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Short Communication

Hemolysis and hypersensitive tests ease culture collection management of antagonistic bacteria

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ABSTRACT

A biosafety assessment is a crucial first step in the management of microbial culture collection to screen and determine unexpected potential plant and human pathogenic bacteria. It is common to collect and store as many fresh culture collections from natural resources before being further evaluated for antagonist bacteria. As a result, a bulk of isolates must be preserved which required more effort and budget. Safety evaluations based on the hemolysis and hypersensitive reactions offer simple tests to ease culture collection management of antagonist bacteria. The study aimed to evaluate the safety of bacterial culture collection for their hemolysis and hypersensitive reactions. Ninety-five isolates of rhizosphere and endophytic bacterial isolates from the culture collections of the Department of Plant Protection-IPB University, Indonesian Industrial and Beverages Crops Research Institute, and Indonesian Spice and Medicinal Crops Research Institute, were evaluated their safety using the hemolysis and hypersensitive tests. The hemolysis test was conducted using blood agar media, from which isolates with a negative (λ) reaction were then tested for the hypersensitivity reaction on tobacco leaves. Bacterial isolates passed from both hemolysis and hypersensitivity tests were then preserved by the lyophilization method for long-term storage of culture collection. Based on the hemolysis test, 68 out of 95 bacterial isolates (71.57%) were found to be positive (α or β) reactions. The hypersensitive test showed that 22 of 27 negative hemolysis isolates did not trigger hypersensitivity reactions in tobacco leaves, therefore, they were preserved by lyophilization. The study indicated that a high number of bacterial isolates in the present collection, 68 positive hemolysis, and 5 hypersensitive, need to be re-evaluated due to their safety concerns. The present study highlights the importance of biosafety tests performed in an early stage before the to permanent collection of antagonist isolates.

Key words: antagonist bacteria, biosafety, early stage, lyophilization

INTRODUCTION

Microbial communities, such as rhizosphere and endophytic, play an important role in the improvement of soil fertility and plant development by enhancing plant growth and health. The roles of these microbes may be through direct and/or indirect mechanisms, such as the production of phytohormones, acquisition of nutrients, aminocyclopropane-1-carboxylic acid (ACC) deaminase production, synthesis of antibiotics, and lytic enzymes, interference in quorum sensing signaling and biofilm formation, and the production of siderophore. However, several studies revealed that the rhizospheres of plants can be a reservoir for opportunistic microbes that could cause disease in humans due to an increase in the number of human infections caused by opportunistic pathogens (Berg et al., 2005; Fletcher et al., 2013). Any microbes that will be released in food production must be safe for the environment (Leahy et al., 2014). Therefore, it is important to evaluate the safety of rhizosphere and endophytic microbial isolates intended to be preserved and used in agriculture production to determine their potential risk as human pathogens (Díaz-Rodríguez et al., 2021).

Before entering permanent preservation and deposit in culture collections, rhizosphere and endophytic microbes are isolated and characterized to fit with the main objectives of the study. As a result, thousands of freshly isolated might be collected and temporarily maintained. Keeping large fresh isolates of microbes needs adequate efforts and budgets which could be a real constraint for people working in culture collections. Therefore, it needs a preliminary screening for selecting beneficial microbes to reduce the number of isolates to be maintained. The first two-safety evaluations are hypersensitivity for determining their potential as plant

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pathogenicity (Wick, 2010) and hemolysis for the early step to evaluate their safety to humans (Thakkar et al., 2015).

