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The Potential of *Rhizophagus intraradices*, *Bacillus thuringiensis Bt* BMKP and Silica for Anthracnose Disease Control in Shallot

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Abstract

Anthracnose caused by *Colletotrichum gloeosporioides* is a prevalent disease that poses a significant threat to shallot production in Indonesia. To mitigate this issue, the use of biological agents presents an alternative for disease control, reducing the inherent risks associated with the use of chemical pesticides. Therefore, this study aimed to determine the potentiality and mechanism of the biological agents (*Rhizophagus intraradices* and *Bacillus thuringiensis*) combined with silica. These agents were evaluated both individually and in combination to suppress the development of anthracnose in Tajuk variety shallot. The study was conducted in the laboratory and greenhouse, arranged in a completely randomized design with six treatments and three replications. The ability to suppress pathogenic fungi was determined based on *in vitro* antagonism tests of *B. thuringiensis*, disease severity, area under disease progress curve (AUDPC), plant height, number of leaves, number of tillers and the percentage of mycorrhizal fungal infections. Furthermore, the mycorrhizal infection on plant roots was observed using staining methods. The results showed that the *Bt* BMKP isolate was included in the *B. thuringiensis* strain RC9 group with the capacity to inhibit *C. gloeosporioides in vitro* by 18.88%. The combination treatment of *R. intraradices*, *B. thuringiensis* and silica reduced infection from anthracnose by 15.52% compared with control. These three treatments also significantly increased the agronomic performance of shallot up to six weeks after planting compared to control and other treatments.

Keywords: *Allium cepa*; biocontrol; *Colletotrichum gloeosporioides*; mycorrhiza; PGPB

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INTRODUCTION

Shallot (*Allium cepa* L. var. *aggregatum*) is one of the horticultural crops playing an essential role in food security. The consumption level is on a persistent upward trajectory, but this surge in demand has not been accompanied by a corresponding increase in production. Shallot production in Indonesia decreased by 22.23 thousand tons or 1.11% in 2022 (Statistic Indonesia, 2023). The decline can be affected by various factors, such as the development of plant disease pathogens.

Anthracnose caused by the fungus *Colletotrichum gloeosporioides* Penz (Galván et al., 1997) is one of the common disease attacking shallot. Typical symptoms of disease are the appearance of white water-soaked spots on the leaves, chlorosis on the entire leaf blade, which turns into necrotic areas with concentric masses of salmon or orange to black spores, drooping leaves, dead shoots, neck elongation or abnormal pseudo stems and slender bulbs formation, and causing plant death (Dutta et al., 2022). Yield losses caused by shallot anthracnose in the field can reach 50 to 100% (Ebenebe, 1980),

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specifically in environmental conditions with high rainfall. This disease poses a substantial threat to production, leading to reduced yields and compromised quality of the bulbs. According to Sarianti and Subandar (2022), the low productivity at the farmer level (4.39 tons ha^{-1}) in Kampong Tanah Bara, Aceh Singkil Regency, is attributed to the high incidence of anthracnose, reaching 96% disease incidence with a severity of 64.93%. Safitri et al. (2019) also reported that the distribution in several regencies in South Kalimantan reached 10 to 26% of the total planting area with different intensities of attack. Generally, the symptoms can be associated with other disease, such as moler disease, purple blotch and leaf blight caused by *Fusarium* spp. (Patil et al., 2018), *Alternaria porri* and *Stemphylium vesicarium* (Korlina et al., 2021).

Various control efforts to minimize the incidence of anthracnose in the field have been performed by referring to integrated control. The use of biological agents that are relatively safe and more environmentally friendly is widely studied as an effective measure to control plant disease (Nega, 2014). The combination of biological agents and other compatible control methods is needed to reduce the use of synthetic chemical fungicides with the potential to cause negative impacts when used continuously for a long time. Biological methods integrated into food production processes can support sustainable agriculture with sufficient crop production without ecological damage (Pretty, 2008). The types of biological agents widely studied for the management of plant pests and disease are the arbuscular mycorrhizal *Rhizophagus intraradices* and the bacterium *Bacillus thuringiensis* known as agricultural pest biocontrol agents.

