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Research Article
Potential of Endophytic and Rhizobacteria as an Effective Biocontrol for *Ralstonia syzygii* subsp. *syzygii*

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Abstract

Background and Objective: Sumatra disease caused by *Ralstonia syzygii* subsp. *syzygii* is reported to generate severe damage to clove plantation in Indonesia. The chemical control of insect vector has partially been successful yet impacted on environmental problems. Endophytic and rhizobacteria have been reported as potential biocontrol agents for many plant diseases due to safety of human and non-target organisms. The objective of this study was to find the potential endophytic and rhizobacteria for biocontrol of *Ralstonia syzygii* subsp. *syzygii*. **Methodology:** The endophytic and rhizobacteria were screened for their indole acetic acid (IAA) production, phosphate solubility, antibiosis activity and followed by molecular characterization. The experiment was arranged in a Completely Randomized Design (CRD) with 3 replications. The data were subjected to statistical analysis using one-way ANOVA and means were separated by DMRT ($p \leq 5\%$). **Results:** Forty six bacterial endophytes were successfully recovered from roots, stems, leaves and flowers of the healthy clove. Also, 66 isolates were isolated from the rhizospheric soil. Among them, 24 endophytic and 21 rhizobacteria produced indole acetic acid (IAA). Only rhizobacterial isolates but not endophytes indicated phosphatase activities. The *in vitro* antibiosis assay against *Ralstonia syzygii* subsp. *syzygii* showed that 23 endophytic and 14 rhizobacteria produced inhibition zone. Furthermore, 16S rDNA analysis confirmed that such endophytic and rhizobacteria were identified as *Bacillus subtilis* subsp. *subtilis* and *Bacillus cereus*, respectively. **Conclusion:** Endophytic and rhizobacteria could produce IAA, dissolve phosphate and generate the biggest inhibition zones against *R. syzygii* subsp. *syzygii* while molecular assay identified them as *B. subtilis* subsp. and *B. cereus*.

Key words: Biocontrol, endophytic bacteria, *Ralstonia syzygii* subsp. *syzygii*, rhizobacteria, rhizosphere

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sumatra disease is one of the diseases that cause a serious problem on the cloves plantation. When there is no cure at present; it is the major disease of clove plantation and, therefore, of great economic importance to the Indonesian clove industry. The disease is caused by xylem-limited bacteria identified as *Ralstonia syzygii* subsp. *syzygii*[1] and is mainly transmitted by tube-building cercopidae, *Hindola* spp. (Homoptera:Machaerotidae). This pathogen affected the xylem of clove so that the generated disease is also known as clove xylem disease[2]. The appearing symptoms of this disease are indicated by yellowing and drying leaves at 28 days after inoculation, as well as falling all leaves of the plant 56 days after inoculation[3]. Until recently, control is basically achieved by insecticides application but not very successful to eliminate insect vector. Rather, a chemical application might impact on insect resurgences and environmental problems. Therefore, alternatives to control Sumatra disease must be sought.

Considering their ability in supporting the plants, the bacteria isolated from the plant tissues and around the rhizospheric area of plants or rhizobacteria could be potential to be developed as biological agents. The former researchers found that those bacteria were capable of supporting the growth and development of plants. This complies with research of Ambawade and Pathad[4], who reported that *Stenotrophomonas maltophilia* BE-25 produced the highest indole-3-acetic acid (IAA) hormones among 22 isolates of endophytic bacteria. That isolate expressed the beneficial effects on crop growth and yield. Patel and Desai[5] stated that the rhizobacteria originated from rhizosphere of paddy had an activity to produce plant growth hormone. Sixty-three isolates were able to produce high IAA hormones around 5.79-43.03 μg mL^{-1} in range. In another study, among 100 bacterial isolates from the rhizosphere and the roots of canola (*Brassica napus* L.) in Northern Iran were reported to produce IAA, 17 isolates solubilized phosphate, 44 isolates produced siderophore, 34 isolates produced 1-Aminocyclopropane-1-carboxylate (ACC) deaminase and 5 isolates produced hydrocyanic acid (HCN)[6].

