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Research Article
Induced Disease Resistance and Promotion of Shallot Growth by *Bacillus velezensis* B-27

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Abstract

Background and Objectives: Shallot is a vegetable crop with high economic value, but its productivity is still relatively low due to various limitations. One of the most hampering factors is moler disease and purple blotch disease caused by *Fusarium* sp. and *Alternaria porri*, respectively. Numerous efforts have been made to control these diseases either using chemical fungicides or through improvement of resistant cultivar. This study aimed to determine moler and purple disease suppression and improvement of plant growth by *Bacillus* as Plant Growth Promoting Rhizobacteria (PGPR) on shallot. **Materials and Methods:** Molecular identification of *Bacillus* was performed by partial *gyrB* gene sequencing using universal gyrB-F/gyrB-R primers. Field observation and experiments were performed using completely randomized factorial block design single factor with 3 blocks for replication. **Results:** The partial *gyrB* gene sequences showed high similarity between *Bacillus* isolate B-27 and *Bacillus velezensis*. The application of *Bacillus* isolate B-27 to shallots was shown to reduce the intensity of moler and purple blotch diseases by 67%. On top of that, *Bacillus* isolate B-27 increased the plant height up to 27.12 cm, the number of leaves up to 23 blades, tillers up to 8 bulbs and the tuber weight during harvest time up to 33.64 kg. **Conclusion:** Molecular identification based on partial *gyrB* gene sequence analysis suggested that *Bacillus* isolate B-27 has close relationship with *Bacillus velezensis*. Besides, the application of *Bacillus* isolate B-27 on shallot could reduce the disease intensity and increase height, number of tillers and plant yield significantly.

Key words: *Bacillus velezensis*, *gyrB*, shallot, moler disease, purple blotch disease

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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

Shallot (*Allium cepa* L.) is considered as a crop of Asteraceae family that is widely cultivated by farmers in Indonesia for its high economic value. The demand of shallot in Indonesia keeps increasing year by year while the productivity has been relatively static. The static productivity happened due to several aspects such as plant-disturbing factors, especially plant diseases.

The major diseases which farmers frequently encounter in shallots are moler disease caused by *Fusarium* sp. and purple blotch disease caused by *Alternaria porri*. The loss caused by those diseases may reach[1] 75%. Conventional control over diseases on shallots is carried out with the application of fungicides such as benomyl, carboxyl, maneb, tebuconazole, mancozeb and metalaxyl[2-4] and through improvement of resistant cultivar as well[5,6]. The misuse of fungicide may potentially cause another problem such as pathogen resistance and residue in the surrounding environment. *Peronospora destructor*, the pathogen of downy mildew disease on shallot has built a resistance against fungicide of active metalaxyl[7]. Therefore, it is necessary to use alternative disease control in shallots, namely the application of biological agents such as mycorrhiza, *Trichoderma*, *Streptomyces* and *Bacillus*.

Bacillus is well-known as Plant Growth Promoting Rhizobacteria (PGPR) that is able to improve plant growth and induce plant systemic resistance towards pathogens[8]. *Bacillus* is able to produce Indole-3-Acetic Acid (IAA) and siderophore, mobilize phosphate and produce antifungal compounds. The presence of *Bacillus* in soil rhizosphere is known to induce ethylene and jasmonic acid production that may protect the plant from pathogen infection[9-13]. Some studies reported that *Bacillus* effectively reduces *Fusarium oxysporum* infection on tomatoes[14], *Botryosphaeria dithidea* on apples[15] and *Colletotrichum gloeosporioides* on strawberries[16].

