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The Potential of Microbes Isolated from Spent Substrate of Shiitake and Oyster Mushrooms to Induce Resistance Against Early Blight Disease in Tomatoes

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ABSTRACT
Early blight disease caused by *Alternaria solani* is an important disease in tomatoes. An environmentally friendly control measure is the use of organic matter, such as spent mushroom substrates (SMS). SMS controls plant diseases primarily through microbial activity that can inhibit the pathogen. The objective of this study is to examine the potential of bacteria and fungi isolated from spent substrate of shiitake mushrooms (*Lentinula edodes*) and oyster mushrooms (*Pleurotus* sp.) to induce resistance against early blight disease in tomatoes. The isolation from SMS of *L. edodes* resulted in seven bacterial isolates and four fungal isolates and the isolation from SMS of *Pleurotus* sp. resulted in five bacterial isolates and two fungal isolates. The isolates were examined for their ability to inhibit early blight disease in tomatoes. The experiment was arranged in randomised complete block design with three replicates. The results showed that the application of the fungal and bacterial isolates in the planting holes inhibited the development of early blight disease in tomatoes by 23.0%–51.6%. The isolates that showed the greatest disease reduction were two bacterial isolates (SB4 and SB7) from spent shiitake substrate. These isolates increased the activities of pathogenesis-related proteins (β-1,3-glucanase and chitinase), indicating the induction of host resistance.

Keywords: *Alternaria solani, Lentinula edodes, Pleurotus, β-1,3-glucanase, chitinase.*

1. INTRODUCTION

One of the limiting factors in tomato production is early blight disease caused by *Alternaria solani*. The pathogen infects all aerial parts of the tomato plant, including the fruits [1, 2]. The infected leaves become necrotic, surrounded by yellowish tissues. In severe infection, the lesions coalesce and the leaves become yellowish and then dry. The infected fruit falls off easily, as the infection usually starts in the area surrounding the petiole [1].

The disease is usually controlled through the application of pesticide. However, the misuse of pesticides may lead to negative impacts such as pathogen resistance, environmental pollution and pesticide residue on the product [3]. Therefore, environmentally-friendly control measures need to be developed.

Control measures that are environmentally friendly include biological control and the use of organic matter. One potentially useful organic matter is a by-product of mushroom cultivation, called spent mushroom substrates (SMS) or spent mushroom compost. In Indonesia, commonly cultivated mushrooms are shiitake mushrooms (*Lentinus edodes*) and oyster mushrooms (*Pleurotus* sp.). The ability of spent substrate from shiitake and oyster mushrooms to control plant disease has been documented. Spent shiitake and/or oyster mushroom substrates have been reported to control bacterial wilt disease (*Ralstonia solancearum*) in potatoes [4, 5], stem-based rot in onions [6], damping off disease (*Phytophthora drechsleri*) in cucumbers [7], damping off disease (*Rhizoctonia solani*) in tomatoes [8] and late blight disease (*Phytophthora capsici*) in peppers [9].

Spent mushroom substrate can control plant diseases through several mechanisms. The efficacy of spent

mushroom compost has been related to the activity of microorganisms that are antagonistic to plant pathogens [7, 10–12]. Spent mushroom substrates also contain antimicrobial substances that inhibit pathogen growth [13, 14]. Water extract from spent mushrooms substrate can induce plant resistance to pathogens as well [9, 15].

Organic matter, including spent mushroom compost, is a source of potential biological control agents for plant diseases. Fungal isolates from spent oyster mushroom substrate were effective at suppressing *Fusarium oxysporum* f.sp. *lycopersici* [11] and *Rhizoctonia solani* [12]. Bacterial isolates from spent oyster mushroom substrate were also effective at inhibiting the growth of plant pathogens in vitro, including *Colletotrichum musae* [10], and *Fusarium solani* [16].

The suppressive effect of antagonistic microbes on plant pathogens can be due to direct antagonism, such as the production of antimicrobial substances, competition for space and nutrients, hyperparasitism, or due to indirect effects through the induction of host resistance [17]. The induction of host resistance by beneficial microbes may be indicated by the separation between the microbes and the pathogen or by the increase in plant defence-related proteins such as β-1,3-glucanase and chitinase in treated plants that are challenged by pathogens [18,19]. The objective of this study is to evaluate the ability of microbes isolated from spent mushroom compost of shiitake and oyster mushrooms to increase resistance to early blight disease in tomatoes.