However, the research in reviewing the influence of bacteria isolated from the cloves plant tissues and bacteria in association with plants especially suppress the clove xylem bacterial disease is very limited. Therefore, this research was aimed to screen isolates of endophytic and rhizobacteria of clove from Kendal, Central Java, as well as to examine their ability in enhancing the growth of cloves and also their antibiosis activity against *Ralstonia syzygii* subsp. *syzygii*.

MATERIALS AND METHODS

Time and place of research: The samples of healthy plant and soil were collected from Kendal, Central Java, Indonesia. The isolation and characterization of endophytic and rhizobacteria was conducted at Laboratory of Plant Pathology, Department of Crop Protection, Faculty of Agriculture, Universitas Gadjah Mada. The research was carried out from August, 2016-April, 2017.

Collection of plant tissue and soil samples: The sampled plants were selected from plant population that showed the best growth condition. Sampling was conducted to obtain the isolates of endophytic and rhizobacteria from healthy plant tissues (stems, flowers, leaves and roots) and rhizosphere (soil).

Samples were put on a paper and into a plastic bag before processed in the Laboratory for Bacterial Isolation[7].

Isolation of endophytic bacteria: Tryptone soya agar media has been used for the isolation of endophytic bacteria and different colonies have been recovered after culture plate. As much as 46 isolates of endophytic bacteria from roots (10 isolates), stems (7 isolates), leaves (12 isolates) and flowers (17 isolates) were recovered from the healthy cloves from Kendal, Central Java, Indonesia. Isolation of endophytic bacteria was conducted as described by Hallmann *et al.*[8]. Roots, stems, flowers and leaves were cut into pieces and washed with running water to eliminate the soil or litter on the plant tissues. The surface of plant tissue was sterilized by soaking in $NaClO_4$ 0.05% for 5 min and rinsed for 4 times with sterile ddH_2O. The thick plant tissues were dipped into a petri dish containing ethanol 70% using sterile forceps and then heat-sterilized above the Bunsen lamp; while soft plant tissues only soaked in ethanol 70%. Afterward, the plant tissues were cultured on tryptone soya agar (TSA) medium to ensure that there was no contaminant on the plant tissues. If there was no microbe growing on TSA within 48 h, the sampled plant tissue samples were crushed and then added with 3 mL of tryptone soya broth (TSB). The suspensions were put on TSA medium, spread with L-glass and then incubated for 48 h.

Isolation of rhizobacteria: The rhizobacteria were isolated using serial dilution method. Ten grams of soil samples were put into 250 mL of Erlenmeyer flask containing 90 mL sterile distilled water, shake for 15 min and then ten fold serially diluted in sterile distilled water. One milliliter of a suspension was pipetted into a test tube containing 9 mL of sterile distilled water to obtain 10^{-1} dilution. The dilution was

prepared up to 10^{-10}. Aliquots of 0.1 mL from 10^{-5} to 10^{-10} were cultured on nutrient agar (NA) medium for 72 h at ambient temperature. The colony isolates were sub-cultured using streak plate method in order to get a pure bacterial single colony.

Estimation of indole acetic acid (IAA) production: IAA production was examined by growing endophytic and rhizobacterial isolates on yeast peptone broth (YPB) medium, then incubated for 24 h at room temperature. Twenty microliters of bacterial suspension was put into test tubes containing 5 mL of YPB medium, added with L-Tryptophan (L-Tryptophan concentration in medium was 500 μg mL^{-1}) and then incubated for 72 h. Then, the suspension was transferred into the tube for centrifugation at 10,000 rpm for 10 min. As much of 0.2 mL aliquot in supernatant was mixed with 0.8 Salkowski's reagent and incubated for 20 min at room temperature. Then, the change of color was observed.

Phosphate solubilization activity: Quantitative screening of phosphate-solubilizing bacteria was carried out by culturing the bacterial isolates on solid National Botanical Research Institute`s Phosphate Growth (NBRIP) medium containing (per liter): 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2.6H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of KCl and 0.1 g of $(NH_4)_2SO_3$ and then incubated for 3 days at room temperature[9]. This assay was conducted to characterize the isolates of rhizobacteria only.

