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Mode of Action of Antimicrobial Substances from *Burkholderia multivorans* and *Microbacterium testaceum* Against *Schizophyllum commune* Fr.

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ABSTRACT

Supernatant of antimicrobial substances from *Burkholderia multivorans* and *Microbacterium testaceum* suppressed *S. commune* is an important factor to understand the right application for bio-control. The mechanism of inhibition of fungal action by antimicrobial substances was observed under electron microscope. Direct contact of the mycelium, spores and basidia of *S. commune* with antimicrobial supernatant under scanning electron microscope showed the loss of hyphae growth and vigor, became wrinkle and were without any injurious of cell wall surface. Under transmission electron microscope the treated hyphae with antimicrobial substances became the damaged internal structural cells such as nucleus, mitochondria and other cell organs, not distinguish the protoplasm organs, often empty cells and the cell wall still remained intact. However, plasma membrane was split out from internal cell wall and collapsed in the middle protoplasm.

Key Words: Antimicrobial; Infiltration; Damaged fungal structures

INTRODUCTION

Schizophyllum commune Fr., an important fungus, causes brown germ and seed rot of oil palm. The occurrence of heavy infection caused seed germination losses up to 60% (Dikin *et al.*, 2003). The mechanism of antagonistic bacteria inhibited fungal pathogens that depend upon biological behavior of antagonist. Recognizing the mode of action of antimicrobial substances is important for the correct application for the control of fungal pathogen and formulation of bio-pesticide. Antagonistic bacteria were known to suppress fungal growth *in vitro* by the production of antimicrobial substances. Some strains of fluorescent *Pseudomonads* produced phenazines and phloroglucinol as major factor of biological control of soilborne plant pathogens (Chin-A-Woeng *et al*., 2002). *Burkholderia cepacia* strain 5.5B produced pyrrolnitrin and phenazine from in liquid medium (nutrient broth) and these compounds suppressed the growth of *Rhizoctonia solani*, the causal agent of *Rhizoctonia* stem rot of poinsettia (Cartwright *et al.*, 1995).

The cell wall of the fungus is made of chitin (poly-glucosamine). The active compound of chitinase in the antimicrobial substances is able to break down the chitin based on the enzymatic mechanism. *Serratia marcescens* as biological control agents produced chitinase and brake down the cell wall of fungus (Iyozumi *et al*., 1996). Antimicrobial substances as secondary metabolites from *Burkholderia multivorans* and *Microbacterium testaceum* have been isolated using sandwich agar plate. These metabolites strongly suppressed seed-borne pathogen of oil palm, *Schizophyllum commune* based on the *in-vitro* screening (Dikin *et al.*, 2005). Application of supernatant of antimicrobial substances for the control of artificially inoculated seeds of oil palm with *S. commune* was promising. So far, the mode of action of the antimicrobial substances has not yet been determined. The purpose of studies was to determine the mechanism of antimicrobial substances from *B. multivorans* and *M. testaceum* against *S. commune*.

MATERIALS AND METHODS

Supernatant of antimicrobial substances. *B. multivorans* and *M. testaceum* were inoculated separately in the sterilized liquid media (neo-peptone 10 g, lactose 10 g & peptone 10 g, maltose 10 g, respectively & dissolved into one liter of distilled water). The fermentation of inoculated liquid media was carried out on the electric shaker (New Brunswick Scientific G-25 KC) at 125 rpm for 5 days and then added 125 mL of absolute methanol. Each supernatant from fermented liquid media was separated using centrifugation at 10,000 rpm for 10 min at 5^{o}C and reduced to 10% of the initial suspension using rotary evaporator (Buchi rotavopor R-200) at 45^{o}C.

Supernatant of antimicrobial substances against *S.*

commune **under SEM.** The mixed supernatant of antimicrobial substances from *B. multivorans* and *M. testaceum* were prepared. Firstly, the basiocarp of *S. commune* and potato dextrose agar plugs of mycelial growth of 1 cm^2 in size were directly dipped into the mixed supernatant of antimicrobial substances solution for 2 h. Secondly, PDA medium seeded with spores of *S. commune* was dropped with 100 µL antimicrobial substances in the centre of plates. The plates were incubated for 5 days for germination of spores. Agar plugs of *S. commune* from the clear area were cut off 1 cm^2 in size. Agar plugs from clear areas, treated agar plugs of mycelial growth and basidiocrap of *S. commune* were prefixed with 4% of gluteraldehyde solution for over night under vacuum and then washed 3 times with 0.1 M sodium cocadylate buffer for 10 min each. The specimens were fixed in 1% of osmium tetraoxide at 4^{o}C for 2 h and then washed 3 times with 0.1 M sodium cacodylate buffer for 10 min each. Specimens were dehydrated in the serial acetone 35, 50, 70 and 95% for 10 min each and 100% for 3 times (15 min each time). Specimens were put into critical point dryer (Baltec 030) for 30 min. Dried specimens were mounted onto stubs using double sided tape of colloidal silver and then coated with gold in sputter coater. The specimens were observed under scanning electron microscope (JEOL JSM-5610LV at 15 kv) (Gupta *et al.,* 1999).