2. MATERIAL AND METHODS

2.1 Isolation and pathogenicity test of A. solani

The pathogen *A. solani* was isolated from tomato leaves showing symptoms of early blight disease. Healthy and infected tissues were cut into small pieces (1 cm^2). The leaf pieces were surface sterilised by soaking in a solution containing 2% chlorine for 3 minutes before being rinsed with sterile water three times. Then the air-dried leaf pieces were placed on potato dextrose agar (PDA) containing chloramphenicol 0.01%. The emerged fungal colonies, characteristic of *A. solani* colonies were purified. The pathogenicity of the fungus was tested by inoculating several plugs of the fungal culture on tomato leaves.

2.2 Isolation of microbes from spent mushroom substrate

The spent mushroom substrates used were obtained from mushroom production in Cikole, Lembang, West Bandung, West Java, Indonesia. The spent substrates from oyster and shiitake mushrooms had been weathered for three months. The mushroom substrates were mainly composed from sawdust and rice bran.

The bacteria and fungi were isolated from spent mushroom substrates using the serial dilution method. The media used for isolation were potato dextrose agar, malt extract agar and nutrient agar. Colonies isolated from different substrates with different characteristics were purified for further selection.

2.3 Effects of the microbes isolated from SMS on early blight disease and tomato growth

The experiment was arranged in a completely randomised design. Treatments consisting of microbial isolates, plant activator (Acibenzolar-S-methyl), and untreated plant (check) were repeated three times. Bacterial suspension (cell density 10^6 cfu mL^{-1}) or conidial suspension (10^6 conidia mL^{-1}) was applied in the planting holes, with a dosage of 20 mL per plant. Tomato seedlings (two weeks old) were then planted in the microbial-treated medium. To examine the effects of the microbes on tomato growth, the medium used was pasteurised soil without organic matter or fertilizer.

The pathogen was inoculated on tomato leaves three weeks after transplanting. A plug of *A. solani* culture (0.8 cm diameter) was inoculated on the tip of each tomato compound leaf. The plug was fastened by plastic wrap. To maintain humidity, inoculated tomato plants were covered by transparent plastic bags for 48 hours. The inoculum plug was removed three days after inoculation. The lesion diameter was measured every three days. The data were used to calculate the area under the disease progress curve (AUDPC) [20]. Tomato growth was determined by measuring the plant's height at one and three weeks before pathogen inoculation.

2.4. β-1,3-glucanase and chitinase enzyme activity

Leaf samples were obtained from tomatoes treated with the microbial isolates and challenged by the pathogen. The leaf samples were taken three days after pathogen inoculation. The β-1,3-glucanase activity was assayed based on the release of reducing sugars from laminarin using Tuzun *et al.*'s method [21] with slight modifications. Each sample extract (250 µl) was mixed equally with 1% laminarin substrate (in a phosphate buffer) and incubated at 30°C for 30 minutes. The reaction was terminated by heating the suspension at 100°C for 10 minutes. The suspension was then mixed with 250 µl of Nelson's reagent and heated at 100°C for 20 minutes. After cooling, arsenomolibdat solution was added to the suspension. The absorbance was measured using a spectrophotometer (510 nm wavelength).

The chitinase activity was assayed using colloidal chitin as a substrate using Siefert and Grossmann's method [22] with slight modifications. The sample extract (250 µl) was mixed equally with a substrate containing 1% colloidal chitin and incubated for an

Table 1. The effects of microbial isolates on tomato growth before pathogen inoculation