Solubilizing efficiency was calculated according to Nguyen *et al.*[10] formulation:

$$\text{Solubilizing efficiency (\%)} = \frac{\text{Diameter of clear zone}}{\text{Diameter of growth}} \times 100$$

***In vitro* antagonism test of endophytic and rhizobacterial isolates against *Ralstonia syzygii* subsp. *syzygii*:** The optical density of the bacterial culture was red by spectrophotometer (Spectronic GENESYS 10 UV-Vis) at OD_{660} nm to estimate the number of bacterial cells[11,12], an antagonistic test of endophytic and rhizobacterial isolates was performed using dual culture method according to Lisboa *et al.*[13]. Five microliters of *R. syzygii* subsp. *syzygii*. suspension from 0.5% water agar (WA) medium at a temperature of 45°C was spread on the surface of yeast peptone agar (YPA) medium. Then, isolates of endophytic and rhizobacteria were inoculated on YPA medium. Those bacterial cultures were incubated for 48 h at a temperature of 28-30°C. The observation was conducted by observing and measuring the inhibition zone which was established by endophytic and rhizobacteria against *Ralstonia syzygii* subsp. *syzygii*.

Molecular identification of endophytic and rhizobacteria based on 16S rRNA gene fragment

DNA extraction: Bacterial genomic DNA was extracted using mini preparation DNA isolation technique with slight modification[14,15]. As much of 1.5 mL of cell culture was centrifuged at 5,000 rpm for 2 min. The pellet of DNA was diluted with 540 μL of TE buffer (0.1 M Tris-HCl, 0.1 M EDTA pH 8), added with 30 μL 10% sodium dodecyl sulfate (SDS) and then incubated at 37°C for 60 min. Afterward, the pellet was added with 100 μL of 5 M NaCl and 80 μL of cetrimonium bromide (CTAB)/NaCl and then incubated at 65°C for 10 min prior to addition of 750 μL of chloroform isoamyl alcohol (24:1) and centrifugation at 12,000 rpm for 5 min. The upper layer was transferred into 1.5 mL Eppendorf tube added with 600 μL of phenol chloroform isoamyl alcohol (25:24:1) and then centrifuged at 12,000 rpm for 5 min. The top layer was transferred again into new 1.5 mL Eppendorf tube. As much of 0.6 times volume of isopropanol was added and centrifuged at 12,000 rpm for 5 min. The pellet was rinsed with ethanol 70%, air-dried and then diluted with 20 μL of TE buffer.

DNA amplification: The extracted DNA was amplified by the PCR technique using 16S rRNA primer according to procedures of Joko *et al.*[16], i.e. 984f (5'-AACGCGAAGAACC TTAC-3') and 1378r (5'-CGGTGTGTACAAGGCCCGGGAACG-3'). PCR reaction was run in a total volume of 25 μL using Go Taq Green Kit with the composition of 2.5 μL of DNA template, 1.5 μL of forward and reverse primers, 12.5 μL of Go Taq Green Kit and 7 μL of nuclease free water[17]. The tubes were placed on PCR machines (thermal cycler) at the temperature of 94°C for 2 min for initial denaturation, followed by 34 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec and extension at 68°C for 30 sec. The PCR was completed with the final extension at 72°C for 5 min and holding at 4°C prior to further analysis. The PCR products were analyzed using electrophoresis in 1% of agarose gel (diluted in 0.5× TBE) containing ethidium bromide. A 1 kb of DNA marker was used for the measurement of the amplified DNA. Electrophoresis was performed at 45 Volt for 50 min. The DNA bands were visualized under ultraviolet trans-illuminator and then documented[18,19].

DNA sequencing and phylogenetic analysis: The PCR products were subjected to nucleotide sequencing at the 1st BASE company, Malaysia. The nucleotide sequence result was then analyzed and edited using Genetix program 7th version (Genetix, Japan)[20].

Phylogenetic data were obtained by alignment of 10 different 16S rDNA sequences retrieved from Basic Local Alignment Search Tool (BLAST) at www.ncbi.nlm.nih.gov.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA 7 program[19]. An unrooted phylogram was obtained by the neighbor joining (NJ) method. An interior branch test was done (heuristic option, 1000 replications) to check the tree topology for robustness. Some reference strains with similarity close to 100% were determined. Additionally, the Poisson correction was applied NJ for distance estimation and the complete deletion option was used in handling gaps or missing data obtained from alignments.

Statistical analysis: The study was conducted using a Complete Random Design (CRD). The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) and means were separated by Duncan's Multiple Range Test (DMRT) ($p \leq 5\%$).