Molecular bacterial identifications are commonly conducted using a genetic marker such as 16S rRNA[17,18]. The 16S rDNA was chosen for its conservative base sequence to construct a universal phylogenetic tree[19,20]. The 16S rRNA genetic marker may generally be used during bacterial classification. However, the downside is that it has a high percentage of similar sequence between species which makes it difficult to differentiate between close relative species. One of the alternatives that can be used as bacterial phylogenetic marker is the use of the gyrase subunit B (*gyrB*) gene sequence[21,22]. The *gyrB* gene is chosen for its higher molecular evolutional level than 16S rRNA which may identify *Bacillus* more specifically[23]. This study aimed to identify *Bacillus* isolate B-27 using *gyrB* gene as molecular marker and its application as PGPR on shallot in the field.

MATERIALS AND METHODS

Study area: The study was carried out at Laboratory of Plant Pathology, Faculty of Agriculture, Universitas Gadjah Mada and continued to a shallot cultivation area located in Gotakan village, Panjatan district, Kulon Progo regency, Special Region of Yogyakarta, Indonesia since January until May, 2019. The bacterial strain used in this study (*Bacillus* isolate B-27) was obtained from Laboratory of Plant Pathology, Faculty of Agriculture, Universitas Gadjah Mada. This research was consisted by several series of experiments, namely (1) Molecular identification of *Bacillus* isolate B-27, (2) Seed treatment with *Bacillus* isolate B-27, (3) Observation upon agronomic, disease and shallot yield parameter in the field, (4) Field preparation and fertilization and (5) Statistical analysis.

Molecular identification of *Bacillus* isolate B-27: Molecular identification of *Bacillus* isolate B-27 was done through DNA extraction, PCR amplification and sequence analysis. Total genomic of bacteria DNA extraction was conducted based on a modified CTAB method[24,25]. The DNA obtained was then amplified using PCR technique using a specific primer pair of gyrB-F (5'-CCC AAG CTT AAC TGC ACT GGG AAA TY-3') and gyrB-R (5'-CGG AAT TCG GAT CCA CRT CGG CRT CB-3'). The gene amplification followed the procedure previously done[26] with the target length of amplicon of ±1500 bp. The PCR was carried out in a total volume of 25 µL, for every reaction covering 12.5 µL master mix (Go*Taq* Green, Promega), 1 µL forward primer, 1 µL reverse primer, 1 µL DNA and nuclease-free water. Afterwards, amplification was conducted using PCR machine (Biorad T100, Germany) with initial denaturation at 95°C for 3 min, followed by 35 cycles including denaturation at 95°C for 1 min, annealing at 57°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. The DNA resulted from the amplification was visualized using electrophoresis through 0.8% agarose gel in 1x TBE buffer[27,28] with 1 kb DNA ladder (Promega) as comparative DNA marker.

Nucleotide sequencing was obtained using direct sequence method by sending to the 1st Base Company (Malaysia). The result of sequencing was then analyzed using BLAST (Basic Local Alignment Search Tool) available in the website of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/Blast.cgi) to discover its percentage of homology. The *gyrB* database of the closely related *Bacillus* retrieved from GenBank with various percentage of homology were used as a comparison during the alignment. The result of

alignment was used for phylogenetic analysis to discover the relationship between the sample and other *Bacillus* spp. using Clustal Omega (www.ebi.ac.uk/Tools/services/web_clustalo/toolform.ebi) and MEGA v.7[29,30]. The phylogenetic tree was created using neighbor-joining algorithm method with classification stability using bootstrap analysis with 1000 times replication.

Field preparation and fertilization: The field preparation consisted of ploughing and bund making. Bunds were made in the size of 0.8×5 m with 0.5 m high and 0.5 m distance between the bunds. In every bund, an experimental plot was made with 120 shallots planted in it. Fertilization was done using organic and inorganic fertilizer to encourage the growth of shallots. The organic fertilizer used was compost with a dose of 1 t/500 m^2, while the inorganic fertilizer used was Phonska of 15 kg/500 m^2, ZA 12.5 kg/500 m^2, NPK 5 kg/500 m^2 and SP-36 15 kg/500 m^2. Shallot bulbs (cv. Crok kuning) treated using *Bacillus* isolate B-27 suspension were planted with a planting distance of 20×20 cm. The next stage was routine watering of once every 1-2 days depending on the bund's condition[31]. This study used a Completely Randomized Factorial Block Design (CRFBD) single factor with 3 blocks for replication. A 15-unit plant samples were made for each replication. The factor used was the application of *Bacillus* isolate B-27.