Treatments	One week after transplanting		Two weeks after transplanting	
	Plant Height (cm)	Increase compared to the check (%)	Plant Height (cm)	Increase compared to the check (%)
Bacterial isolate SB1	10.2 ± 0.8 [c]	1.3	15.7 ± 1.5 [cdef]	1.5
Bacterial isolate SB2	10.3 ± 1.5 [c]	1.3	16.2 ± 1.2 [def]	1.5
Bacterial isolate SB3	9.7 ± 1.5 [bc]	1.3	15.7 ± 1.0 [cdef]	1.5
Bacterial isolate SB4	10.2 ± 0.8 [c]	1.3	17.0 ± 1.5 [ef]	1.6
Bacterial isolate SB5	9.7 ± 0.9 [bc]	1.3	14.8 ± 1.0 [bcdef]	1.4
Bacterial isolate SB6	14.8 ± 1.0 [d]	1.9	19.5 ± 1.2 f	1.8
Bacterial isolate SB7	10.3 ± 0.9 [c]	1.3	17.7 ± 1.3 ef	1.7
Bacterial isolate TB1	8.5 ± 0.5 [abc]	1.1	14.5 ± 0.8 [bcdef]	1.4
Bacterial isolate TB2	6.3 ± 0.8 [a]	0.8	9.5 ± 1.1 [a]	0.9
Bacterial isolate TB3	7.3 ± 0.6 [abc]	1.0	10.7 ± 0.8 [abc]	1.0
Bacterial isolate TB4	8.5 ± 1.2 [abc]	1.1	12.7 ± 1.5 [abcde]	1.2
Bacterial isolate TB5	6.8 ± 1.6 [ab]	0.9	10.8 ± 1.8 [abc]	1.1
Fungal isolate SJ1	8.3 ± 1.0 [abc]	1.1	13.5 ± 0.8 [abcde]	1.3
Fungal isolate SJ2	8.7 ± 1.3 [abc]	1.1	13.7 ± 1.3 [abcde]	1.0
Fungal isolate SJ3	7.7 ± 1.2 [abc]	1.0	11.7± 0.7 [abcd]	1.1
Fungal isolate SJ4	8.2 ± 0.7 [abc]	1.1	10.8 ± 1.4 [abc]	1.3
Fungal isolate TJ1	7.8 ± 1.5 [abc]	1.0	9.9 ± 1.3 [ab]	0.9
Fungal isolate TJ2	7.8 ± 1.3 [abc]	1.0	11.2 ± 0.7 [abcd]	1.0
Plant activator	8.5 ± 1.2 [abc]	1.1	11.0 ± 1.1 [abcd]	1.0
Untreated plant (Check)	7.7 ± 0.9 [abc]	1.0	10.7 ± 1.2 [abc]	1.0

[a] Data in one column followed by different letters were significantly different ($p < 0.05$) based on Tukey's HSD test

hour. The reaction was terminated by the addition of *trichlorocetic acid* 20%, and the suspension was centrifuged at 5000 rpm for five minutes. The supernatant (0.3 ml) was mixed with 0.7 ml NaOH 0.5 mM. The solution was incubated for 30 minutes and then its absorbance was measured using a spectrophotometer (585 nm wavelength). The chitinase activity was measured as the amount of N-acetylglucosamine GlcNac released per hour per mg protein.

3. RESULT AND DISCUSSION

3.1 Effect of the microbial isolates on tomato growth

The isolation of microbes from spent substrate of shiitake mushrooms (*L. edodes*) resulted in seven bacterial isolates and four fungal isolates, while isolation from spent oyster mushroom substrate (*Pleurotus* sp.) resulted in five bacterial isolates and two fungal isolates. Based on their morphological characteristics, the fungal isolates from shiitake mushrooms were from genera *Trichoderma, Aspergillus, Rhizopus* and *Penicilium*, while the fungal isolates from oyster mushrooms were from genera *Aspergillus* and *Rhizopus*. Adedeji and Modupe [11] also isolated *Trichoderma viridae*, *Penicillum* spp. and *Aspergillus terrus* from spent oyster mushroom substrate. Zulfikar *et al.* [16] found bacterial isolates from spent oyster mushroom substrate belonging to genera *Bacillus*, *Pseudomonas*, *Chyryseobactrium*, *Ochrobactrum*, *Paraburkholderia* and *Serratia.*

Among the microbial isolates, there was only one isolate (SB6) that significantly improved tomato growth at one week after planting. However, at two weeks after planting, there were four isolates (SB2, SB4, SB6 and SB7) that improved tomato growth. The height of the tomatoes treated by those isolates was 1.51–1.82 times higher than untreated plants (Table 1). All isolates that improved the tomato growth were bacteria from spent shiitake substrate. The isolates may be plant growth promoting bacteria. Zulfikar *et al.* [16] also found a bacterial isolate, *Bacillus subtilis*, from spent oyster mushroom compost that increased tomato growth.