Supernatant of antimicrobial substances against *S. commune* under TEM. Agar plugs from clear areas and agar plugs of mycelial growth of *S. commune* were prefixed with 4% of gluteraldehyde solution for overnight under vacuum and then washed 3 times with 0.1 M sodium cocadylate buffer for 10 min each. The specimens were fixed in 1% of osmium tetraoxide at 4^{o}C for 2 h and then washed 3 times with 0.1 M sodium cacodylate buffer for 10 min each. Specimens were dehydrated in the serial acetone 35, 50, 70 and 95% for 10 min each and 100% for 3 times for 15 min each. Specimens were infiltrated with serial mixed resin and acetone, resin: acetone with ratio 1:1 for 1 h, resin: acetone with ration 1:3 for 2 h; 100% of resin for aver night, 100% of resin for 2 h. Specimens were embedded in resin with beam capsule and then polymerized in an oven at 60^{o}C for 48 h. The specimens were sectioned with glass knife of 1 µm in size to search the expected area of specimen by observation sectioned under the light microscope. The right area of specimen was ultra-thin sectioned using diamond knife to obtain the thinnest golden specimens, picked up with grids and dried with filter paper. Ultra-thin sectioned specimen on grid was stained with uranylacetate for 10 min and washed with 50% filtrated alcohol and then washed with double distilled water. The specimens were observed under transmission electron microscope EFTEM LEO 912AB (Pan *et al.,* 1997).

RESULTS

The treated hyphae and basidiocarp of *S. commune* with supernatant containing antimicrobial substances from *B. multivorans* and *M. testaceum* were observed under SEM, revealed that all parts of *S. commune* such as mycelia, spores, basidia became lysed. Direct effect of antimicrobial supernatant were loss vigorous of spores, hyphae became flat, not able to grow, wrinkle, although no injurious were noted on cell wall surface. The effect of antimicrobial supernatant at the edge area of PDA clear zone showed some spores did not germinate and whatever germinated spores showed retarded growth. The germinated spores also formed the hyphae tubes beyond area of clear zone, the tip of these hyphae was un-able to extend. These hyphae became retarded, thicken cell wall, rough surface of hypha and wrinkle. The branches of the hyphae were thickened cell wall of hyphae, the septa of which blocked the infiltration of antimicrobial substances from one cell to other cells. There were some empty cells between the cells but with thickened wall (Fig. 1).

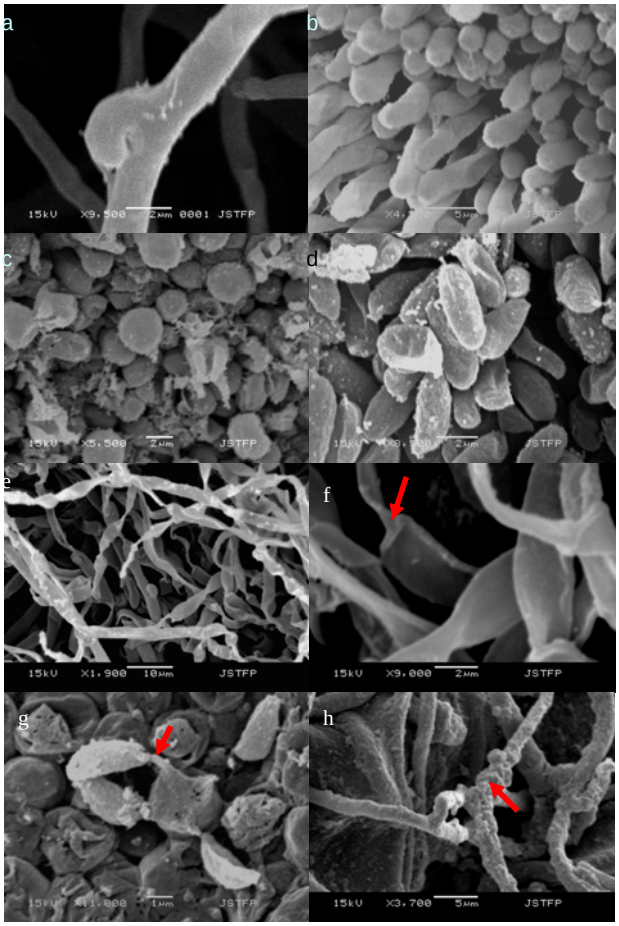
As observed under TEM, the sectioned hyphae after treated with supernatant of antimicrobial substances damaged protoplasm including nucleus, mitochondria and other structures. In some cells these structures were not distinguishable. The plasma membrane split out from the internal cell wall and collapsed in the central part of protoplasm. In contrast, the mycelia and spores of *S. commune* treated with liquid medium itself showed that the mycelia and spores did not show any change in the morphological structures and still able grew on PDA medium (Fig. 2).

