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Biological Control of Seedborne Pathogen of Oil Palm, *Schizopyllum commune* Fr. with Antagonistic Bacteria

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ABSTRACT

Schizophyllum commune Fr. is one of important pathogen of oil palm. The fungus was confirmed as causal agents of brown germ and seed rot of oil palm. The inoculated seeds of oil palm may cause loss of germination and reach up to 65%. Seed treatment was applied by infiltration of antagonistic bacteria that isolated from rotted fruits and infected seeds of oil palm. Preliminary screening was done by dual culture assays included radial growth inhibition, diameter growth of mycelia, spore germination, potential mycelial growth and antifungal substances production. The purposed study was to control seedborne pathogen, *S. commune* with antagonistic bacteria as biological control agents. There were 4 species of antagonistic bacteria in the biological seed treatment namely *Burkholderia cepacia, Pseudomonas aeruginosa, Serratia marcescens*, and *Serratia* sp. Seed treatment application was done by dipping-vacuum oil palm seeds in bacterial solution with concentration of 10^9 CFU per mL at 400-500 mm Hg Vac., and 100-150 mm Hg Vac. for 2 minutes to control the inoculated un-germinated and the inoculated germinated seeds respectively. The results showed that *B. cepacia* and *Serratia* sp. significantly suppressed seedborne pathogen, *S. commune* by indicating on the increased seed germination, recovery of seedling growth and non-phytotoxin effects on seedling growth.

Key Words: *Schizophyllum commune* Fr.; Biological treatment; *B.cepacia Serratia* sp.

INTRODUCTION

Malaysia significantly contributes oil palm production and the trading oils fats of the world. The country currently has more than 2.9 million ha of oil palm plantation area, which equals to more than one third of the cultivated land in the country. In 2002, the palm oil, palm kernel oil and palm kernel cake production were 11.9, 1.4 and 1.7 million tones respectively (Davidson, 2003).

The cultivation of oil palm in the new area and replanting on old area is still used the seeds as plant propagation materials, although tissue culture technology has been establishing in breeding program. Certified seeds including purity, high germination and seed health standard are the priority to succeed in the development of oil palm industry. However, there are many evidences of yield losses due to pathogen infection in the field from different stages of plant growth. The routine seed health testing is the early step to detect the presence of seedborne pathogens associated with oil palm seeds.

Zubir *et al*. (1995) intercepted numbers of fungi in the seed consignment under plant quarantine inspection from Costa Rica and Papua New Guinea *i.e. Fusarium solani, Fusarium moniliforme, Aspergillus* spp., *Monosporium* sp., *Penicillium* spp., *Colletotrichum gloeosporioides, Bipolaris* sp., *S. commune, Cephalosporium* sp., *Botryodiplodia theobromae, Verticillium* sp., *Sporitrichum sp., Haplosporella* sp., *Chaetomium* sp., *Thielaviopsis* sp., *Graphium* sp., *Mycotipa* sp., *Cylindrocladium* sp. and *Absida* sp. Turner (1981) recognized that *S. commune* was one of detected fungi in the oil palm seeds caused brown germ and seed rot.

S. commune Fr. was confirmed to cause loss of germination rate of oil palm that may reach up to 65 percent through pathogenicity test (Dikin *et al.,* 2003). On the other hand, fungal status is contradictive in the categorizing as edible mushroom, and plant pathogen. In some countries, basidiocarp of fungus is used as traditional extra power drink and dishes. The fungus was not only seedborne disease of oil palm, but also caused decay and heart rot of apples, wood rot and decay of forestry in USA (Lamey & Stack ,1993; Anonymous, 1999).

Fungal infection takes place during seed germination process in germinator, and basically the early infection in fruit bunches was started during harvesting. Fruit bunches harvesting can cause damage to the mesocarp due to bunches falling from tall trunk. The established fungus in the top soil, plant debris and any other organic matters around the plant plate is the source of inoculum to contaminate fruit bunches. Fungus grew in the fermented fruits, mycelia penetrated fibrous to reach the seed.

