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To cite this article: M Hoesain *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **759** 012069

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In vitro studies on *Bacillus* sp. and *Pseudomonas* sp. compatibility with botanical pesticide

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Abstract. The effect of botanical pesticides as an insect pest control agent has been proven in several studies. Despite capable of controlling pests, botanical pesticides have not been reported to promote plant growth. One technique for making botanical pesticides served in dual function is to combine them with beneficial microbes. This study was aimed to determine the compatibility of 3 isolates of *Bacillus* sp. and 3 isolates of *Pseudomonas* sp. against botanical pesticides. The botanical pesticides were made from a mixture of *Ageratum conyzoides*, *Aglaia odorata*, and *Azadirachta indica* leaves. The solvent used 96% ethanol, and the process of making botanical pesticides was performed using a rotary evaporator at 45°C. The compatibility test was performed *in vitro* by dropping 10 µl of botanical pesticides on the bacterial culture. Tests were carried out using various concentrations, namely 0%, 25%, 50%, 75%, and 100%. The test results showed that *Bacillus* sp. A and *Pseudomonas* sp. E strain were compatible on all assay concentrations. *Bacillus* sp. A and *Pseudomonas* sp. D and *Pseudomonas* sp. E were compatible with the concentrations of 0 to 75%. Furthermore, *Bacillus* sp. B, *Bacillus* sp. C, and *Pseudomonas* sp. F were compatible with the assay concentrations of 0 to 50%.

1. Introduction

Plant pest organism control can be performed using various pesticides. However, the use of excessive and continuous chemical pesticides is reported to have a negative impact on human health and environmental sustainability [1, 2]. The negative impact will be more severe when farmers do not carefully choose the chemical pesticides and the way to use them. Moreover, the negative impact of excessive and continuous chemical pesticide use is residual pesticide accumulation on the agricultural product [3, 4]. Besides, chemical pesticides' active compounds are also reported to be hardly degraded and accumulated in the soil [5]. One solution to overcome this problem is through employing botanical pesticides [6].



Botanical pesticides are pesticides with their active compounds originating from plants or parts of plants, such as roots, leaves, stems, or fruits. These materials are processed into various forms, namely raw materials in powder, extract, or resin, as secondary metabolite liquid retrieval from plants' parts [7]. Pesticides from botanical materials are not new materials but have been long used. Since agriculture still performs traditionally, farmers worldwide have been used to applying the available materials in nature to control the plant pest organisms [8-10].

Botanical pesticides utilized the plant secondary metabolites as their active compounds. These compounds have functioned as pest repellent, attractant, and killer as well as pest appetite inhibitor [11, 12]. The use of plant materials known to have those characteristics, specifically as active botanical compounds are expected to substitute the use of synthetic pesticides; therefore, the synthetic chemical residue compounds on various agricultural products that are known to carry various negative impact for nature and life around them can be suppressed as low as possible [13, 14].

The application of botanical pesticides has still been lately performed individually. The mixture product of plant secondary metabolites enriched with the endophytic bacteria (referred to as biopesticides) is still unavailable in Indonesia. The combined application of biological control agents can provide a better result than the individual application [15, 16]. Endophytic bacteria are bacteria that live in plant tissue and beneficial for their host. Endophytic bacteria can play double roles as biological control and growth promoter [17]. As a control agent, endophytic bacteria can produce extracellular enzymes (protease, chitinase, lipase, cellulase), HCN volatile compounds, and siderophore compounds [18-20]. As a growth promoter, endophytic bacteria can fix nitrogen (N) from the environment, dissolve phosphate (P), and produce phytohormones (auxins, cytokinins, gibberellins) [21, 22].

The combination of botanical pesticide and endophytic bacteria can suppress important pests and diseases in rice plants. Afzal et al. [23] reported that the combination of biopesticide with active plant metabolites and endophytic bacteria could suppress blight disease caused by *Xanthomonas oryzae*. This disease is one of the important diseases in rice plants in almost all rice-producing countries. Also, the combination of some biopesticide active compounds are reported to suppress *Meloidogyne graminicola* nematode population in rice plant effectively. The decreased population of *M. graminicola* is reported to positively correlate with decreased root damage in rice [24]. Another study reported that the combination of an endophytic bacterial consortium with botanical carrying material effectively suppressed *N. lugens* [25].