RESULTS

Isolation of endophytic and rhizobacteria from healthy clove plant tissues and rhizosphere: The bacterial endophytes isolates could be categorized into 6 different morphotypes according to the morphology, color and colony shape, i.e. circular slimy entire bright yellow colony, irregular not slimy undulate pale white colony, circular slimy entire transparent colony, circular not slimy entire pale white colony, irregular not slimy undulate brownish pale white colony and circular slimy entire dark yellow to orange colony. The endophytic bacteria having irregular, not slimy undulate pale white colony were dominantly found, with the frequency of 43.47%.

Meanwhile, there were 66 isolates of rhizobacteria which were successfully explored from the soil around rooting area of healthy cloves. Based on the morphology, color and colony shape, those isolates belonged to one morphotype, i.e. white irregular pale not a slimy undulate colony.

Indole acetic acid (IAA) production and phosphate solubilization: The production of IAA from tested isolates was qualitatively indicated by changes of the color to red when bacterial filtrate was dropped with Salkowski's reagent (Fig. 1). The results revealed that 24 isolates of endophytic and 21 rhizobacteria were capable of producing IAA.

The production of clear zones around the colonies was an indication for the presence of phosphate-solubilizing bacteria (Fig. 2). Such cultures dissolving phosphate was quantitatively determined by a biochemical method. Among 21 isolates of rhizobacteria, 20 isolates positively reacted by producing clear zone on NBRIP medium after 3 days incubation at room temperature, with the efficiency of the solubility reached about 50-150%.

***In vitro* antagonism test of endophytic and rhizobacterial isolates:** *In vitro* antagonism test of endophytic and rhizobacteria against *R. syzygii* subsp. *syzygii* on YPA medium showed that among 44 tested isolates, an isolate of

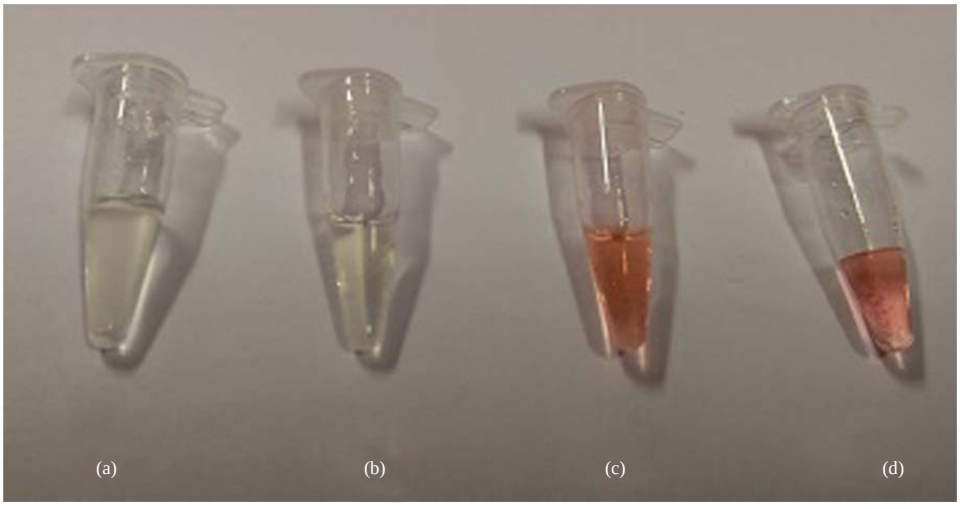

Fig. 1(a-d): IAA production ability by endophytic and rhizobacterial isolates which were demonstrated by the existence of color change to red after addition of Salkowski's reagent into the medium. Bacterial supernatant (a) Without Salkowski's reagent, (b) Without color change, (c) Supernatants of rhizobacteria and (d) Endophytic bacteria showing color change

