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GROWTH INHIBITION OF FUNGAL PLANT PATHOGENS BY ANTAGONIST BACTERIA USING DUAL CULTURE ASSAYS

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ABSTRACT

There has been excessive use of chemical-based pesticides globally resulting in significant environmental consequences with adverse effects on human health. Therefore, more sustainable environment friendly alternative solutions are needed to counter such environmental and health challenges. Development of biocontrol method is a sustainable solution to these challenges. The main objective of this research was to investigate the efficacy of potential antagonist bacteria to inhibit important fungal plant pathogens *in vitro* by applying dual culture assays on potato dextrose agar (PDA) or trypticase soya agar (TSA) media, with a view to isolate and screen potential antagonists for development of future biocontrol agents. The target pathogens were *Fusarium oxysporum, Ceratocystis* sp., *Aspergillus flavus* and *Aspergillus niger*, commonly found to infect horticultural plants in Bedugul village in north Bali island. The antagonist bacteria were isolated from various sources such as soil of rhizosphere zone and roots of lettuce plants and mature compost. The potential of antagonist candidates was screened on the basis of inhibitory activity against targeted fungal pathogens. Bacterial antagonists with highest zone of inhibition were identified up to genus level using biochemical tests, and the results were matched with those specified in the Bergey's Manual of determinative bacteriology. Fifteen bacterial isolates were successfully isolated from various sources, and 60% of these isolates showed antagonistic activity *in vitro* against fungal pathogens with various degree of inhibition. This indicated the initial potential to develop as biocontrol agents. Based on preliminary identification, genera of *Bacillus* and *Pseudomonas* were found to be the predominant isolates and in addition genus *Acinetobacter* was also identified in this study.

Keywords: Antagonistic, *Aspergillus flavus, Aspergillus niger, Ceratocystis* sp., Dual Culture Assay, *Fusarium oxysporum*

INTRODUCTION

Chemical-based pesticides have been used to control plant pathogens in farming practices for decades, and its application in agricultural sector into the future will continue in the short to the medium term (Bueno *et al.* 2017), despite the pollution challenges. These chemical-based solutions such as pesticides are used to protect agricultural crops from attack by fungi, insects, weeds, and rodents as well as lengthen the lifespan of farming products or prevent spoilage (Kumar *et al.* 2012). Application of chemical pesticides is also common in non-agricultural sectors, such as sport facilities, shampoos for animals, and boats (Nicolopoulou-Stamati *et al.* 2016). Long term application of chemical based pesticides in agricultural and non-agricultural sectors has resulted environment and health impact in everyday life. The residue of such harmful compounds is now found in soil, waterways, agricultural crops, milk and meat causing human health challenges (Kim *et al.* 2016). The presence of these chemical contaminations in soil also frequently kills beneficial microbiota of soil, such as mycorrhiza and plant growth promoting rhizobacteria beyond the target pathogens. Furthermore, increase in resistance of plant pathogens may occur as a result of excessive application of such

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chemical compounds in farming practices (Lucas *et al.* 2015). This may also result in increased dose or innovations in new pesticides that are needed to control the same pathogens (Fernandes *et al.* 2010). Other studies have also reported negative impacts of pesticide exposure to the consumer and community (Andersson 2014). In terms of health impacts pesticides studies indicate neurological effects, diabetes, respiratory diseases, fetal diseases, genetic disorders and cancer in humans (Andersson 2014; Kim *et al.* 2017). Methyl bromide is an example of a widely used broad spectrum pesticide which was phased out in developed countries without an alternative (UNEP 2014). In addition it is claimed that these chemicals contribute to greenhouse gas emission (Heimpel *et al.* 2013).

Although above serious challenges exist, pesticides use in farming practices will continue and totally phasing out of such compounds will take time until better sustainable non-toxic alternatives are found (Bueno *et al.* 2017). Biological based sustainable technologies are being intensively researched towards the development of alternative methods to control plant pathogens worldwide. Integrated Pest Management (IPM), including the use of biological control agents to control pests and pathogens is one of the most favorable and sustainable solution paths globally.