The combination formula of macerated plant metabolites and endophytic bacteria has the potential to become a new type of biopesticides with double functions as pest and disease control in organic rice plants. The mode of actions on the combination formula can be either directly or indirectly. Directly, the insecticidal compounds obtained from plant maceration can poison the target pest insects [26]. Moreover, indirectly, the combined endophytic bacteria can induce the plant resistance through Induced Systemic Resistance (ISR) mechanism. The ISR mechanism is a plant resistance form against various plant pests with a broad spectrum [27].

Based on the data above, there is a potential to develop a new type of biopesticides with the active ingredient of endophytic bacteria and botanical pesticide. This research is a preliminary study which aimed to test the compatibility of endophytic bacteria with botanical pesticides. The results of this study will provide a new information regarding the compatibility of endophytic bacteria with vegetable pesticides which are still rarely reported.

2. Material and Methods

2.1 Time and Location

This study was performed on September 2020 in the laboratory of Plant Pest Organism Control Technology, Plant Protection Study Program, Faculty of Agriculture, Jember University, Indonesia.

2.2 Endophytic Bacterial Isolate Source

The endophytic bacterial isolate source used in this study was from the previous study. The endophytic bacteria used were from *Bacillus* sp. and *Pseudomonas* sp. group that have been identified and characterized as capable of producing extracellular enzymes, volatile compounds, and contributing to plant growth promoter. Bacteria were preserved on Tryptic Soy Broth (TSB) media + 40% glycerol at 4 °C [20, 21].

2.3 Botanical Pesticide Material

The botanical pesticide was made from a mixture of some plant types. Plants used as botanical pesticide ingredient materials were *Azadirachta indica*, *Aglaia odorata*, and *Ageratum conyzoides* leaves. All samples were obtained around Jember University without requiring special permission.

2.4 Simplicia Production

All materials obtained were air-dried in the greenhouse, Plant Protection Study Program, Faculty of Agriculture, Jember University. Materials were dried for four days or until the mixture content reached 20%. Drying was performed carefully; therefore, the plant materials were directly exposed to sunlight. Dried materials were blended using a blender and filtered using a filter with a size of 150 mesh.

2.5 Botanical Pesticide Material Extraction

As much as 50 g powder from each material type was collected in a glass tube. All materials were soaked in 750 ml ethanol 96%. The soaking suspension was mixed with *Tween 80* as much as 3 ml and stirred until evenly distributed. The suspension formed was then incubated for 24 hours. The 24-hour soaked suspension was filtered using a filter paper, then supernatant formed was evaporated using a rotary evaporator at 45 °C. The suspension formed was then preserved for the next experiment [28].

2.6 Compatibility Test of Endophytic Bacteria with Botanical Pesticides

The endophytic bacteria were tested their compatibility using the disk diffusion method. The endophytic bacteria were grown on TSB liquid media in the form of suspension. Suspension preparation was performed by growing the single colony of 24 – 48-hour bacterial isolate on 100 ml TSB and incubated for 24 hours. The compatibility test was performed by dropping 200 µl endophytic bacteria suspension on a Petri dish given Tryptic Soy Agar (TSA) media, then spreaded using a bacterial glass bead. 300 µl bioinsecticide formula suspension was dropped on a 5 mm diameter sterile filter paper. The combination was compatible as no clear zone formed. The test was performed in various botanical pesticide concentrations, namely 25, 50, 75, and 100%. As a control, the filter paper was dropped a sterile aqua dest. Each treatment was repeated four times and moved on to the uniform environment [29].

3. Results and Discussion

3.1 Endophytic Bacteria Density

The endophytic bacteria used in this study had various cell densities. Based on the counting result in the laboratory, it was identified that the endophytic bacteria density used in this study was ranged from 3.6×10^8 until 8.7×10^8 . Moreover, each endophytic bacteria density used for the compatibility test is presented in Table 1.

Table 1. Endophytic bacteria cell density used for compatibility test

Code	Genus	Cell density
A	<i>Bacillus</i> sp.	4.2×10^8
B	<i>Bacillus</i> sp.	4.4×10^8
C	<i>Bacillus</i> sp.	3.6×10^8
D	<i>Pseudomonas</i> sp.	8.7×10^8

E	<i>Pseudomonas</i> sp.	6.0×10^8
F	<i>Pseudomonas</i> sp.	5.1×10^8

3.2 Endophytic Bacteria Compatibility with Botanical Pesticide

The test results showed that all bacteria did not form clear zones on control treatment, which means that bacteria are compatible when combined with water. On the concentration tests of 25% until 50%, all bacteria showed the same pattern, namely compatible. Compatibility was presented by the occurrence of a clear zone around paper disk dropped with botanical pesticide. Furthermore, on the concentration test of 75%, the result had a quite variety. The B, C, and F bacteria were incompatible when tested with 75% concentration of botanical pesticide, as shown from the occurrence of clear zone around paper disk dropped with botanical pesticide. Nevertheless, A, D, and E bacteria were compatible with the concentration test of 75%. On the concentration test of 100% botanical pesticide, the study result showed that A and E bacteria were compatible. The test results are presented in Table 2 and Figure 1.