Fungal infection on oil palm seed occurred mostly in high humid chambers and unclean removing the fibrous layer on the shell surface. The initial growth of *S. commune* appeared as small white patches of mycelium over the outer seed surface. In the favourable conditions, mycelia gradually grow over the whole shell to cover the germ pores of seeds and penetrate the germ plugs to reach the surface of

testa near the embryo, hence affected respiration activity in the seed germination.

Seed treatment of oil palm is commonly carried out by dipping in sodium hypochlorite solution, application of fungicide and pre-heat treatment at 39-40°C for 40 days. Unfortunately, thus treatments did not reduce the fungal infection in the oil palm seeds, particularly *S. commune* and the chemical toxic residue contaminated the environment around oil palm seed processing area. Development of antagonistic bacteria for the control of pathogenic fungi was based on the environment friendly approach. Many antagonistic bacteria formulations definitely suppressed pathogenic fungi up to field application. *Pseudomonas fluorescens* is one of antagonistic bacteria, used for seed treatment of cotton to suppress *Pythium ultimum* with dosage 10 µg per seed (Howell & Stipanovic, 1980).

The objective of study was to use potential antagonistic bacteria from rotted fruits and seed rot of oil palm as biological control agents for the control of seedborne pathogen, *S. commune.*

MATERIALS AND METHODS

Isolation, identification and pathogenicity test of *S. commune* Fr. *S. commune* was isolated from rotted fruits of oil palm, obtained from the infected plantation and infected seeds with white patches. The sliced mesocarps in small size (0.5-1.5 cm) were plated on moistened filter paper and Water Agar medium (5 g Agar in 1 L of distilled water), and incubated for 7 days at room temperature. Identification based on morphological characteristics were papery, leathery compact mycelia, white mycelia, the presence of clamped connections on the hyphae, spinulose projection, often production of fruiting bodies with white cap less than 1-2 cm in diameter, and gills. Pathogencity test was to inoculate un-germinated seeds by placing oil palm seeds on top of *S. commune* culture on PDA medium in 9 cm diameter petri dish containing 10 seeds. Petri dishes were incubated in transparent polythene bags for 7days to permit the mycelia to colonize the seeds (Dikin *et al*., 2003).

Isolation, screening, and identification of antagonistic bacteria. Antagonistic bacteria were isolated from rotted fruits and infected seeds of oil palm caused by *S. commune* on Nutrient Agar and King's B Agar media. The preliminary screening was done including radial growth inhibition zone, potential mycelial growth, inhibition of mycelial growth, inhibition of spore germination and antifungal substances production. Four species of identified antagonistic bacteria were used in this study and bacteria were confirmed with Biolog ®Identification System namely *Burkholderia cepacia, Pseudomonas aeruginosa, Serratia marcescens,* and *Serratia* sp. (Dikin *et al*., 2002).

Efficacy of antagonistic bacteria against inoculated un-germinated seeds of oil palm. Pre-heated oil palm seeds were soaked in distilled water for 7 days. Water-soaked seeds were air-dried in laminar flow cabinet for 3-4 hours at room temperature (26±2°C) to release the excess water. 25 seeds were inoculated with *S. commune* by placing the seeds on top of 7 day's old *S. commune* culture on PDA medium. Each of *S. commune* culture in 9 cm diameter Petri dish contained 10 oil palm seeds that arranged in good enough distance. Petri dishes were incubated for 7 days in polythene bags to permit the mycelia to colonize the seeds. Bacterial solution was prepared from four different species with concentration of 10^9 CFU per mL and added antifungal substances from antagonistic bacterial itself (Dikin *et al.,* 2002). The inoculated seeds were dipped into prepared antagonistic bacteria solution of 10^9 CFU per mL added antifungal substances, and vacuumed at 400-500 mm Hg Vac. for 2 minutes using EYELA Aspirator type A-3S. Treated seeds were placed on sterilized filter paper in laminar flow cabinet to release the excess bacterial solution. Treated seeds were placed in a polythene bag and incubated at 26±2°C for germination. Germinated seeds were recorded at 7 days interval. Each treatment was replicated 4 times.