DISCUSSION

Dipping treatment of the hyphae and spores of *S. commune* in the supernatant of antimicrobial substances caused loss of vigorous of cell, plasmolysis, malformed fungal structures and retained cell wall and any degradation. Antimicrobial substances infiltrated cell wall to reach the cytoplasm by osmosis. Fungal cell mainly consists of cell wall and cytoplasm (Alexopoulos *et al.,* 1996). The cell wall of the fungus is constructed from chitin (poly-glucosamine). The remained cell wall of *S. commune* after treatment was due to the reason that the active enzyme, chitinase did not reach there (Iyozumi *et al*., 1996).

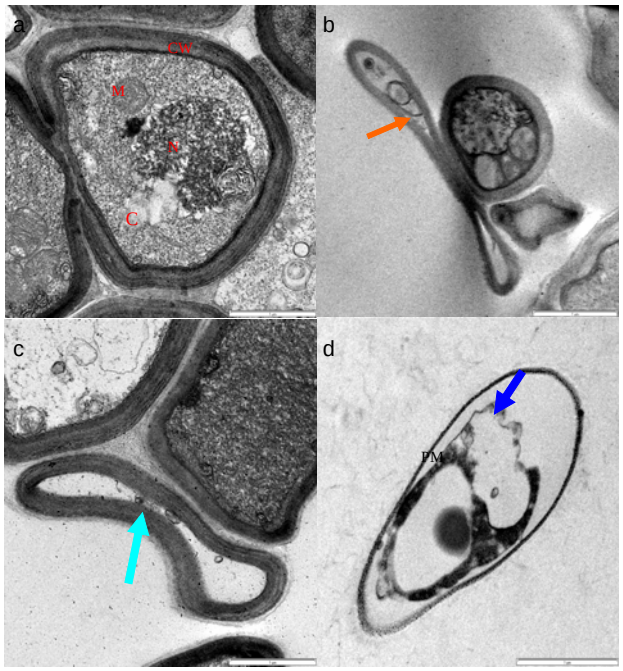
Supernatant from the fermented liquid media contained antimicrobial substances against *S. commune* as determined by autography thin layer chromatography. Supernatant from *B. multivorans* and *M. testaceum* was separated elution profiles using high performance liquid chromatography with UV detector at 254 nm and based on the comparison with some external standards of active compounds. The supernatant of antimicrobial substances from *B. multivorans* and *M. testaceum* were found to contain pyrrolnitrin, phenylpyrrol, pheazine, aminopyrrol and other un-known compounds. Destruction of the cytoplasm of fungal cells was caused related to these compounds. Chernin *et al.* (1996) reported the loss of

Fig. 1. SEM images of hyphae, basidia, sterigmata of *S. commune;* (a.) Hyphae of *S. commune* with clump connection without treatment with supernatant of antimicrobial substances (scale bar = 2 µ) (b.) Basidia of *S. commune* without treatment with supernatant of antimicrobial substances (scale bar = 5µ) (c.) Basidia with sterigmata of *S. commune* without treatment with supernatant of antimicrobial substances (scale bar = 2µ) (d.) Basiospores of *S. commune* without treatment with supernatant of antimicrobial substances (scale bar = 2µ) (e.) Tapped mycelia of *S. commune* after dipped into supernatant of antimicrobial substances suspension (scale bar = 10µ) (f.) Tapped hyphae of *S. commune* after dipped into supernatant of antimicrobial substances suspension (scale bar = 2µ), arrow head = septa (g.) Flatted basidia, collapsed sterigmata (arrow head), collapsed basidiospores of *S. commune* after dipped into supernatant of antimicrobial substances suspension (scale bar = 1µ) (h.) Rough surface of hyphae was taken from the area nearby antimicrobial substances on PDA seeded with spores (scale bar = 5µ)



mitochondria activity in the fungal cytoplasm caused by pyrrolnitrin from bacteria, inhibited succinate oxidase, NADH oxidase succinate cythocrome C reductase and