In Bali, particularly in Bedugul Village, crop rotation in farming practices has been applied for years. Horticultural crops planted in this village include tomato, lettuce, cabbage, potato, and broccoli. At the time of sample collection, this farm was planted with lettuce plants, and therefore, the bacterial antagonists were isolated from soil in the rhizosphere zone and roots of lettuce plants from this area. In addition to these sources, we also isolated antagonists from vermi compost collected from a composting site in Denpasar city where earth worms were used as starter in the process of composting. Previous literatures for example in Gehan *et al.* (2018) have reported that mature composts (including vermi compost) are rich in antagonists of fungal pathogens.

Based on the above rationale, the goal of this study focused on isolation, screening, and identification of potential of bacterial biocontrol agents to inhibit plant pathogens, with a view toward the development of biocontrol agents, so that chemical-based fungicide use in the future can be reduced. The primary objective of this study was to investigate the effectiveness of bacterial antagonist isolates to inhibit the *in vitro* growth of several fungal plant pathogens (*Fusarium oxysporum*, *Ceratocystis* sp., *Aspergillus flavus*, and *Aspergillus niger*), commonly found to infect horticultural plants (including lettuce plants) in Bali, particularly in Bedugul area (Suryanti *et al.* 2013) and neighboring villages of Bedugul, including Kembang Merta village (Wulansari *et al.* 2015).

MATERIALS AND METHODS

Fungal Pathogens

Fungal pathogens (*Fusarium oxysporum, Ceratocystis* sp., *Aspergillus flavus,* and *Aspergillus niger*) were obtained from stock culture collections of the Mycology Laboratory, School of Biology, Universitas Udayana, Bali. These fungal isolates were identified in our previous study from samples collected in Bedugul Village (Suryanti *et al.* 2013). For regular use, these pathogens were subcultured on fresh PDA. For long-term storage, they were grown for 48 hours at ambient temperature on potato dextrose agar, cut into 1 x 1 cm^2, placed in sterile distilled water, and stored at 4 °C.

Sample Collection and Isolation of Potential Bacterial Antagonists

Antagonists of plant pathogens were isolated from several sources, namely mature vermi-compost (a composting site in Denpasar City), soils sampled from rhizhosphere in lettuce farm in Bedugul, Bali, and rhizoplane (roots) of lettuce plants from Bedugul, Bali. Isolation of bacterial antagonists from these sources was conducted by applying serial dilution and plating method on potato dextrose agar (PDA) or trypticase soya agar (TSA). Samples weighing 10 g were dissolved in saline solution (0.85% w/v NaCl) and volume was adjusted to 100 mL to obtain dilution rate of 10^{-1}, and stomached for 20 minutes to release attached microbes on particles of soil or other samples. These samples were then further diluted in saline solution up to 10^{-6}, and 0.1 mL of sample from each dilution

was then spread onto either potato dextrose agar (PDA) or trypticase soya agar (TSA), followed by incubation at ambient temperature for 24 -72 hours. Colonies that could be distinguished appeared on media (at dilution rates of 10^{-3} - 10^{-6}) following 48 hours incubation at ambient temperature and were streaked for single colony isolation to obtain pure culture isolates. These pure cultures were then suspended in trypticase soya broth (TSB) or potato dextrose broth (PDB) with 30% glycerol in the medium, and stored at -80°C (as stock cultures) until needed for further experiments.

In vitro Dual Culture Assay for Screening Potential Antagonists

Isolates of bacteria obtained previously were spot inoculated in triplicates onto the periphery of PDA or TSA plates (corresponding to the medium on which they were isolated). Plugs (1 cm^2) of fungal pathogens were then placed in the center of the plates followed by incubation at ambient temperature or approximately at 25 °C for 1 to 7 days, after which time the inhibition zone between the antagonists and pathogens were measured from three different angles using a vernier calipers, and then averaged. Three replications were made in the experiment to obtain representative data. Isolates showing inhibition zone were subcultured on fresh PDA or TSA and stored at 4 °C for further study. For long term storage, these isolates were stored in TSB with 30% glycerol and stored at -80 °C.