Various studies had used these two criteria in selecting microbes before testing their particular benefits as biopesticides. These studies proved that adopting these two safety criteria significantly reduced the number of microbes to be preserved. The previous study (Oktafiyanto et al., 2018) reported a total of 843 endophytic bacterial isolates isolated from mangroves, only 403 isolates (47.80%) showed negative reactions in hypersensitive and hemolytic tests, of which 19 isolates could effectively suppress the growth *Ralstonia solanacearum* and killed *Meloidogyne* based on in vitro test. Another study by Akhdiya et al. (2018) screened 529 colonies of bacteria, but 9 isolates were safe based on the hypersensitivity and hemolysis reactions. Amaria et al. (2019) showed that 3 out of 35 bacteria (9%) isolated from the Philippine-thung plant (*Reutealis trisperma* (Blanco) *Airy Shaw*) were hemolysis reactions and 17 isolates (47.2%) were hypersensitive. Nine (15.25%) of 59 endophytic bacteria isolated from *Bambusa vulgaris*, *Gliricidia sepium*, and *Ocimum tenuiflorum* were hypersensitive and 14 isolates (23.72%) were hemolysis (Triwidodo & Listihani, 2021). The study aimed to evaluate the safety of bacterial culture collection for their hemolysis and hypersensitive reactions, before being lyophilized as a permanent collection.

MATERIALS AND METHODS

Research Site. The research was carried out at the Plant Bacteriology Laboratory, Department of Plant Protection-IPB University, Bogor, West Java, Indonesia.

Source of Isolates. Ninety-five of the bacterial isolates used in this study were from the culture collections of the Department of Plant Protection-IPB University, Indonesian Industrial and Beverages Crops Research Institute (IIBCRI), and Indonesian Spice and Medicinal Crops Research Institute (ISMCRI). All bacterial isolates were kept in sterile water and subjected to evaluation of their potential as biocontrol agents (Table 1). These isolates were cultured on tryptic soy agar (TSA) (Merck, Germany) for 24–48 hours at 28 ºC.

Hemolysis Tests. The hemolysis test followed the method of Gilligan (2013) and Buxton (2016). A loop -full of the individual bacterial isolate from pure cultures was streaked on blood agar media containing 5% (v/v) fresh goat/sheep blood. After 24–48 hours of incubation at 28 ºC, a clear zone or color change observed around the bacterial colonies indicated a positive reaction for hemolysis. Different colors were noted to specify reaction types of hemolysis, i.e., a dark or greenish-grey colored zone around the bacteria indicated partial lysis of the blood agar or known as α hemolysis reaction, a distinct clear zone referred to complete lysis of the blood agar or β hemolysis reaction, and if no zone or color change observed around the bacterial colony indicated negative or γ hemolysis reaction.

Hypersensitive reaction test. The hypersensitive test followed the method described by Klement & Goodman (1967). Bacterial isolates tested were those that showed negative reactions in the hemolysis tests, and a positive control was *Xanthomonas oryzae* pv. *oryzae*, the cause of bacterial leaf blight disease of rice. Bacterial isolates were grown on tryptic soy broth (TSB) (Merck, Germany) and incubated in a shaker at 120 rpm for 24–48 hours at 28 ºC. One mL of bacterial suspension was injected using a syringe into the lower surface tissue of the White barley variety of tobacco leaves. The inoculated tobacco plants were incubated outdoors for 24–48 hours followed by the observation of hypersensitive reaction. Bacterial isolates which showed necrosis symptoms on tobacco plants were categorized to be potential plant pathogens.

Those bacterial isolates that passed both the hemolysis and hypersensitive tests were further preserved as lyophilized for long-term storage of culture collection for up to 50 years (Kupletskaya & Netrusov, 2011) and characterized their potency as biocontrol agents.

RESULTS AND DISCUSSION

Sixty-eight out of 95 bacterial isolates (71.57%) showed positive hemolysis reactions (α and β) on the blood agar medium (Table 1; Figure 1). It means that only 27 bacterial isolates passed the hemolysis and needed the hypersensitive test. The results of this study revealed that the bacterial isolates detected as positive hemolysis were mostly β hemolysis, while only 5 isolates of α hemolysis were found. The transparent clear zone surrounding the bacterial colony is a characteristic of β hemolysis that formed as a result of the complete lysis of red blood cells in the medium. The occurrence of lysis is caused by toxic hemolysin produced by bacteria to destroy red blood cells, causing the denaturation of hemoglobin to form a colorless product (Gilligan, 2013; Buxton, 2016).