Efficacy of antagonistic bacteria against inoculated germinated seeds of oil palm. The emerging seeds were selected from germinator with germ tube 1-2 mm in length. 15 germinated seeds were inoculated with *S. commune* by placing on top of 7 days old culture. Each of *S. commune* culture in 9 cm diameter Petri dish contained 5 seeds, and then Petri dishes were placed in polythene bags to permit the mycelia to colonize the seeds. Bacterial solution was prepared from four different species with a concentration of 10^9 CFU per mL. The inoculated germinated seeds were treated by dipping into antagonistic bacterial solution of 10^9 CFU per mL and vacuumed at 150-200 mm Hg Vac. for 2 minutes using EYELA Aspirator type A-3S. Treated seeds were air-dried in laminar flow cabinet to release the excess bacterial solution. 15 seeds were sowed in sterilized soil of 300 mL plastic pots containing one seed per pot. Daily watering was done to maintain plant growth. Each treatment was replicated 4 times. The growth of seedlings with parameters height of seedling, width of leaf, and length of root were recorded at 4 weeks after sowing. The measuring dry weight of seedlings was done after seedlings air dried in oven at 60°C for 7 days

The effects of antagonistic bacteria on the plant growth of oil palm. Healthy germinated seeds of oil palm were plated in the sterilized soil of 300 mL plastic pots containing one seed per pot, and watered regularly in the glass house. Fresh antagonistic bacterial solution of 48 h was prepared from four different species with concentration of 10^9 CFU per mL. Every week, each pot was poured with 10 mL of antagonistic bacterial solution. The control was germinated seeds, poured with sterilized water. Each treatment contained 15 plant pots and was replicated 4 times. Daily watering was done to maintain the plant growth. Observation included height of seedling upper the soil and width of leaf at 7 days interval. Final observation on wet weight and dry weight was done at 12 weeks after sowing.

RESULTS AND DISCUSSION

Isolation of *S. commune* from rotted fruit on moistened filter paper appeared white patches of mycelia on the surface of fruit at 5 days after incubation. *S. commune* produced basidiocarp from harden mesocarp with the extended incubation more than 7 days. *S. commune* was isolated from rotted fruits of oil palm on WA medium, appeared visually white mycelia that covered fruit surface of oil palm. The fruits became soften, mesocarp discolored, the fibrous discolored and turned black in color. The mycelia entered into the fruit through the fibrous layer. In advanced symptoms, fruits became black in color, dry, and hard. In the humid condition, fruiting bodies were often produced from mummified fruits that appeared small balls 1-2 mm in size, and white color. Fruiting bodies developed and formed fan shape 1-2 cm wide. Upper surface of fruiting bodies was white in color, covered with small messy hairs. The margin of fruiting body cap was pale brown. The under side of the fruiting bodies appeared gills, split longitudinally, light brown in color. Stalk of fruiting body was about 2-5 mm long and laterally attached to the cap.

Isolation of *S. commune* on WA medium from infected seeds showed white mycelia which grew from the kernel, mycelia grow on the WA medium with clamp connections, and hyphae (2-4 µ in width). The fungus did not produce basidiocarp on WA medium. The advanced growth of *S. commune* produced basidiocarp on PDA medium around the edge of Petri dish. *S. commune* culture has characteristics white color of culture on both sides of agar plate, papery, leathery compact mycelia. The mycelia is white in colour with clamp connections and often produced basidiocarp with the white cap (less than 1-2 cm in diameter). Spores were produced on the under side of part of basidiocarp. Spores were hyaline in color, cylindrical, and single cell (Plate 1.).

Artificial inoculation of *S. commune* to the un-germinated oil palm seeds by direct contact resulted in significantly decreased seed germination ($P < 0.05$). *S. commune* colonized un-germinated seeds at 7 days after inoculation and mycelial growth covered surface of endocarp. The mycelia blocked germ pores of seed and penetrated the germ pores to reach the surface of endosperm (kernel). Infection of *S. commune* lossed the percentage of seed germination. The inoculated seeds resulted in decreased germination rate and elongation of germ tube was inhibited. Brown discoloration was common on plumule and radicle. Final record of seed germination rate at 28 days after incubation showed that percentage of germination of inoculated un-germinated seeds was 20.7% compared to un-inoculated seeds reached 85%. Recorded percentage of seed germination is presented in Table I.