Preliminary Identification of Biocontrol Agent Candidates

Bacterial isolates showing *in vitro* antagonistic activity against tested plant fungal pathogens were preliminarily identified up to genus level on the basis of their morphological characteristics and specific selected tests (for soil bacterial identification) and the results were matched with those indicated in Bergey's Manual of Systematic Bacteriology (Krieg & Holt 1984; Holt *et al.* 1994). The tested characteristics of bacterial antagonist candidates were: Gram stain and cell morphology, oxidative/fermentative properties, spore stain, motility, flagella stain, oxidase, urease, and catalase production, ability to hydrolyze casein, UV fluorescence, levan production, gelatin hydrolysis, casein hydrolysis, and starch hydrolysis. The procedures (preliminary identification of soil bacteria up to genus level) of all tests followed the steps specified in Ramona (2003), Suryanti *et al.* (2013) and Wulansari *et al.* (2015).

RESULTS AND DISCUSSION

A total of fifteen (15) distinguishable bacterial isolates were successfully isolated from this study. Of these, 60% of the isolates showed antagonistic activity *in vitro* against one or more tested fungal pathogens with various degree of inhibition (Fig. 1 and Table 1). Rhizosphere zone of lettuce plants appeared to be the best source of potential antagonists. Most bacterial antagonists isolated in this study were obtained from such zone and they showed various degree of *in vitro* inhibition of plant pathogens (Table 1). Surprisingly, only 2 isolates from lettuce roots inhibited the plant pathogen *in vitro*. Bacterial isolates obtained from vermi-compost inhibited the *in vitro* growth of all fungal pathogens used in the study with various degree of inhibition (Table 1). It can be seen in Table 1 that isolates A4, A5, T1, and T2 which were identified as *Bacillus* or *Pseudomonas* (Table 2 and 3) appeared to have the widest spectrum of *in vitro* inhibition. These isolates inhibited the growth of all tested fungal pathogens with various degree of inhibition, depending on the species of pathogens. These results provided us with initial indication of antagonists with potential to be developed as biocontrol agents. This can now be targeted for further greenhouse or field trial scale studies for validation and effective application. The inhibition zone produced by these bacterial antagonists against the targeted fungal pathogens varied between 0.86 ± 0.11 cm and 3.20 ± 0.00 cm (Table 1). Isolate A1 which was identified to belong to genus of Pseudomonas only inhibited the growth of *Fusarium oxysporum*, with inhibition zone of 1.83 ± 0.15 cm (Table 1 and 2). The origin of this isolate was the rhizosphere zone of lettuce plants.

The limited number of isolates obtained in this study (Table 1, 2, and 3) was partly due to the primary focus on building overall knowledge and basic tools related to *in vitro* cultivation of

beneficial antagonistic microbes against fungal pathogens of Horticultural crops and from this foundation then subsequently build a wider screening system for extensive isolation of biocontrol agents. Previous reports indicated that an optimal optimized screening system is essential for *in vitro* cultivation from wider substrates (Weller 1988) and this study advanced this objective. Our results from this initial study were in line with Malleswari (2014) and Suthar *et al.* (2016) who reported similar numbers of antagonist isolates from soil samples. In synthetic media, such as TSA or PDA (considered as general media for bacteria and fungi, respectively) genera of *Bacillus*, *Pseudomonas*, and some genera belonging to enterobacteriaceae commonly grow well on such media and dominate during culturing. Bacteria or fungi with specific or unidentified need will not show growth response on such media and has been reviewed previously by Pham and Kim (2012) and as suggested only 1% of soil bacteria may be culturable on synthetic media. Therefore, there is a need for further improvement of methods as well as content and quality of synthetic media so that wider range of soil bacteria become culturable and therefore increase the probability to obtain potential isolates for biocontrol development. Pham and Kim (2016) proposed a novel method for bacterial cultivation by applying polycarbonate permeable membrane of trans well plates in liquid cultures with common synthetic media to mimic the actual environmental conditions. By applying such method they increased numbers of culturable isolates by more than 20%. Such methods will be built on the foundations of this study in future strategies.

