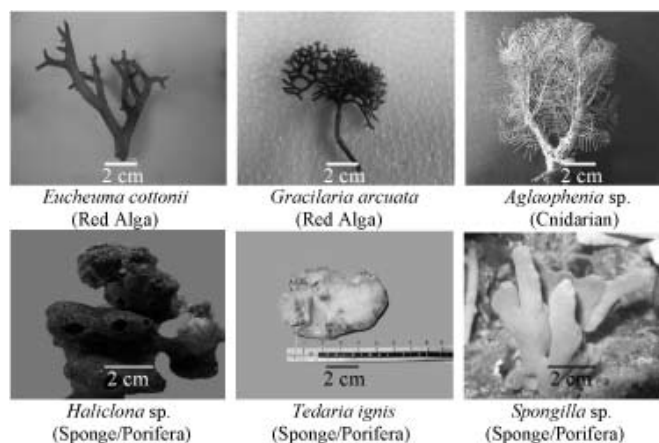


Figure 1. Some marine organisms that exhibited antifungal activity against *Fusarium*.

Table 1. Diameter of inhibition zones and MIC values of marine biota extract against *Fusarium*

Number	Species	Diameter of inhibition zone (mm)	MIC value (%)	Beaches of collections
Ethanol extracts of marine algae				
1	<i>Eucheuma cottonii</i>	9 (fare)	0.4	Serangan
2	<i>Gracilaria arcuata</i>	18 (strong)	0.2	Serangan
3	<i>Gracilaria arcuata</i>	19 (strong)	0.2	Batu Bolong
4	<i>Gracilaria arcuata</i>	17 (strong)	0.2	Tanah Lot
Methanol extract of sponges (Porifera)				
1	<i>Amorphinopsis</i> sp.	15 (strong)	0,5	Tukad Abu
2	Family:Tetillidae	10 (fare)	5.0	Menjangan
3	<i>Haliclona</i> sp.	18 (strong)	0.2	Tukad Abu
4	Family:Raspailiidae	19 (strong)	0.4	Menjangan
5	<i>Tedaria ignis</i>	18 (strong)	0.2	Tukad Abu
6	<i>Spongilla</i> sp.	23 (very strong)	0.2	Tukad Abu
7	<i>Clathrina</i> sp.	9 (fare)	5.0	Tukad Abu
Methanol extract of coral (Cnidaria)				
1	<i>Aglaophenia</i> sp.	58 (very strong)	0.05	Tukad Abu

Legends :

- Inhibition zones was based on concentration of 50%
- Inhibition zones (diameter of inhibition zone subtracted by diameter of extract well) in PDA were incubated for 24 hours at room temperature.
- Category in bracket ( ) was inhibition ability based on Davis Stout Categories (Ardiansyah, 2005).
- Results were based on 5 replicates
- Standard MIC used for antimicrobe was  $\leq 0.1\%$  (w/v) (Hoffmann *et al.*, 1993; Andrews, 2006).

Crude extract of *Aglaophenia* sp. was able to inhibit the patogen growth *in-vitro* on PDA medium. Well diffusion method exhibited its MIC against *Fusarium* spores was 0.05% after two days incubation.

According to LIPI (The Indonesian Institute of Science ) identification, the *Aglaophenia* sp. contains the most active compound as the marine animal, and its classification is as follows: Kingdom Animalia, Phylum Cnidaria (Coelenterata), Class Hydrozoa, Ordo Hydroida, Subordo Thecata, Family Aglaopheniidae, Genus *Aglaophenia*, Spesies *Aglaophenia* sp.

**The Effect of Extract Against Colony Growth**

The inhibition of the extract against the colony growth differed significantly among concentrations of the treatments. The percentage of inhibition increased in increase of the extract concentrations.

The colony was significantly inhibited on 0.05% extract concentration, and the entire inhibition occurred on 0.15% (w/v) as the value of MIC inhibition criteria based on mycelium (Figure 2; Table 2).

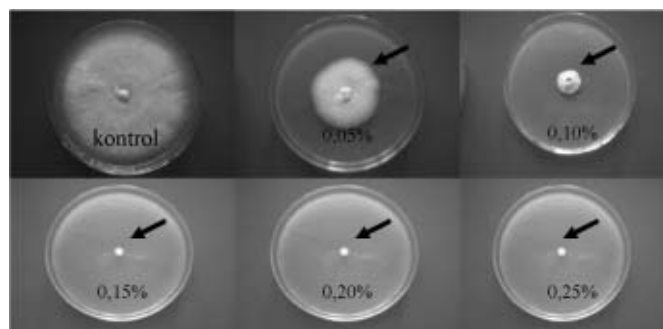


Figure 2. Effect of crude extracts against mycelium of *Fusarium* in PDA medium. %= tested concentration. Sign (♣) is the affected colony after 7 days incubation.

Table 2. Development of *Fusarium* colony diameters affected by various concentrations of *Aglaophenia* sp.

Concentration (%)	Diameter of colony (mm) in days after inoculation						
	1	2	3	4	5	6	7
0.00	6.00 a	13.33 a	38.67 a	54.67 a	68.33 a	75.67 a	82.67 a
0.05	1.00 b	6.33 b	18.00 b	24.67 b	30.33 b	35.33 b	39.33 b
0.10	0.00 c	1.33 c	8.00 c	10.33 c	12.00 c	13.67 c	16.00 c
0.15	0.00 c	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d
0.20	0.00 c	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d

Legends:

Numbers followed by the same letters in one column were not significantly different according to Duncan Multiple Ring Test of 5%. Data analyzed were  $\sqrt{(x+0,5)}$  transformed.