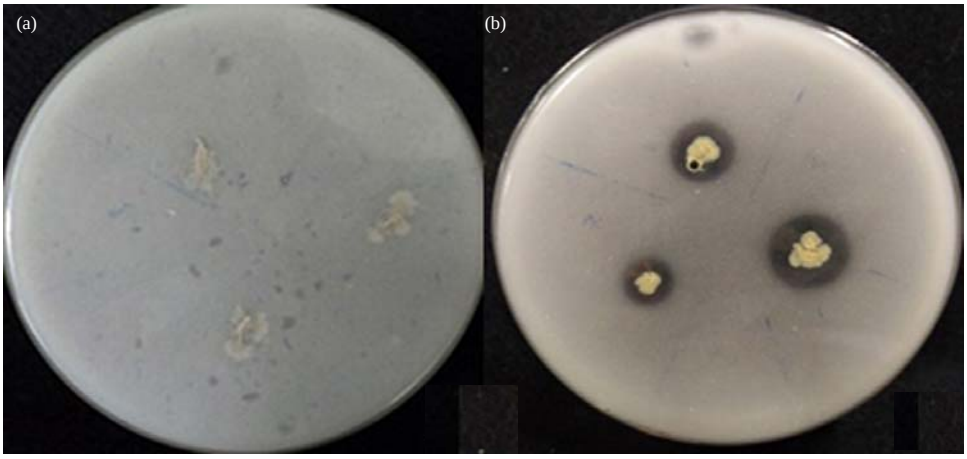

Fig. 2(a-b): Phosphate solubilization with (a) Negative response and (b) Positive response indicated by clear halo zone of rhizobacteria on NBRIP medium

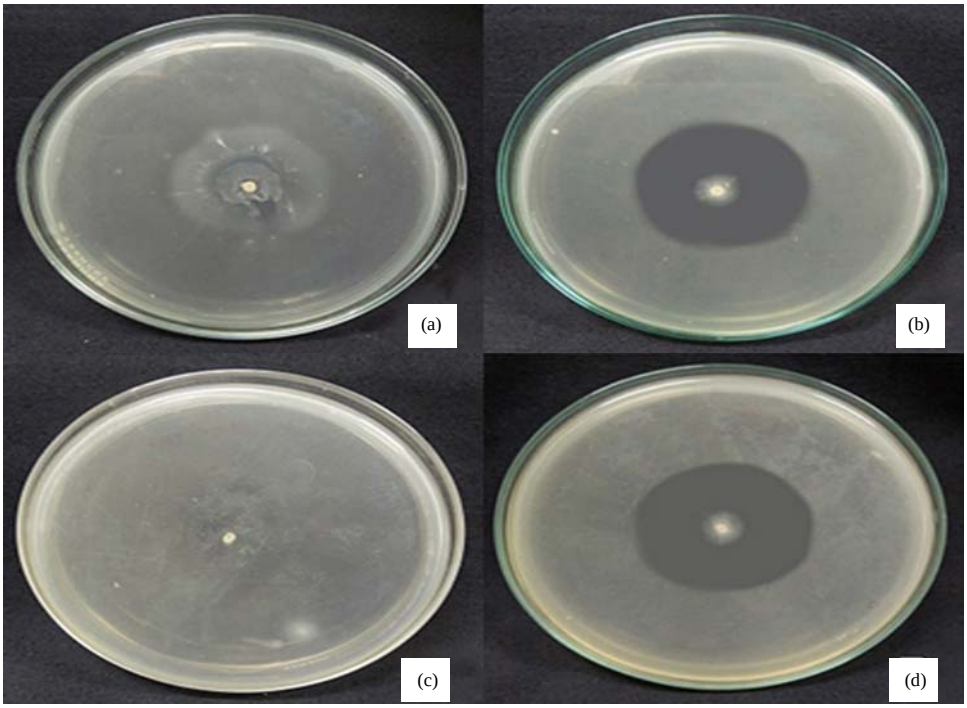

Fig. 3(a-d): Antagonistic assay of endophytic and rhizobacterial isolates against *R. syzygii* subsp. *syzygii* with dual culture method for 7 days incubation on YPA medium. Isolates of endophytic bacteria (a) Without and (b) With inhibition zone. Isolates of rhizobacteria (c) Without and (d) With inhibition zone

endophytic and rhizobacteria could produce the largest inhibition zone (Fig. 3). The result illustrated that antibiosis was one of the antagonistic mechanisms of endophytic and rhizobacteria in controlling *R. syzygii* subsp. *syzygii* on cloves.