At 28 days after incubation, germination rate decreased 64.3%. The compact mycelia of *S. commune* colonized the oil palm seeds to reach the internal seeds in the advanced incubation. The spinulose projection on hyphae was more develop in the internal seeds compared to the mycelial growth on PDA or WA medium. It was believed that the spinulose projections indicated the dormant stage of hyphae. Later, in the high humid condition the spinulose projections became swollen and extended to form new hyphae.

Table I. Mean percent seed germination of oil palm after incubation at 26±2°C

Treatment	Seed germination (%)		
	14DAI	21DAI	28DAI
Inoculated un-germinated seeds with S. commune	9.5b	17.0b	20.7b
Un-inoculated un-germinated seeds (Control)	24.2a	79.0a	85.0a

Mean within a column with the same letter are not significantly different at $P < 0.01$ using DMRT; DAI = day after incubation

A total of 40 bacterial isolates were collected from rotted fruits and infected seeds of oil palm and screened on dual culture assay. Eight out of 40 bacterial isolates inhibited the radial growth of *S. commune* on PDA medium. Three species of bacterial isolates grown on NA medium appeared with different colony color, form, and elevation (Plate 2.). *B. cepacia* was glistening, yellow in color, mucoid having entire margin. *Serratia* sp. was glistening, white in color, mucoid with entire margin. *S. marcescens* was glistening, red in color, mucoid having entire margin.
Dual culture inhibition for screening antagonistic bacteria showed that the bacterial isolate inhibited mycelial growth, blocked mycelial penetration and free zone between bacterial isolates and fungal culture on PDA medium at 7 days after incubation at 26±2°C (Plate 3.).

Four species of selected antagonistic bacteria were *B. cepacia, P. fluorescens, Serratia marcescens* and *Serratia* sp. that used in seed treatment of inoculated un-germinated oil palm seeds. Dipping-vacuum treatment of the inoculated un-germinated seeds with antagonistic bacterial solution significantly reduced the fungal infection ($P>0.01$). Among four species studied, *B. cepacia* and *Serratia* sp. were more effective in reducing fungal infection than other species. It was indicated by the increased percentage of seed germination of oil palm (Table II).

Double impact of dipping-vacuum seed treatment was in addition to enhance antagonistic bacterial penetration into the seeds also increased seed germination, which are presented on the treatment between vacuum and non vacuum on the infected seeds. Dipping-vacuum treatment physically broke the compact mycelia around the germ pores, induced the growth of antagonistic bacteria in the seed thereby inhibiting the mycelial growth. Microscopic observation of kernel from inoculated seeds treated with antagonistic bacteria revealed that the bacterial penetration reached the surface of testa and colonized the mycelia. This cauesed retardation of hypae and wrinkle along the hyphae.

Plate 1. a. The culture of S. commune on PDA at 6 days after incubation. b. Mycelium with clamp connections (cc) and spinulose projections (sp). c. Basidiocarp in fan-shaped; d. Basidiospores of *S. commune*