Table 2. Endophytic bacteria compatibility with botanical pesticide on various concentrations

Code	Test Concentration				
	0%	25%	50%	75%	100%
A	+	+	+	+	+
B	+	+	+	-	-
C	+	+	+	-	-
D	+	+	+	+	-
E	+	+	+	+	+
F	+	+	+	-	-

Note: (+) compatible, (-) not compatible

3.3 Discussion

Botanical pesticide formulation can be done by mixing various materials, as well as mixed with bacteria. The botanical pesticide formulation is an active compound combination with other materials on a certain level and form. The formulation is commonly performed to increase the botanical pesticide quality [12]. Formulation commonly contains three types, namely active ingredient, adjuvant, and carrier. The active ingredient is the main component of botanical pesticide that can be solid, liquid, or gas [30, 31]. Adjuvants commonly used are solvent, emulsifier, wetting agent or diluents, stain, and odor enhancer. Adjuvants aim to improve pesticide efficacy and make the botanical pesticide applicable [32].

Compatibility tests should be performed before ingredient formulation to prevent a dangerous and ineffective formula. The plant ingredient sources used in this study were reported to have some antimicrobial compounds; therefore, the compatibility test should be performed first before formulation [33]. Based on their active compounds, seeds and leaves of *A. indica* contain azadirachtin, meliantriol, salannin, and nimbin as secondary metabolites of *A. indica*. The active compounds of *A. indica* do not kill pest rapidly but influencing the appetite, growth, reproduction level, molting process, mating and sexual communication inhibition, low hatching rate, and chitin forming inhibition. Moreover, it also plays a role as a sterile agent. Besides characterized as an insecticide, this plant also has fungicide, virucide, nematocidal, bactericide, miticide, and rodenticide. These active compounds have been reported to affect about 400 insects [34].