Table 2. The ability of bacterial and fungal isolates from spent mushroom substrate from shiitake and oyster mushrooms to inhibit early blight disease in tomatoes

Treatments	Type of mushroom substrate	The first appearance of symptoms (days after inoculation)	AUDPC	Disease inhibition (%)
Bacterial isolate SB1	Shiitake mushroom	3	28.6 ± 3.7 [a]	45.9
Bacterial isolate SB2	Shiitake mushroom	3	34.5 ± 3.1 [abc]	34.7
Bacterial isolate SB3	Shiitake mushroom	3	38.1 ± 1.6 [bc]	28.0
Bacterial isolate SB4	Shiitake mushroom	**3**	**26.2 ± 1.9 [a]**	**50.5**
Bacterial isolate SB5	Shiitake mushroom	3	40.4 ± 3.9 [c]	23.8
Bacterial isolate SB6	Shiitake mushroom	3	29.7 ± 2.3 [ab]	43.8
Bacterial isolate SB7	Shiitake mushroom	**3**	**25.6** ± 1.9 [a]	**51.6**
Bacterial isolate TB1	Oyster mushroom	3	37.3 ± 1.7 [bc]	29.5
Bacterial isolate TB2	Oyster mushroom	3	38.8 ± 3.9 [bc]	26.7
Bacterial isolate TB3	Oyster mushroom	3	39.6 ± 0.1 [c]	25.2
Bacterial isolate TB4	Oyster mushroom	3	40.8 ± 3.1 [c]	23.0
Bacterial isolate TB5	Oyster mushroom	3	38.7 ± 2.7 [bc]	27.0
Fungal isolat SJ1	Shiitake mushroom	3	40.5 ± 3.4 [c]	23.6
Fungal isolat SJ2	Shiitake mushroom	3	40.7 ± 1.7 [c]	23.1
Fungal isolat SJ3	Shiitake mushroom	3	38.7 ± 1.8 [bc]	26.8
Fungal isolat SJ4	Shiitake mushroom	3	36.2 ± 2.8 [bc]	31.6
Fungal isolat TJ1	Oyster mushroom	3	38.9 ± 2.9 [bc]	26.6
Fungal isolat TJ2	Oyster mushroom	3	37.1 ± 1.1 [bc]	30.0
Plant activator	-	3	31.3 ± 2.1 [abc]	40.8
Untreated plants (check)	-	3	52.9 ± 3.6 [d]	-

[a] Data in one column followed by different letters were significantly different ($p < 0.05$) based on Tukey's HSD test

The ability of bacteria to promote plant growth can be due to several mechanisms, such as their abilities to facilitate the availability of nutrients essential for plant growth or produce plant hormones such as auxin, cytokinin or gibberellin [23]. Hameeda *et al*. [24] suggested that the growth-promoting effects of composts and their water extracts could be due to nutrient availability and the presence of plant growth-promoting microbes in the compost. The presence of bacterial isolates with growth promoting effects in spent shiitake mushroom compost may involve or support substrates' ability to improve plant growth. Yusidah and Istifadah [6] found that spent shiitake mushroom compost supported shallot growth and yield as well as a farmer's practice that used fertilizer.

3.2 Effects of the microbial isolates on early blight diseases

Application of microbes isolated from spent mushroom substrate from shiitake and oyster mushrooms into the planting medium may suppress early blight disease in tomato leaves. Most isolates (16 isolates) did not effectively reduce early blight diseases, only reducing disease by 23%–46%. Only two isolates suppressed the disease 50.5%–51.6%. Those isolates were bacterial isolates from spent shiitake substrate (Table 2).