Fig. 2. TEM images of the cellular ultrastructure of *S. commune* (Scale bar = 1 µ) (a.) Cross-section of hyphae untreated with supernatant of antimicrobial substances (M = mitochondria; N = Nucleous; CW = Cell Wall; C = Cytoplasm) (b.) Cross-section of flatted hyphae after dipped into supernatant of antimicrobial substances, and the remind cytoplasm in the cell (c.) Cross-section of flatted hyphae after dipped into supernatant of antimicrobial substances, empty cell with remind cell wall. (d.) Cross-section of spore with plasma membrane split out from internal cell wall (PM) and collapsed cytoplasm



NADH-cytochrome reductase. Pyrrolnitrin also involved in cellular processes such as oxidative stress, blocked electron transport, inhibition of DNA and RNA synthesis.

Indirect contact of supernatant of antimicrobial substances against *S. commune* caused retardation and rough surface of hyphae. The retarded hyphae recovered for growth after transferred to PDA plate. This indicated that supernatant contained antimicrobial substances such as fungistatical bio-pesticide. It also showed the fungus protected itself from any disruption substances from out side.

CONCLUSIONS

Antimicrobial substances produced from *B. multivorans* RU50 and *M. testaceum* RU7 inhibited the growth of *S. commune*. Active compounds of antimicrobial substances destroyed the protoplasm of hyphae and spores including mitochondria, nucleus, internal plasma membrane, un-distinguishable internal organ in cytoplasm and hyphae and spores collapsed. The treated hyphae and

spores with antimicrobial substances were not able to grow.

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REFERENCES

Alexopoulos, C.J., C.M. Mims and M. Blackwell, 1996. Introductory Mycology. Phyllum: Basidiomycota. Order: Aphyllophorales. *Polypores Chantharelles Tooth Fungi Coral Fungi and Corticioids*, 19: 563–97

Cartwright, D.K., W.S. Chilton and D.M. Benson, 1995. Pyrrolnitrin and phenazine production by *Pseudomonas cepacia*, strain 5.5B, a bio-control agent of *Rhizoctonia solani*. *Appl. Microbiol. Biotechnol*., 43: 211–6

Chin-A-Woeng, T.F.C., G.V. Bloemberg and B.J.J. Lugtenberg, 2002. Phenazines and their role in bio-control by *Pseudomonas* bacteria. *New Phytol*., 157: 503–23

Chernin, L., A. Brandis, Z. Ismailov and I. Chet, 1996. Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. *Current Microbiol*., 32: 208–12

Dikin, A., K. Sijam, Z.A.M. Ahmad and I.A. Seman, 2003. Association of *Schizophyllum commune* Fr. with oil palm seeds. *The 3rd National Seed Symposium, Putrajaya, Malaysian.* April 8-9 2003

Dikin, A., K. Sijam, J. Kadir and I.A. Seman, 2005. Extraction of Antimicrobial Substances from Antagonistic Bacteria Against *Schyzophyllum commune* Fr. *Proceedings 27th Malaysian Microbiology Symposium, Innovation Through Microbes*. Grand Plaza Parkroyal Penang, Malaysia, 24-27 November 2005

Gupta, V.P., S.K. Tewart, Govindaiah and A.K. Bajpai, 1999. Ultrastructure of mycoparasitism of *Trichoderma, Gliocladium* and *Laetisaria* species on *Botryodiplodia theobromae*. *J. Phytopathol*., 147: 19–24

Iyozumi, H., T. Komagata, K. Hirayae, K. Tsuchiya, T. Hibi and K. Akutsu, 1996. Biological control of cyclamen gray mould (*Botrytis cinerea*) by *Serratia marcescens* B2. *Annals Phytopathol. Soc. Japan*, 62: 559–65

Pan, M.J., S. Rademan, K. Kunert and J.W. Hastings, 1997. Ultra-structural studies on the colonization of banana tissue and *Fusarium oxysporum* f.sp. *cubens* race 4 by the endophytic bacterium *Burkholderia cepacia*. *J. Phytopathol*., 145: 479–86

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