Seed treatment with *Bacillus* isolate B-27: *Bacillus* isolate B-27 was grown in Yeast Peptone Agar (YPA) medium (0.5% yeast extract, 1% polypeptone, 1.5% agar) and put into incubation for 48 h. *Bacillus* propagation was acquired by harvesting its grown colony. Afterwards, it was suspended in 1000 mL sterile water of 10^8 CFU mL^{-1}. The application of *Bacillus* on shallots was done by soaking it for 30 min before planting it in the field as well as watering the plant once a week using *Bacillus* suspension[32]. In Dripping and Drenching (DD) treatment seeds were soaked and drenched with *Bacillus* isolate B-27. While, seeds were soaked but not drenched in B-27 isolate in Tuber Dripping (TD). In Soil Drenching (SD), seed were not soaked but drenched while in control no soaking and drenching was performed.

Observation upon agronomic, disease and shallot yield parameter in the field: Observation variables consist of the intensity of moler and purple blotch diseases, Area Under the Disease Progress Curve (AUDPC), agronomic parameters i.e., plant height, number of leaves, number of tillers and tuber weight. Disease intensity, AUDPC and agronomic parameters observations were conducted every week starting from the 2-7 weeks of the plant age. Meanwhile, the observation of tuber weight was conducted at the 8th week of planting. Moler disease symptom could be traced from yellowing and twisting on the shallot's leaves. Scoring was made for the yellowing and twisting percentage and the disease intensity was then calculated based on a score as follows: Score 0 = Leaves are all healthy, score 1 = 1-20% yellowing and twisting on the leaves, score 2 = 21-40% yellowing and twisting on the leaves, score 3 = 41-60% yellowing and twisting on the leaves, score 4 = 61-80% yellowing and twisting on the leaves and score 5 = 81-100% yellowing and twisting on the leaves[2]. Meanwhile, purple blotch caused by *A. porri* was observed from the purple blotch symptoms on the shallot's leaves. Scoring was made for the purple blotch percentage and the disease intensity was then calculated based on a score as follows: Score 0 = healthy plant, score 1 = 1-10% of the leaves covered in purple blotch, score 2 = 11-20% of the leaves covered in purple blotch, score 3 = 21-40% of the leaves covered in purple blotch, score 4 = 41-75% of the leaves covered in purple blotch and score 5 = 76-100% of the leaves covered in purple blotch[33,34]. Disease intensity can be calculated using the following equation[35,36]:

$$\text{Disease intensity (\%)} = \frac{\sum (n \times v)}{N \times V} \times 100$$

Where:

n = Number of plants with the same damage category
v = Score of each damage category
N = Total number of plants observed
V = Score for the most severe damage

Meanwhile, the Area Under the Disease Progress Curve (AUDPC) was calculated based on a equation[37]:

$$AUDPC = \sum_{i}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) \left(t_{i+1} - t_i \right)$$

Where:

y_{i+1} = Observation data i+1
y_i = Observation data i
t_{i+1} = Observations time i+1
y_i = Observation time i

Statistical analysis: Data on observation of the disease occurred in the field was analyzed using Analysis of Variance (ANOVA) with 95% confidence interval. If a significant difference appeared, post-secondary test might be held using Duncan Multiple Range Test (DMRT) with 95% confidence interval. Additionally, the data were analyzed descriptively and shown in graphics and figures.

RESULTS

Molecular identification of *Bacillus* isolate B-27: Molecular identification of *Bacillus* isolate B-27 conducted using universal gyrB primers. Approximately 1500 bp amplicon of *Bacillus* isolate B-27 was observed by PCR with the primers of gyrB-F/gyrB-R (Fig. 1a). The corrected nucleotide sequences of the PCR product were determined with BLAST and showed high identity with *Bacillus velezensis*. The nucleotide sequence was then deposited in the GenBank under the accession number MN905547.