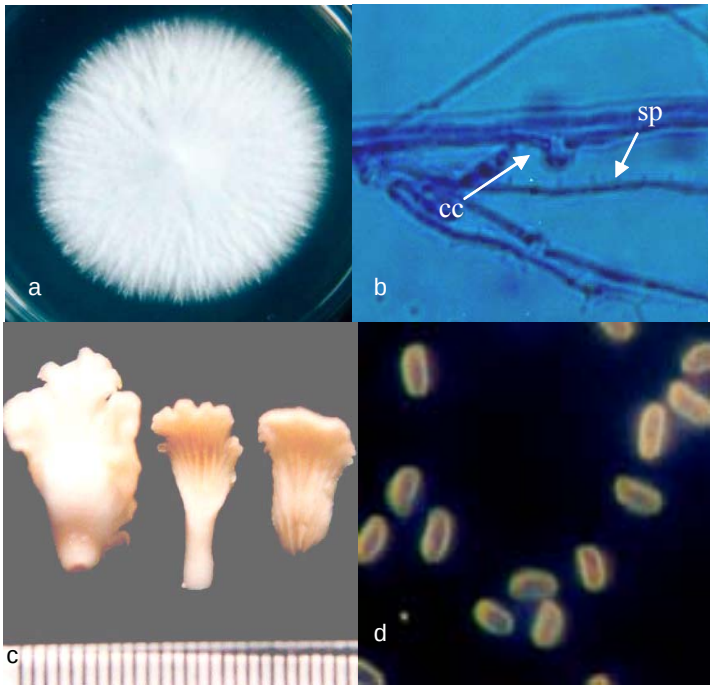


Plate 2. Antagonistic bacterial isolates grow on NA medium at 48 hours after incubation at 26±2°C (a). *B. cepacia*, (b). *Serratia* sp. (c). *S. marcescens*

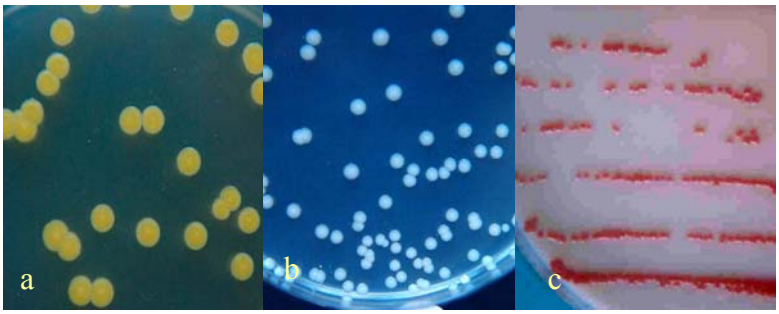
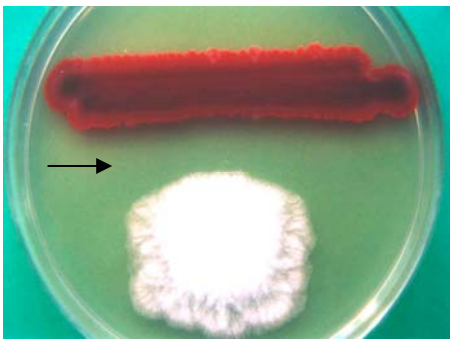


Plate 3. Dual culture inhibition between *S. commune* (bottom) and *S. marcescens* (top) on PDA medium at 7 days after incubation (→, free zone)



Re-isolation of *B. cepacia* and *Serratia* sp. from the treated seeds with antagonistic bacteria on NA medium, particularly on the kernel has proved the potency of both bacterial isolates attacks *S. commune*.

Germinated seeds were inoculated with *S. commune* and then treated with 4 species of selected antagonistic bacteria. It showed that antagonistic bacteria able to eliminate the progress of symptoms on seedlings. Potential antagonistic bacteria controlled the fungus in inoculated germinated seeds and showed statistically significant effect ($P>0.05$) on the growth of plant parts i.e. height of seedling, width of leaf, length of root, wet weight and dry weight of seedlings compared to un-inoculated germinated seeds (Table III.). Dipping-vacuum treatment with antagonistic bacterial solution on inoculated germinated seeds was statistically significantly different (Table III) on wide of leaf, length of root and dry weight. The inoculated germinated seeds were still able to grow and oil palm seedlings were resistance against the colonization of *S. commune*.

Antagonistic bacteria were able to penetrate compact mycelia, to colonize oil palm seeds by the precise vacuum tension. This was required in application of seed treatment to break compact mycelia of *S. commune* and without damage of seedlings. Dipping-vacuum treatment for germinated seeds can cause the injuried seedlings if the vacuum tension was higher than 200 mm Hg. Vac. The vacuum treatment has also been applied to induce *Penicillium* spp. into the maize seeds for the control of seed and root rot due to *Fusarium* sp. and *Rhizoctonia solani* (Sarbini & Noormita, 2003).