The dual culture assays applied in our study (following isolation of antagonist candidates) appeared to be very convenient to initially indicate antagonism of bacterial antagonists against plant fungal pathogens. These results will have to be evaluated and correlated for their effectiveness in further greenhouse or field scales experiments. Overall, this is a classical method and has been used effectively to isolate and initially screen bacterial antagonists of fungal plant pathogens *in vitro*. Other studies recently applied this method to obtain bacterial antagonist candidates and subsequently identified the same to target fungal pathogens (Suthar *et al.* 2016; Wang *et al.* 2019); and Manandhar *et al.* 2019). This indicates that dual culture method is still a reliable approach to initially screen and identify antagonistic activity of a bacterial isolate against fungal or bacterial pathogens.

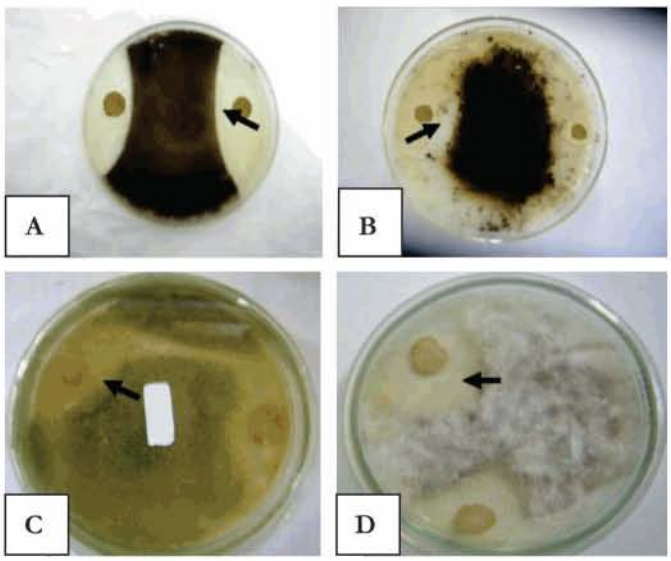

Figure 1 Some examples of *in vitro* antagonism between bacterial isolates obtained in this study and the tested fungal pathogens. (a) *Pseudomonas* sp. vs *Ceratocystis* sp., (b) *Pseudomonas* sp. vs *Aspergillus niger*, (c), *Bacillus* sp. vs *Aspergillus flavus* and (d). *Bacillus* sp. vs *Fusarium oxysporum*
Note: Arrowheads show the inhibition zones between the bacterial antagonists and tested fungal pathogens.

Table 1 Average diameter of *in vitro* inhibition zones (on PDA or TSA media) of bacterial antagonist candidates on several plant pathogens (*Fusarium oxysporum, Ceratocystis* sp., *Aspergillus flavus,* and *Aspergillus niger*)