Taxonomic identification of selected endophytic and rhizobacteria: In this study, DNA was extracted from selected isolates of endophytic and rhizobacteria showing the largest inhibition zone against *R. syzygii* subsp. *syzygii*. The nucleotide sequence was analyzed using BLAST. Phylogenetic analysis indicated that endophytic bacteria (EA) was closely related to *Bacillus subtilis* subsp. *subtilis* JCM 1465 with 93% maximum identity and rhizobacteria (RA) was to *Bacillus cereus* JCM 2152 with 90% maximum identity.

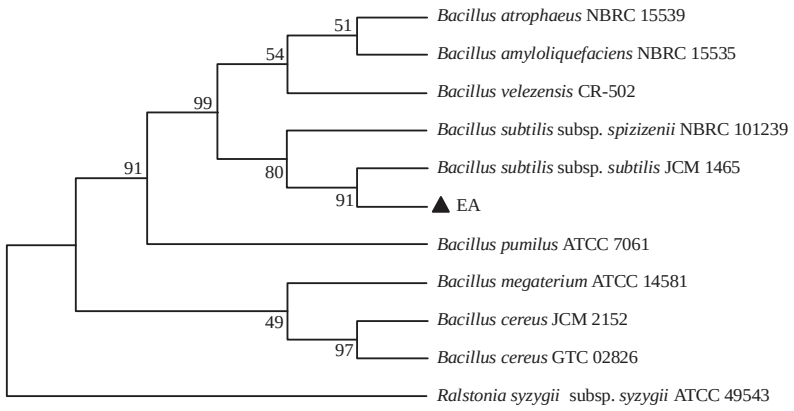

Fig. 4: Phylogenetic tree construction of endophytic bacterial isolates (EA) based on 16S rRNA gene partial sequence. The phylogenetic tree construction use neighbor-joining method with 1000 times of bootstrap

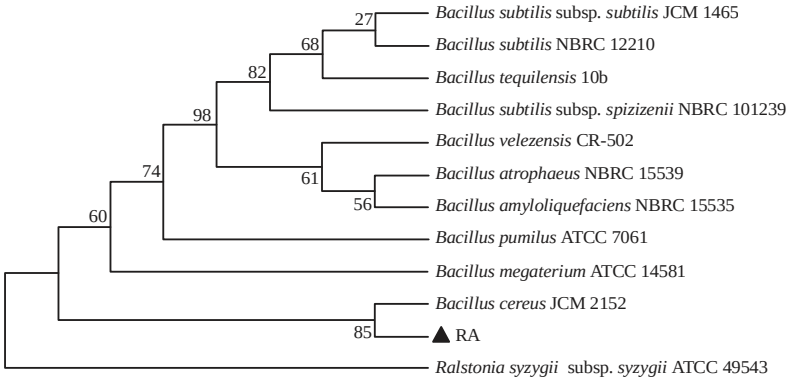

Fig. 5: Phylogenetic tree of rhizobacterial isolates (RA) based on sequence of 16S rRNA gene fragment using neighbor-joining method with 1000 times of bootstrap

Meanwhile, *R. syzygii* subsp. *syzygii*. ATCC 49543 was categorized into out-group of *Bacillus* spp. Relationship of endophytic and rhizobacterial isolates with the other isolates could be presented on a phylogenetic tree. The phylogenetic tree which was formed by neighbour joining (NJ) using MEGA 7 program was presented in Fig. 4 and 5 for bacterial endophyte and rhizobacteria, respectively.

DISCUSSION

These studies demonstrated the occurrence and diversity of culturable endophytic and rhizobacteria. Endophytic bacteria colonized inner host tissues, sometimes in high numbers, without damaging the host or eliciting strong defense responses[21]. Endophytes were defined as those bacteria that could be isolated from surface-disinfested plant tissue or extracted from the plant and that did not visibly harm the plant[6]. Zinniel *et al.*[22] successfully isolated 853 endophytic strains from aerial tissues of 4 species of agronomic crops and 27 prairie grass. On the other hand, Pastel and Desai[5] explored about 63 isolates on different media from rhizosphere soil, ecto and endorhizospheric regions as well as the bulk soil of paddy field. Bacteria could develop under abundant nutrients in the rhizosphere and some of them were beneficial in stimulating plant growth[23]. Joko *et al.*[24] reported that the community structure of aerobic rhizobacteria exhibited 60% similarity between healthy and infected plants; while anaerobic one occurred between healthy and infected plants with 50% similarity.