Alpha hemolysis with a greenish-black zone

surrounding the bacterial colony occurred due mainly to bacterial isolates producing H_2O_2 can cause hemoglobin in red blood cells to be converted into methemoglobin. This partial lysis occurs because the red blood cells are not completely destroyed, and therefore, the zone still contains red blood cells. Microscopic observations have shown that the blood cell membranes are intact (Gilligan, 2013). However, in biosafety screening, alpha hemolysis is still categorized as positive for hemolysis such as β hemolysis which has the potential to cause human and animal diseases (Naomi et al., 2019). Alpha and β hemolysis are considered determinants or assess the level of virulence and clinical relevance related to their potential as human and animal pathogens. Hemolysin compounds produced by bacteria are important virulence factors (Mogrovejo et al., 2020).

Following the hypersensitivity test on tobacco leaves (Table 1; Figure 2), only 22 bacterial isolates failed to induce a hypersensitivity reaction suggesting that these isolates are not considered plant pathogens. Therefore, they could be further characterized and well-maintained as candidate biocontrol agents. Bacterial isolates that induced hypersensitivity reactions are indicated by the appearance of necrotic symptoms. It is reported that such symptoms resulted from the death of plant tissue at the infection site, and the limited multiplication and spread of the pathogen (Klement & Goodman, 1967; Heath, 2000). In contrast, the absence of symptoms on tobacco leaves indicates the bacterial isolates are non-pathogenic. Bacterial suspension infiltrated in tobacco leaves induced the activation of defense genes and the production of antimicrobial secondary metabolites which act as plant barriers against pathogen infection (Wang et al., 2016). The study of Wang et al. (2016) revealed that there was no direct correlation between the origins of the bacterial isolates (rhizosphere or endophyte) with the biosafety reactions (hemolysis and hypersensitive).

The present study highlights the importance of conducting the two safety tests in the early stage

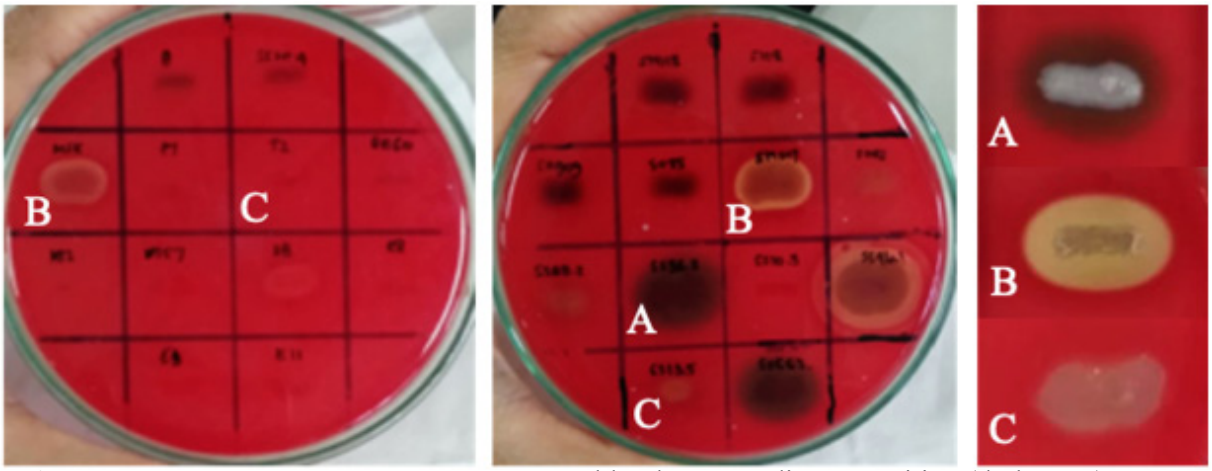

Figure 1. Hemolysis reaction of bacterial isolates on blood agar media. A. Positive (dark zone)= α; B. Clear zone= β, and C. Negative (no zone)= γ.