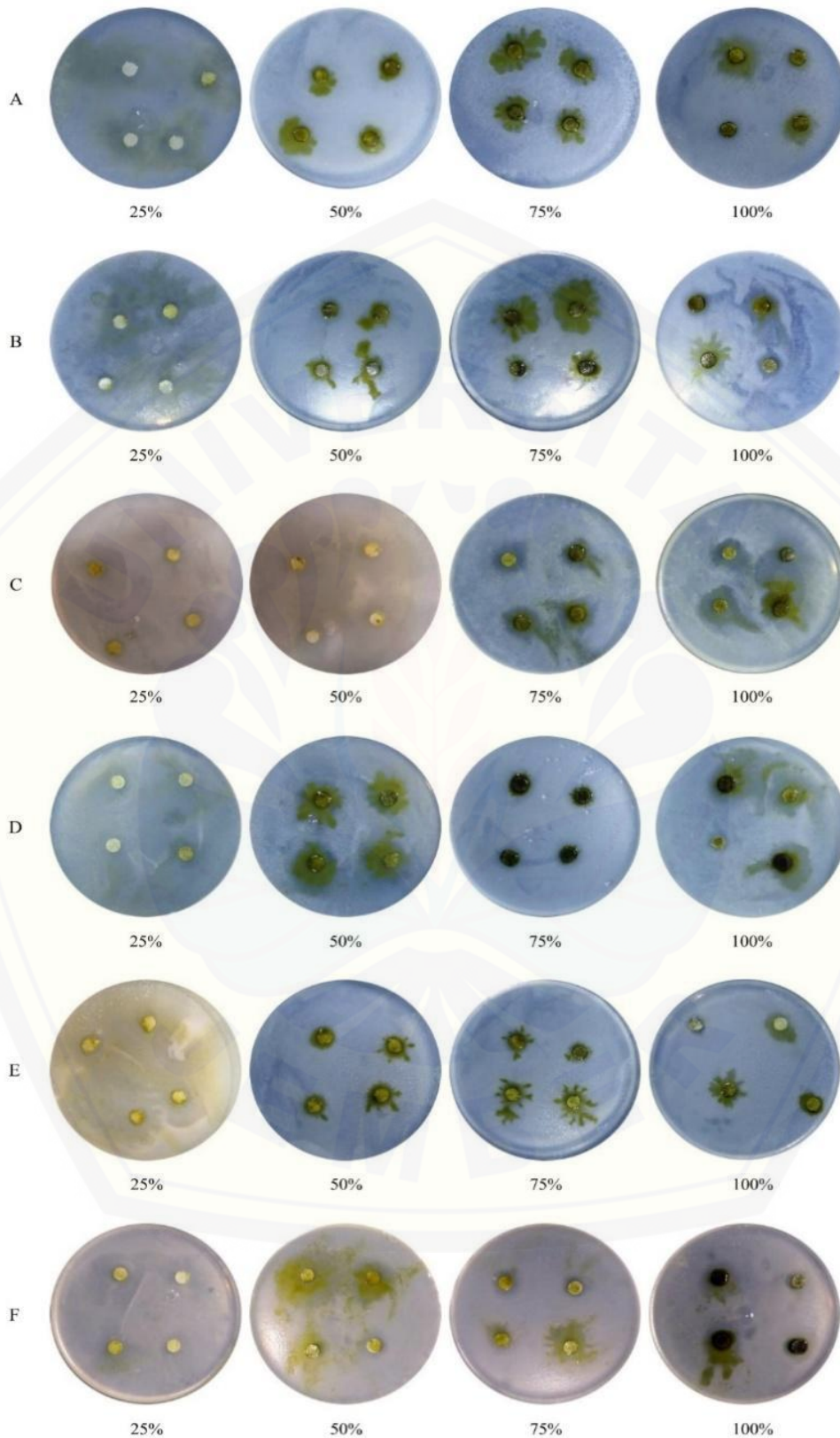


Figure 1. Endophytic bacteria compatibility with botanical pesticide on various concentration

Moreover, the secondary metabolites contained in *A. conyzoides* are saponins, flavonoids, polyphenols, coumarins, 5% eugenol, hydrogen cyanide (HCN), and essential oils. *A. conyzoides* as a botanical pesticide is specifically reported for insect pests, as the bioactive compounds in this plant have resistant and inhibition characteristics on insect proliferation. In *A. conyzoides*, the plant parts extracted were leaves [35]. Furthermore, According to Ewete et al. [36], *A. odorata* leaves contained alkaloids, saponins, flavonoids, tannins, and essential oils. *A. odorata* also reported contained secondary metabolites that played the roles as either insecticide or fungicide.

Although botanical pesticide's main ingredients contain some antimicrobial compounds, some bacteria can survive on these compounds. This survival is one of which is caused by the bacteria's capability of producing biofilm. Biofilm is the collection of microorganisms or microbial cells, especially bacteria attached to a surface and covered with polysaccharide adhesive excreted by the bacteria (exopolysaccharides/EPS) [37]. This attachment is followed by organic material accumulation covered with an extracellular polymer matrix produced by the bacteria. This matrix is in the form of a cross-threaded structure as an adhesive device for biofilm. This biofilm can be formed from various microorganism species that form a consortium. The biofilm matrix plays a role as the external digestive system, protecting from enzymes that can disrupt cell metabolism and dissolved, colloid, and solid biopolymer compound metabolism agent. Biofilm is also more resistant than planktonic cells against antibacterial agents [38]. The existence of microorganism physiological activity in biofilm causes the developed chemical gradient to create different micro-habitats that can support diversity. Biofilm is formed due to the existence of a less beneficial environment [39].

In a separate study, Berbers et al. [40] reported that bacteria from *Bacillus* sp. and *Pseudomonas* sp. group had good resistance against some types of antibiotics. Resistance can be formed due to bacterial mechanisms to protect the DNA, therefore unaffected by antimicrobial compounds in the environment. Another report by Thomas et al. [41] also mentioned that endophytic bacteria could be combined with botanical pesticides. The combination of endophytic bacteria and botanical pesticides will induce the effect of botanical pesticide use.

4. Conclusion

The endophytic bacteria from *Bacillus* sp. and *Pseudomonas* sp. group had the potential to be combined with botanical pesticides extracted from *A. indica*, *A. odorata*, and *A. conyzoides* leaves using ethanol 96% solvent. The maximum concentration recommended to perform this combination is 50% or 1:1 (v/v).

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Acknowledgements

The authors would like to thank Jember University for funding this study through the *Hibah Penelitian Pendukung IDB* scheme in 2020.