One of examined characteristic in this investigation was IAA production. Selected strains showing prolific growth on the medium also produced IAA on YPB medium containing 200 μg mL^{-1} of L-tryptophan. On the previous research, Ngoma *et al.*[25] reported that 11 isolates of endophytic bacteria were potential in producing IAA. The results of their experiments revealed that most of the bacterial isolates,

especially *Ochrobactrum intermedium* (KC010522 and KC010521), *Ochrobactrum* sp. (TOA62 and TOA64), *Ochrobactrum anthropi* (KC010524 andKC010523) and *Pseudomonas* (KC010520), were able to produce IAA. The ability of isolates in producing IAA was indicated by discoloration of isolates to pink. However, such color changes varied for each isolate. The investigation of Tsavkelova *et al*.[26] observed that the highest auxin level was detected during the stationary phase of bacterial growth. They also found that biological activity of IAA-producing microbes was demonstrated considerable stimulation of root formation and plant growth.

Sharma[27] explained that phosphate solubility of rhizobacteria was the indicator for an activity of phosphatase enzyme. This enzyme could produce clear zone around the colony. Phosphorus was the second major nutrients after nitrogen for plants requirement. Phosphate played important roles in plant physiological activities such as cell division, photosynthesis and development of root systems. In general, plant absorbed phosphate from the soil in form of ion ($H_2PO_4^-$); while the phosphate availability in soil was frequently lacking for the plant since it was bond with other cation. Some microorganisms were involved in a range of process affecting the transformation of soil phosphorus (P) and thus they were integral components of the soil 'P' cycle. Phosphate-solubilizing microorganisms generally converted insoluble phosphates into soluble forms through acidification process, chelation and exchange reaction. The antibacterial activity of the test isolates might be due to the production of antibiotics. Endophytic and rhizobacterial isolates showed positive inhibition on growth of *Ralstonia syzygii* subsp. *syzygii*.

Isolated bacteria were firstly grouped using classic microbiological methods and they were then distinguished by 16S rDNA analysis. One strain from each group of Gram-positive bacteria was continued with phylogenetic analysis. Our findings identified those isolates as *B. subtilis* subsp. *subtilis* and *B. cereus*, respectively. Recently, Malleswari and Bagyanarayana[28] explored 219 bacterial strains from the rhizosphere of various medicinal and aromatic plants in different locations of Andhra Pradesh (India) for initial screening of their PGPR activities. Among the 219 isolates, four bacterial strains showed maximum PGPR activities and were identified as *Pantoea* sp., *Bacillus* sp. and *Pseudomonas* sp. On the other case, Kloepper *et al*.[29] reported that *Bacillus* spp. could elicit induced systemic resistance (ISR) and promote plant growth. They found that elicitation of ISR by *Bacillus* spp. was associated with ultrastructural changes in plants during pathogen attack and cytochemical alterations. Due to its wide host range, *B. subtilis* had the ability to form endospores, produced different biologically active compounds and was potential to be biocontrol and PGPR agents[30]. Rhizobacteria of pigeon pea rhizosphere were isolated by Rani *et al*.[31] from different areas in India which were Samalkot, Pithapuram, Peddapuram and Kakinada. Two isolates that could produce PGPR were identified by molecular assay using 16S rRNA and sequencing. These two isolates were identified as *Bacillus cereus* and *Enterobacter cancerogenus*.

CONCLUSION

Endophytic and rhizobacteria isolated from healthy clove and its rhizosphere could produce IAA, dissolve phosphate, generate the biggest inhibition zones through the antagonistic test against *Ralstonia syzygii* subsp. *syzygii*, while 16S rDNA analysis on endophytic and rhizobacteria identified them as *B. subtilis* subsp. *subtilis* and *B. cereus*, respectively.

SIGNIFICANCE STATEMENTS

This study discovers the potency of endophytic and rhizobacteria to control Sumatra disease caused by *R. syzygii* subsp. *syzygii*. The study showed that *B. subtilis* subsp. *subtilis* and *B. cereus* were identified as potential biocontrol as well as plant growth promoting agents that will support the development of eco-friendly disease management in clove plantation.

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