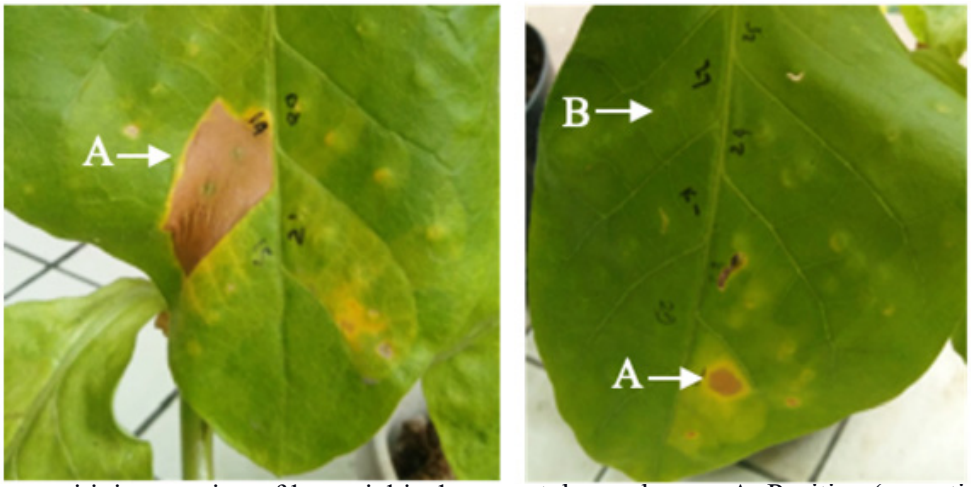

Figure 2. Hypersensitivity reaction of bacterial isolates on tobacco leaves. A. Positive (necrotic symptom); B. Negative (no necrotic symptom).

Table 1. The origin and biosafety evaluation based on the hemolysis and hypersensitivity reactions of 95

No	Origin	Code	HA[1)]	HR[2)]	No	Origin	Code	HA	HR	No	Origin	Code	HA	HR
1	Rubber rhizosphere	S018	γ	-	33	Patchouli endophyte	TT21	β	nt	65	Coffee endophyte	L19	β	nt
2	Rubber rhizosphere	S085	γ	-	34	Patchouli endophyte	TT22	β	nt	66	Coffee endophyte	L20	β	nt
3	Rubber rhizosphere	S108	γ	-	35	Patchouli endophyte	TT24	β	nt	67	Coffee endophyte	BPKN1	β	nt
4	Rubber rhizosphere	SK017	β	nt[3)]	36	Patchouli endophyte	TT25	β	nt	68	Coffee endophyte	BPKN3	β	nt
5	Rubber rhizosphere	SK0I8	γ	-	37	Patchouli endophyte	TT26	β	nt	69	Coffee endophyte	BPKN4	β	nt
6	Rubber rhizosphere	SK909	γ	-	38	Patchouli endophyte	TT27	β	nt	70	Coffee endophyte	BPKN5	β	nt
7	Philippine-thung rhizosphere	B	γ	-	39	Patchouli endophyte	TT28	β	nt	71	Coffee endophyte	BPKN6	β	nt
8	Cacao rhizosphere	SS1.2	γ	-	40	Patchouli endophyte	EH33	β	nt	72	Black pepper endophyte	LPKN1	β	nt
9	Cacao rhizosphere	SS10.3	γ	-	41	Patchouli endophyte	EH34	β	nt	73	Black pepper endophyte	LPKN2	β	nt
10	Cacao rhizosphere	SS19.7	γ	-	42	Patchouli endophyte	EH37	β	nt	74	Black pepper endophyte	LPKN3	β	nt
11	Cacao rhizosphere	SS20.4	γ	-	43	Patchouli endophyte	EH39	β	nt	75	Black pepper endophyte	LPKN5	β	nt
12	Cacao rhizosphere	SS4b.1	γ	+	44	Patchouli endophyte	EH40	β	nt	76	Black pepper endophyte	LPKN6	β	nt
13	Cacao rhizosphere	SS5b.3	γ	+	45	Patchouli endophyte	EH41	β	nt	77	Weed endophyte	E8	γ	-
14	Cacao rhizosphere	SSG6.1	γ	+	46	Patchouli endophyte	TT21	β	nt	78	Weed endophyte	E9	γ	-
15	Cacao rhizosphere	SSG6.2	α	nt	47	Nutmeg endophyte	BE60	γ	-	79	Weed endophyte	E11	γ	+
16	Cacao rhizosphere	SS3G.2	α	nt	48	Coffee endophyte	L1	β	nt	80	Weed endophyte	E12	β	nt

[1)] HA= hemolysis reaction based on the blood agar; [2)] HR = hypersensitive reaction based on the infiltration on tobacco leaf; - = negative reaction.