The phylogenetic analysis using neighbor-joining method with 1000 bootstraps revealed that *Bacillus* isolate B-27 was in the same cluster as to *B. amyloliquefaciens* operational group and possessed a high similarity to 99% with *B. velezensis* which is recognized as being synonymous to *B. amyloliquefaciens* subsp. *plantarum* (Fig. 1b). High sequence similarity of the *gyrB* gene of *Bacillus* isolate B-27 and *B. velezensis* FZB42 meant that yielded a branch with the *B. amyloliquefaciens* strain. In Fig. 1b, it can be seen that *B. subtilis* and *B. amyloliquefaciens* have different cluster based on the *gyrB* gene. This may happen due to the different genetic variety between *gyrB* sequences when they are used to identify bacteria from *B. subtilis* group.

Suppression of moler and purple blotch disease in the field: Moler disease without dipping and drenching treatment with *Bacillus* isolate B-27 has the highest intensity of moler disease of 0.27% followed by soil drenching treatment with 0.18%, tuber dipping with 0.15% as well as dipping and drenching treatment with 0.09%. The application of *Bacillus* isolate B-27 on shallots could suppress moler disease intensity by 33-67% (Table 1). *Fusarium* attack causing moler disease in this shallot cultivation is considered low. This research was conducted during dry season which influence the moler disease to hardly spread. Table 1 also showed the intensity of the second observed disease, namely purple blotch disease. The result indicated that the highest score was found in control treatment with 0.18% followed by soil drenching with 0.15%,

Table 1: Intensity of moler and purple blotch disease after *Bacillus* isolate B-27 treatment

Treatments	Moler disease intensity (%)	Purple blotch disease intensity (%)
DD	0.09[a]	0.06[a]
TD	0.15[a]	0.09[a]
SD	0.18[a]	0.15[a]
Control	0.27[a]	0.18[a]

DD: Dipping and drenching, TD: Tuber dipping, SD: Soil drenching, Data were analyzed using DMRT, data notation followed by the same letter showed no significant difference at 95% confidence interval