Furthermore, the evaluation of antagonistic bacteria on plant growth performance showed that antagonistic bacteria did not cause any negative impact on the plant growth at different stages compared to non-infestation of antagonistic bacteria as presented in Table IV. There was better plant performance interest of the height of seedlings treated with *B. cepacia, Serratia* sp., which was higher than non-antagonistic bacterial infestation at 12 weeks after sowing. On the other hand, *S. marcescens* and *P. aeruginosa* did not affect the height of plant growth, compared to non-infestation of antagonistic bacteria. The wet weight of seedling was statistically non-significant, while dry weight of seedlings at 12 weeks after sowing showed that infestation of *P. aeruginosa* into oil palm seedlings caused the increased dry weight followed by *Serratia* sp. and *B. cepacia* (1.73, 1.63, and 1.61 respectively). The increased dry weight of seedling particularly infestation of *P. aeruginosa* and *B. cepacia* due to the fact that both bacteria were known as plant growth promoting (Sigee, 1993; Reddy, 1996).

Table II. Mean percentage of seed germination of oil palm after treatment with antagonistic bacteria by dipping-vacuum treatment

Treatments	Germination rate (%)			
	14DAI	21DAI	28DAI	35DAI
Inoculated un-germinated seeds + S. marcescens(dipping- vacuum)	40b	53b	67b	67b
Inoculated un-germinated seeds + P. aeruginosa(dipping- vacuum)	43.7b	53b	69b	69b
Inoculated un-germinated seeds + B. cepacia(dipping- vacuum)	60a	64a	81a	81a
Inoculated un-germinated seeds + Serratia sp. (dipping- vacuum)	58a	70a	83a	83a
Un-inoculated un-germinated seeds	46b	69a	80a	80a
Inoculated un-germinated seeds (dipping- vacuum)	18c	26c	36c	36c
Inoculated un-germinated seeds (without dipping-vacuum)	9c	10d	19.5d	19.5d

Table III. The effect of dipping-vacuum treatment of antagonistic bacteria on the inoculated germinated seeds of oil palm at 4 weeks after incubation

Treatment	Height of seedling (cm)	Width of leaf (cm)	Length of root (cm)	Wet weight(g)	Dry weight(mg)
Un-inoculated germinated seeds	12.90a	2.23a	11.12a	1.19a	160.85a
Inoculated germinated seeds + dipping-vacuum	10.81c	1.46c	9.83b	0.97b	134.18b
Inoculated germinated seeds + dipping-vacuum S. marcescens	11.65bc	1.89ab	11.27a	1.10ab	167.80a
Inoculated germinated seeds + dipping-vacuum P. aeruginosa	10.99c	1.79bc	10.65ab	0.96b	152.05ab
Inoculated germinated seeds + dipping-vacuum B. cepacia	11.52bc	2.05ab	11.06a	1.08ab	170.68a
Inoculated germinated seeds + dipping-vacuum +Serratia sp	11.99b	1.76bc	10.84ab	1.03ab	161.93a

Table IV. Mean height and weight of seedling after treatment with antagonistic bacteria at 12 weeks after incubation

Treatment	Height of seedling after sowing (cm)			Weight of 12 weeks old seedling (g)	
	4 weeks	8 weeks	12 weeks	Wet	Dry
Germinated seeds	8.7bc	20.5a	27.2b	3.87a	1.33c
Germinated seeds + S. marcescens	8.6bc	20.4a	27.4b	4.02a	1.47bc
Germinated seeds + P. aeruginosa	8.9b	21.2a	28.3ab	4.14a	1.73a
Germinated seeds + B.cepacia	9.4a	21.6a	28.8a	4.22a	1.61ab
Germinated seeds + Serratia sp.	9.5a	21.4a	28.8a	4.24a	1.63ab

Means within a column with the same letter are not significantly different at $p<0.05$ using DMRT; DAI = days after incubation

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