Table 1. Continued

No	Origin	Code	HA[1)]	HR[2)]	No	Origin	Code	HA	HR	No	Origin	Code	HA	HR
17	Cacao rhizosphere	SSG11.2	γ	+	49	Coffee endophyte	L2	β	nt	81	Weed endophyte	E14	β	nt
18	Patchouli endophyte	NJ2	γ	-	50	Coffee endophyte	L3	β	nt	82	Weed endophyte	E15	β	nt
19	Patchouli endophyte	NJ57	γ	-	51	Coffee endophyte	L4	β	nt	83	Weed endophyte	E16	β	nt
20	Patchouli endophyte	P7	γ	-	52	Coffee endophyte	L6	β	nt	84	Weed endophyte	E17	β	nt
21	Patchouli endophyte	T2	γ	-	53	Coffee endophyte	L7	β	nt	85	Weed endophyte	E19	β	nt
22	Patchouli endophyte	3B	β	nt	54	Coffee endophyte	L8	β	nt	86	Weed endophyte	E8	γ	-
23	Patchouli endophyte	TT3	β	nt	55	Coffee endophyte	L9	β	nt	87	Weed endophyte	E9	γ	-
24	Patchouli endophyte	TT4	β	nt	56	Coffee endophyte	L10	β	nt	88	Weed endophyte	E21	γ	-
25	Patchouli endophyte	TT5	β	nt	57	Coffee endophyte	L11	γ	-	89	Weed endophyte	E22	β	nt
26	Patchouli endophyte	TT7	β	nt	58	Coffee endophyte	L12	γ	-	90	Weed endophyte	E23	β	nt
27	Patchouli endophyte	TT10	β	nt	59	Coffee endophyte	L13	β	nt	91	Weed endophyte	E24	β	nt
28	Patchouli endophyte	TT11	β	nt	60	Coffee endophyte	L14	γ	-	92	Weed endophyte	E25	β	nt
29	Patchouli endophyte	TT12	β	nt	61	Coffee endophyte	L15	γ	-	93	Weed endophyte	E26	β	nt
30	Patchouli endophyte	TT15	β	nt	62	Coffee endophyte	L16	β	nt	94	Weed endophyte	E27	β	nt
31	Patchouli endophyte	TT16	β	nt	63	Coffee endophyte	L17	β	nt	95	Weed endophyte	E28	β	nt
32	Patchouli endophyte	TT17	β	nt	64	Coffee endophyte	L18	β	nt					

[1)] HA= hemolysis reaction based on the blood agar; [2)] HR = hypersensitive reaction based on the infiltration on tobacco leaf; - = negative reaction.

of a biocontrol research study to eliminate bacteria antagonists that are the potential to harm environments. Both hemolysis and hypersensitive tests are easily conducted in a standard laboratory. Overall, the results of the present study imply that 68 positive hemolysis and 5 hypersensitive bacterial isolates tested out of 95 isolates (76.8%) need to be re-evaluated their present in the present culture collection due to their safety concerns. The present study highlights the importance of biosafety tests performed in an early stage to manage culture collection before being preserved as a permanent collection of antagonist isolates.

CONCLUSIONS

Hemolysis and hypersensitivity are simple tests to evaluate the safety of bacterial culture collection from unexpected potential plant and human pathogenic bacteria in the culture collection. The tests could eliminate a large number of positive harmful isolates from a culture collection

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AUTHORS' CONTRIBUTIONS

WA, MSS, KHM, S, and W contributed to the design and conduct of the research, analysis of the results, and drafting of the manuscript. The final manuscript was read, revised, and approved by all authors.

COMPETING INTEREST

The authors have no conflict of interest to declare.

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