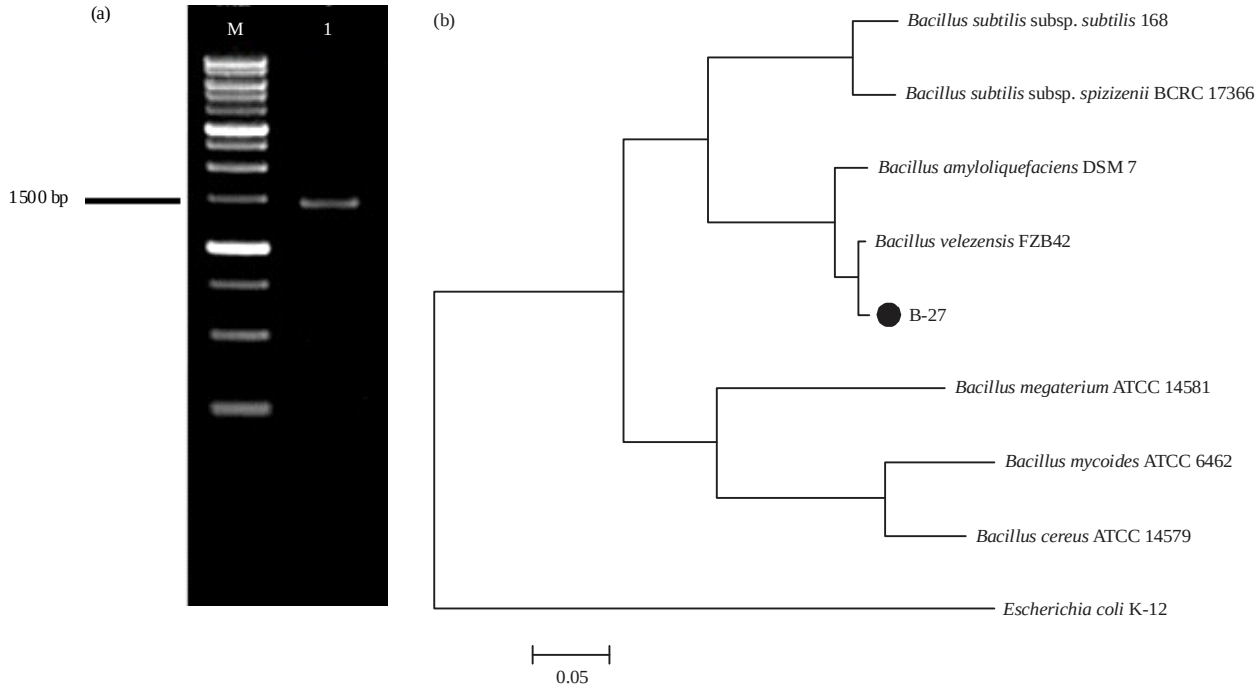

Fig. 1(a-b): (a) PCR result of *gyrB* gene of *Bacillus* isolate B-27 on 0.8% agarose gel with target size of ±1500 bp and (b) Phylogenetic tree from *Bacillus* isolate B-27 and the closest relatives of other species of the genus *Bacillus* based on *gyrB* gene sequences
M: Marker 1 kb, 1: *Bacillus* isolate B-27

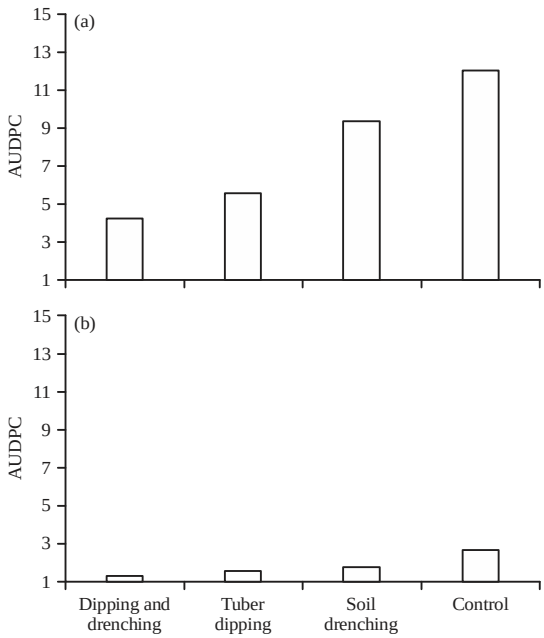

Fig. 2(a-b): AUDPC score of (a) Moler disease and (b) Purple blotch disease after *Bacillus* isolate B-27 treatment
AUDPC: Area under the disease progress curve

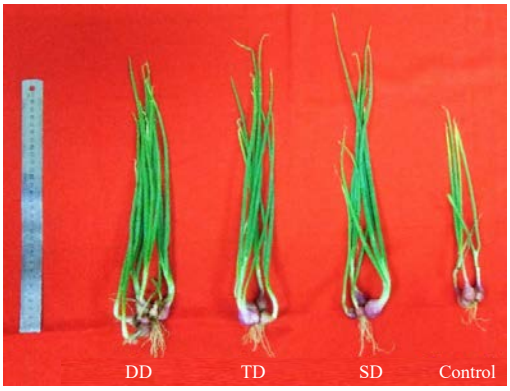

Fig. 3: Effect of *Bacillus* isolate B-27 treatment on shallot growth
DD: Dipping and drenching, TD: Tuber dipping, SD: Soil drenching

tuber dipping with 0.09% and dipping drenching treatments with 0.06%. Treatment with *Bacillus* on shallots may inhibit the intensity of purple blotch disease in the field from 17-67%. Similar to moler disease intensity, the appearance of purple blotch disease in this study is considered low. This was also thought to happen due to dry season during the time of research. The condition of area with poor drainage and rainfall influenced the spore growth of *A. porri* that caused purple blotch disease.