Isolate codes	Source of antagonists	Diameter of inhibition zones on fungal pathogens (cm)*			
		F. oxysporum	*Ceratocystis* sp.	*A. flavus*	*A. niger*
A1 (PDA)	Lettuce rhizosphere	1.83 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
A4 (PDA)	Lettuce rhizosphere	1.06 ± 0,06	2.20 ± 0.10	1.50 ± 0.10	1.60 ± 0.11
A5 (PDA)	Lettuce rhizosphere	3.20 ± 0.00	2.46 ± 0.11	1.33 ± 0.11	1.30 ± 0.10
A6 (PDA)	Lettuce rhizosphere	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T1 (PDA)	Vermi-compost	0.96 ± 0.06	1.80 ± 0.06	1.13 ± 0.11	1.13 ± 0.11
T2 (PDA)	Vermi-compost	0.86 ± 0.11	1.83 ± 0.06	1.26 ± 0.06	0.90 ± 0.10
S1 (TSA)	Lettuce rhizosphere	1.63 ± 0.06	0.00 ± 0.00	3.36 ± 0.30	0.00 ± 0.00
S2 (TSA)	Lettuce rhizosphere	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
S3 (TSA)	Lettuce rhizosphere	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.13 ± 0.15
F1 (TSA)	Roots of lettuce	1.50 ± 010	0.00 ± 0.00	2.70 ± 0.10	0.00 ± 0.00
F2 (TSA)	Roots of lettuce	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
F3 (TSA)	Roots of lettuce	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
R1 (TSA)	Roots of lettuce	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
R2 (TSA)	Roots of lettuce	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
R3 (TSA)	Roots of lettuce	0.00 ± 0.00	0.00 ± 0.00	1.60 ± 0.17	0.00 ± 0.00

Notes: * Values in Table 1 ± standard deviation are averages of triplicates; PDA (Potato Dextrose Agar) and TSA (Trypticase Soy Agar) were media used in the dual culture assays.

The relative average inhibition zones shown in Table 1 varied from 0.0 to 3.36 cm, and these apparently were affected by types and the origin of the isolates. The zone of inhibition was also determined by species of fungal pathogens tested. Generally, bacterial isolates obtained from rhizosphere of lettuce plants was found to be antagonistic against one or more fungal pathogens tested in this study (Table 1). Similar results were also reported by Ramona (2003) and Wang *et al.* (2019) who found abundant antagonist isolates in the rhizosphere zones of various plants. This indicates that rhizosphere area can still be considered as a reliable source of bacterial antagonists of plant pathogens for development of biocontrol agents. In addition to rhizosphere zone, antagonists can also be isolated from many sources, such as mature compost or surface of plant roots (rhizoplane) (Table 1). Some researchers have also often isolated these antagonists from suppressive soil (Schlatter *et al.* 2017), suppressive compost (Gehan *et al.* 2018), or from contaminated media in the laboratory (Ramona 2003).

The origin of the antagonist isolates is not the most important aspect in the development of biocontrol agents. The effectiveness of these antagonists in protecting horticultural plants from attack by certain pathogens either in greenhouse or field applications is apparently more important consideration than their origins. To increase the effectiveness of antagonist isolations, ideally these antagonists should be isolated from places where these agents are to be applied, as they are already well-adapted in such areas of plant systems (Whipps 2001; Ganeshan & Kumar 2005; Dukare *et al.* 2018).

Spectrum of fungal pathogen inhibition *in vitro* of the bacterial isolates obtained from this study varied among species (Table 1). Some isolates were able to control only one tested fungal pathogen, while the other controlled two or more pathogens (Table 1). Generally, bacterial antagonists isolated from vermi-compost and rhizosphere zone of lettuce plants appeared to have wider spectrum of fungal pathogen inhibition *in vitro* when compared to those isolated from other sources. Surprisingly, isolates obtained from Rhizoplane (hair-root surfaces) of lettuce plants were found to be less effective in inhibiting the four fungal pathogens *in vitro* than isolates obtained from other sources (Table 1). This phenomenon was contradictory to that reported by Wei *et al.* (1996) and Walia *et al.* (2013), but in line with that reported by Oka (2004). Although some isolates in Table 1 showed a wide spectrum of fungal pathogen inhibition, these isolates will have to be further

tested to study their effectiveness when applied in glasshouse or field trials. Ramona (2003) reported that a positive correlation between the results of *in vitro* bioassay and those of glasshouse or field trials may not be necessary. The reasons for these were extensively reviewed by Whipps (2001) and O'Brien (2016) and wider testing is essential following the screening for practical application.