The separation between the microbe application (in the soil) and the pathogen application (on the tomato leaves) indicates that disease reduction occurred due to an increase in plant resistance rather than direct antagonism of the microbes on the pathogen. Lopez *et al*. [25] stated that spent mushroom composts are sources of microbes that have the potential to induce

plant resistance. Zang *et al.* [26] suggested that various microbes in the compost were involved in the induction of plant resistance. Ahlawat and Sagar [27] stated that the most common bacteria in spent mushroom substrates are bacteria from genera *Bacillus* and *Pseudomonas*. The ability of these bacteria to induce plant resistance have been reported [28]. The induction of systemic resistance by microbial isolates was confirmed by the increase in plant defence-related protein activity**.**

Table 3. Chitinase and glucanase enzyme activity in tomato plants inoculated by *A. solani* in different treatments

Treatments	β-1.3-Glucanase		Chitinase	
	Enzyme activities (U μg^{-1} protein)	Increases in enzyme activity (times)	Enzyme activity (U μg^{-1} protein)	Increases in enzyme activity (times)
Bacterial isolate SB4 + pathogen	0.166	3.4	0.0307	1.8
Bacterial isolate SB7 + pathogen	0.182	3.7	0.0275	1.6
Bacterial isolate TB4 + pathogen	0.110	2.2	0.0244	1.4
Pathogen	0.077	1.6	0.0257	1.5
Healthy plant	0.049	-	0.0174	

3.3. β-1,3-glucanase and chitinase enzyme activity

The results show that inoculating microbes in the soil and challenging tomatoes with the pathogen increases the activity of pathogenesis-related proteins such as β-1,3-glucanase and chitinase (Table 3). In treatments using the effective isolates (SB4 and SB7), the activity levels of these enzymes were higher than when using the least effective isolate (TB4). This indicates that the increase of these enzymes' activity played an important role in inhibiting the development of early blight disease. Chitinase and β-1,3-glucanase are hydrolytic enzymes that can degrade cell wall components in plant pathogens [19]. Khanal *et al.* [29] reported that plant defence-enzymes such as chitinase and β-1,3-glucanase possess antifungal activity and therefore inhibit *Alternaria alternata* and *Fusarium oxysporum*. In the induction of plant resistance by beneficial microbes, enzyme production increases considerably after pathogen infection. The colonisation of microbes in the rhizosphere or roots can sensitise plants enhancing their defence capacities and helping them react more rapidly to pathogen infection [17,18].

Pathogen inoculation also enhanced the activity of pathogenesis-related proteins such as glucanase and chitinase. Lawrence *et al.* [30] found that *A. solani* inoculation increased the activity of β-1,3-glucanase and chitinase. However, these enzymes increased less in susceptible varieties than in resistant varieties. In this study, the increase of pathogenesis-related proteins was not sufficient to protect the plant from pathogen infection.

In this study, the increase of β-1,3-glucanase activity was more pronounced than the increase in chitinase activity. Kang *et al*. [9] found that the application of shiitake mushroom water extract increased the expression of genes responsible for the production of PR protein genes including glucanase. Zang *et al.* [26] further found that systemic resistance induced by compost was indicated by an increase of β-1,3-glucanase production. They suggested that the microbes in the compost were involved in the induction of resistance, as the effect was reduced or eliminated by autoclaving.

The results of this study show that spent substrates, particularly from shiitake mushroom cultivation, contain beneficial microbes, which have potential as biofertilizer and biocontrol agents. Application of two bacterial isolates (SB4 and SB7) in the planting medium increased tomato growth and suppressed early blight diseases. The isolates increased the activity of plant defence enzymes such as β-1,3-glucanase and chitinase after pathogen inoculation, indicating the activation of plant defence reactions. In this study, these isolates have not yet been identified, and hence need to be further characterised. The efficacy of the isolates in suppressing other tomato diseases, particularly soil-borne diseases, also needs to be studied further. The use of microbial isolates that possess direct antagonistic effects on plant pathogens as well as those that induce plant resistance will provide comprehensive plant disease control**.**

4. CONCLUSION

Isolation from SMS from shiitake and oyster mushrooms resulted in 12 bacterial isolates and six fungal isolates. Among these isolates, four bacterial isolates from shiitake mushrooms (SB2, SB4, SB6 and SB7) increased plant growth. Two bacterial isolates (SB4 and SB7) suppressed early blight disease by 50.6–51.6% and increased β-1,3-glucanase and chitinase

activity, indicating their ability to induce resistance against the disease in tomatoes.

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