Table 2: Shallot growth after treatments using *Bacillus* isolate B-27

Treatments	Plant height (cm)	Number of leaves (blade)	Number of tillers
DD	27.12^{a}	23.13^{a}	7.51^{a}
TD	25.49^{b}	20.83^{b}	7.02^{ab}
SD	24.84^{b}	19.78^{bc}	6.60^{b}
Control	24.57^{b}	18.44^{c}	5.82^{c}

DD: Dipping and drenching, TD: Tuber dipping, SD: Soil drenching, data were analyzed using DMRT, data notation followed by the same letter showed no significant difference at the 95% confidence interval

Table 3: Shallot tuber weight after treatments using *Bacillus* isolate B-27

Treatments	Tuber weight (g/plant)
DD	33.64^{a}
TD	26.23^{c}
SD	23.87^{d}
Control	29.08^{b}

DD: Dipping and drenching, TD: Tuber dipping, SD: Soil drenching, data were analyzed using DMRT, data notation followed by the same letter showed no significant difference at the 95% confidence interval

Figure 2a showed that moler disease with the highest score of AUDPC was found in control treatment with a score of 12 followed by soil drenching, tuber dipping and dipping and drenching. Purple blotch disease with the highest score of AUDPC was found in soil drenching with 1.7 followed by tuber dipping and dipping and drenching. Out of the 4 treatments using *Bacillus* isolate B-27 on shallots, dipping and drenching treatment had a lowest score of AUDPC (Fig. 2b).

Agronomic parameter of shallot after treatments: Agronomic parameters observed in this study comprised of plant height, number of leaves and number of tillers. As shown in Fig. 3, there was an increase of the agronomic growth after application of *Bacillus* isolate B-27 compared to that of control plant. Table 2 showed the detail results on growth promotion of shallot after treatment with the application of *Bacillus* isolate B-27 after planting in every week. The Dipping and Drenching (DD) treatment has the highest score in plant height, number of leaves and number of tillers then followed by Tuber Dipping (TD), Soil Drenching (SD) and control treatment.

Shallot yield after treatments: This study showed that there was an impact in the application of *Bacillus* isolates B-27 towards shallot yield. Based on the overall treatments, the best result was found in dipping and drenching treatment which incorporates a pre-planting dip and *Bacillus* isolate B-27 application every week with the score of 33.64 g/plant (Table 3). In control treatment without dipping and drenching by *Bacillus* isolate B-27, the score was 29.08 g/plant while tuber dipping and soil drenching scored below control treatment with 26.23 and 23.87 g/plant each.

DISCUSSION

Recently, different strains of *B. velezensis*, which is a typical PGPR have received considerable attention. For example, living spores of *B. amyloliquefaciens* FZB42, now reclassified as a strain of *B. velezensis* have been formulated into the commercially available bio-inoculant RhizoVital®, which is used to control a variety of soil-borne diseases[38]. The strain is capable of stimulating plant growth and producing different types of biologically active secondary metabolites that suppress plant pathogenic microflora. Meanwhile, *B. velezensis* has been formulated into the commercially available fungicide Botrybel (Agricaldes, Spain) owing to its activity against *Botrytis cinerea*, the etiological agent of gray mold, which reportedly infects over 200 plant species worldwide[39]. The beneficial species *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus* are representing a group of phylogenetically and phonetically homogeneous species called the *B. subtilis* species complex[40,41] based on the phylogenomic analysis.