Although observable inhibition zones were clearly indicated in the *in vitro* bioassay (Table 1 and Fig. 1), the mechanisms by which bacterial antagonists may inhibit the fungal pathogens (for examples: antibiosis, siderophore production, or other mechanisms) was still inconclusive. Besides that, the effect of this antagonism (whether it was lethal or fungi static) has not yet been answered and will be considered in future studies.

Bacterial isolates with ability to inhibit the growth of at least one tested fungal pathogen were initially identified up to genus level, based on the method of soil bacterial identification specified in Ramona (2003) and Suryanti *et al.* (2013). The results are shown in Table 2 and Table 3. Based on these results, the genus name of the isolates was determined. One of the *Pseudomonas* isolates showed fluorescence properties under UV light, in addition to the results of other tests generally used in the initial preliminary identification of soil bacteria, and therefore, it was identified as *P. fluorescence* (Table 2). Among the 9 bacterial antagonists, 5 isolates belong to *Bacillus* sp. as all of them produce endospore and showed characteristics of genus *Bacillus* as shown in Table 3.

Preliminary identification of the isolates obtained from this study showed that genera of *Bacillus* and *Pseudomonas* were predominant bacterial isolates contained in all samples (Table 2). This is in line with that reported by Kumari and Khanna (2016) and Soare *et al.* (2019). The presence of these two bacterial genera in the soil or other samples is relatively more abundant than others and is partly due to simpler requirements for growth of these groups when compared to others (Raaijmakers *et al.* 2010). The ability of *Bacillus* sp. to produce endospore also contributes to the survival of this genus in adverse environmental conditions (Kumari & Khanna 2016). Besides *Bacillus* and *Pseudomonas*, genus of *Acinetobacter* was also isolated in this study (Table 2).

Table 2 Preliminary identification of Gram-negative bacterial antagonists

Isolate codes	Gram stain	O/F	Motility	Flagella Position	Oxidase	UV fluorescence	Starch hydrolysis	Levan production	Gelatin hydrolysis	Pigment	Preliminary Identity
A1	negative	O	+	Polar	-	-	+	-	+	-	*Pseudomonas* sp.
A4	negative	O	+	Polar	+	+	-	-	+	-	*P. fluorescence*
T1	negative	O	+	Polar	+	-	-	+	-	-	*Pseudomonas* sp.
S1	negative	O	-	-	-	-	-	ND	+	Creamy	*Acinetobacter* sp.

Note: O/F: Oxidative/Fermentative.

Table 3 Preliminary identification of Gram-positive bacterial antagonists

Isolate Codes	Gram stain	O/F	Motility	Flagella position	Catalase	Pigment	Spore	Starch Hydrolysis	Urease	Casein hydrolysis	colony	Preliminary Identity
A5	positive	O	+	Polar	+	-	+	+	+	+	dry	*Bacillus* sp.
T2	positive	O	+	Polar	+	-	+	+	+	+	dry	*Bacillus* sp.
S3	positive	O	+	Polar	+	-	+	+	-	+	dry	*Bacillus* sp.
F1	positive	O	-	-	+	-	+	-	+	+	dry	*Bacillus* sp.
R3	positive	O	+	polar	+	-	+	+	+	+	dry	*Bacillus* sp.

Note: O/F: Oxidative/Fermentative.

CONCLUSION

Nine out of 15 (60 %) bacterial isolates showed antagonistic activities against several tested fungal pathogens in the *in vitro* dual culture assay, although the mechanisms by which these biocontrol candidates inhibit the fungal pathogen is still inconclusive, and therefore further study is needed to specifically elucidate the mechanisms (such as antibiosis, production of siderophore, etc.) of biocontrol. In the initial and preliminary identification of these antagonists, it was found that genera of *Bacillus* and *Pseudomonas* were predominant isolates found in all samples. Besides these, genus of *Acinetobacter* was also isolated and identified in this study.

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