The inhibition patterns of the extract against *Fusarium* colony growth were presented in Figure 2. The inhibition ability was stable above 50% from the second days to seven days of observations. The inhibition patterns indicated that the bioactive compound was stable for long periods of time which means that the extract protection for the plants was potentially good.

**Effect of The Extract Against Mycelia Dry Weight**

Effect of extract against mycelia dry weight is listed on Table 3. The extract was significantly influenced the dry weight of mycelia.

The higher extract concentration applied to the fungi, the higher inhibition happened. For example, the inhibition ability of mycelia dry weight on concentration of 0,15% was significantly higher than the lower concentration (e.g. 0.10%).

Table 3. Inhibition ability of *Aglaophenia* sp. extract of mycelia dry weight of the *Fusarium*

Concentration (%)	Tested in PDA		Tested in PDB	
	Dry weight (mg)	Inhibition ability (%)	Dry weight (mg)	Inhibition ability (%)
0.00	59.91 a	0.00 a	23.93 a	0.00 a
0.05	8.72 b	85.50 b	15.63 b	34.33 b
0.10	6.86 c	88.61 c	7.20 c	69.85 c
0.15	1.34 d	97.83 d	0.63 d	97.33 d
0.20	0.82 d	98.72 d	0.83 d	96.55 d

Legends:

Numbers followed by the same letters in one column were not significantly different according to DMRT 5%. Data analyzed were  $\log(x+1)$  transformed.

**The Form of Extract Inhibitions on *Fusarium***

One symptom of extract inhibition detected on mycelium was wrinkled. The symptom was caused by plasmolysis processes on mycelium, especially on young mycelium (Figure 3).

**DISCUSSION**

Biodiversity of marine biota found at Bali beaches containing bioactivity compounds were 12 species that comprised of algae, sponges and corals. The most effective one was animal marine biota *Aglaophenia* sp. with MIC value of 0.05% based on inhibition on germination spore of *Fusarium*. Based on Hoffmann *et al.* (1993) and Andrews (2006) criterias, the extract with MIC less than 0.1% is reliable to be tested, therefore

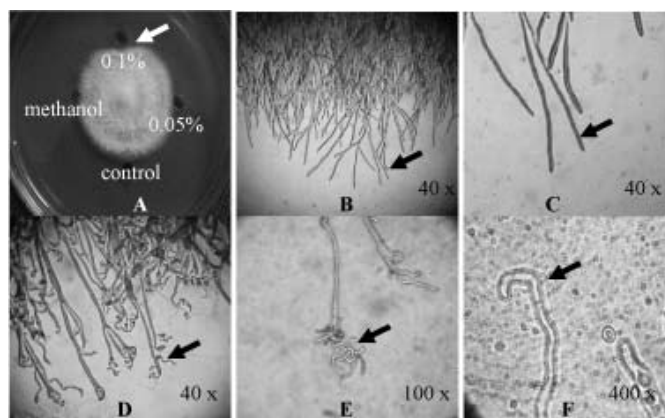


Figure 3. The effect of *Aglaophenia* sp. extract on *Fusarium* mycelia. A: Colony affected by extract at concentrations of 0%, 0.05%, 0.1% (w/v) and methanol; B and C: Control; D, E, and F: abnormal mycelia: spirally wrinkled at 0.1% concentration.

methanol extract of *Aglaophenia* sp. could be tested for the next steps.

Figure 2 and Table 2 showed that the extract significantly inhibited the colony growth and total inhibition (100%) occurred at concentration of 0.15%. The inhibition occurred up to day seventh of treatments. This result suggested that the extract is fungitoxic.

The extract significantly suppressed the dry weight of mycelia compared to control. The higher the concentration of treatments, the higher the suppression against mycelial dry weight. The mycelial dry weight at 0.15% was significantly lower than other treatments. The mycelia on treatments 0.15% and above were not able to grow. Therefore, the mycelia dry weight counted at treatments 0.15% and 0.20% was the mycelia from the original inoculums. This means that the extract was able to inhibit *Fusarium* at the mycelium stages.

One symptom of inhibition on mycelium growth was the wrinkled of mycelium which was caused by plasmolysis that occurred particularly on young mycelia (Figure 3). According to Sugiharso (1980) that bioactive compounds of fungicides commonly cause plasmolysis on *Fusarium*. The bioactive compound acts as chelating agent of membrane molecule and caused leaking in membrane cell. Consequently,  $K^+$ ,  $Na^+$ ,  $Cl^-$ ,  $Ca^{+2}$  ions from cytoplasm come out of the cell. This could happen due to the outer cell osmotic pressure was higher than inner cell. Therefore water run out of cell and cell then becomes dry and wrinkled. Furthermore, Stenersen (2004) stated that the common symptoms caused by antifungi compound are distortion of cell, swelling, and spirally wrinkling cell.

## CONCLUSION

Among all marine biotas collected from Bali island beaches there were 12 species showed inhibition activity against *Fusarium oxysporum* f.sp. *vanillae*. The most active extract was from *Aglaophenia* sp. with MIC of 0.05%. The extract was able to suppress colony growth and mycelial dry weight. The mycelia affected by the extract were wrinkled.

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