The *gyrB* gene encodes the protein DNA gyrase subunit B that plays an important role in the replication of DNA found in species of bacteria in universal. Molecular evolution from *gyrB* gene sequence runs faster than 16S rRNA gene sequence[42]. Study regarding the use of *gyrB* gene in phylogenetic bacteria sequences has been conducted numerous times, namely upon *Pseudomonas*[26]; *Acinetobacter*[43,44], *Salmonella*, *Shigella* and *Eschericia coli*[45], *Bacillus* group[46] and *Pantoea ananatis*[47]. Some of the study results above suggested that *gyrB* is a suitable phylogenetic marker for taxonomy relationship study on species level. In the previous study, the *gyrB* sequence and DNA hybridization was successfully used to discriminate species of the *B. subtilis* group[21]. The *gyrB* sequence can be used to identify up to the subspecies level.

Treatment using *Bacillus* isolate B-27 on shallots may reduce the development of moler disease in the field. This low intensity of moler disease might be influenced by compounds produced. *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 was reported to produce antifungal compounds such as surfactant, fengycin and bacillomycin D. The most powerful compound produced by *B. amyloliquefaciens* subsp. *plantarum* FZB42 is bacillomycin D[48]. During *in vitro* test, bacillomycin D may inhibit *Fusarium graminearum* growth by causing swelling at the tip of the hyphae and conidia as well as reducing the germination down to 5.44%. Wheat plants infected with *F. graminearum* when treated with bacillomycin D of 90 μg mL^{-1} concentration showed a significant decrease of symptom appearance, which characterized by the less growth of mycelia at the tip of plants and 45% uninfected wheat seeds unlike the wheat seeds without treatment that are mostly covered in mycelia and turn grey[49].

Dipping and drenching treatment using *Bacillus* isolate B-27 on shallots may inhibit the intensity of purple blotch. *Bacillus* as beneficial microbes may inhibit pathogen growth by producing antifungal compound and inducing plant resistance and therefore pathogens can not infect their host plant[50]. Application of *B. subtilis* may suppress the spread of purple blotch down[51] to 37.29%. While the disease could be suppressed and minimized, the plant production could be maximized. This indicated that the lower the score of AUDPC acquired, the more effective the treatment in controlling pathogen[52].

Treatment using *Bacillus* isolate B-27 on shallot plant gave higher score of agronomic parameters than control plants. This could happen due to the high metabolism of *Bacillus* that produces higher number of CO_2 in rhizosphere. High production of CO_2 triggers carbonate acid formation that may affect to low soil pH in rhizosphere. This process occurred because microorganisms act as a booster in nutrients absorption that helps plant to grow higher. The use of *B. subtilis* on shallots may increase the content of phosphorus that plays an important role in plant growth and nitrogen absorption[53]. *Bacillus* activity in colonizing the roots caused an increase in nutrients of chlorophyll absorption that supports photosynthesis process and affects plant production. Nutrients from the organic fertilizer coincided with the treatment using *Bacillus* were absorbed by the plants for metabolism. This metabolism consists of carbohydrate formation that is mostly translocated for tuber and potassium that contributes to tuber formation and growth[54].

The current finding in this research showed several benefits that may obtained from PGPR application on shallot. The application of *B. velezensis* B-27 proved to significantly reduced moler and purple blotch disease, while also improved plant growth. Therefore, it might be an effective strategy on plant disease management in the future. It is also compromising more eco-friendly approach to disease control compared with conventional method. However, it is still needed to be evaluated on different location and different season to ensure the accurate dose and response.

CONCLUSION

Bacillus isolate B-27 upon shallot reduced moler disease and purple blotch disease in the field by 67%. In addition, it also improved plant height, number of leaves, number of tillers and tuber weight. The result of identification revealed that *Bacillus* isolate B-27 has a close relation with *Bacillus velezensis.*

SIGNIFICANCE STATEMENT

This study discovers the effectiveness of *Bacillus velezensis* as plant growth promoting bacteria as well as biocontrol agent that will support the development of eco-friendly disease management in shallot cultivation. Furthermore, the *gyrB* target gene appears to be an ideal molecular marker for the accurate discrimination and identification of *Bacillus* spp. Thus, a new theory on these biocontrol applications may be arrived at.

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