Arbuscular mycorrhizal fungi (AMF) play a role in assisting plants to deal with biotic stress due to pathogen infection through the mechanisms of competing for nutrients, space and photosynthesis, the changes in the rhizosphere, and the induction of host resistance (Dowarah et al., 2022). The application of AMF to plants has been widely reported to reduce the incidence of moler disease in shallot caused by *Fusarium* spp. (Fitriani et al., 2020). AMF is also known to play a role in spurring plant growth as well as increasing height and number of leaves (Shuab et al., 2014) with production yields of 50% in shallot plants (Saleh et al., 2021).

The application of *R. intraradices* as an AMF species has been reported to extend the incubation period of the pathogen *Fusarium solani*, which causes moler disease in shallot (Artanti et al., 2022). The species also suppressed various other plant pathogens, such as reducing root rot caused by *Fusarium pseudograminearum* on wheat (Spagnoletti et al., 2021) and *Macrophomina phaseolina* on soybeans (Spagnoletti et al., 2020). Inoculation of tomato plants with *R. intraradices* reduced the impact of verticillium wilt and improved plant growth (Dey and Ghosh, 2022). However, information regarding the ability to control air-borne pathogens such as *C. gloeosporioides* in shallot is limited and has not been widely studied.

B. thuringiensis, which has long been known as an agricultural pest biocontrol agent with its entomopathogenic properties, is also starting to become the attention of studies related to plant disease control because of its antimicrobial activity. The plant growth promoting bacteria (PGPB) can indirectly produce plant growth-promoting compounds. Furthermore, it produces bacteriocins, fengycin, chitinase and cell wall-degrading enzymes, which have an impact on the development of phytopathogens (Jouzani et al., 2017), indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate-deaminase (ACC-deaminase), phosphate-solubilizing enzyme (PSE) and siderophores (Azizoglu, 2019). The antagonistic effect of *B. thuringiensis* due to the chitinase activity has been reported to inhibit the growth and development of phytopathogenic fungi such as *Rhizoctonia solani*, *Fusarium oxysporum*, *A. porri* and *Pyricularia oryzae in vitro* (Subbanna et al., 2019). The potential of these two biological agents is better when combined with other control methods.

Good cultivation methods are one of the efforts made in controlling plant disease to reduce the incidence and severity of pathogen infections (Navitasari et al., 2020). Additional fertilization is applied to fulfill the nutritional needs of plants, specifically nutrients that cannot be directly absorbed by plants. Meanwhile, silica is one of the non-essential nutrients known to help plants deal with pathogen infections and pest attacks (Zargar et al., 2019) as well as environmental stresses such as drought, inundation, salinity, nutrient deficiencies and excesses (Luyckx et al., 2017; Etesami and Jeong, 2018).

The advantages of both biological agents and a single application of silica in controlling plant disease have been widely reported, but the combination of the three has not been thoroughly studied. The combination with biological agents can potentially offer a multifaceted method to addressing agricultural challenges. Silica enhances plant resistance and provides physical barriers against pathogens, while biological control agents can actively suppress pests or diseases. Therefore, this study aimed to determine the potentiality and mechanism of biological agents *R. intraradices* and *B. thuringiensis* combined with silica in suppressing the development of shallot anthracnose.

MATERIALS AND METHOD

Study area

The study was conducted in the laboratory and greenhouse of the Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, D.I. Yogyakarta, Indonesia, from January to April 2023.

Isolation and identification of the fungus causing shallot anthracnose

Shallot leaves showing symptoms of anthracnose disease were obtained from the production centers in Gotakan Village, Panjatan Sub-district, Kulon Progo Regency, D.I. Yogyakarta (7°53'33.3"S 110°09'19.8"E). Meanwhile, the pathogenic fungi were isolated using tissue culture based on Reecha et al. (2022). The part between the infected shallot leaf and the healthy ones was cut about 1 cm x 1 cm using a scalpel. The leaf pieces were sterilized by immersion in 1% clorox (disinfecting bleach) for 1 minute, rinsed using sterile water three times for 1 minute and then dried. After drying, the leaf pieces were grown on potato dextrose agar (PDA) in a petri dish and incubated at room temperature for 5 to 10 days. The postulates of Koch were conducted using shallot plants to ensure the isolated pathogen was the causal agent of anthracnose. A fungal suspension with a density of 10^6 spores ml^{-1} was directly inoculated by spraying onto the leaves (Safitri et al., 2019). The plants were kept under homogeneous conditions and observations were carried out until symptoms appeared. Plants showing symptoms were then re-isolated to produce pure isolates and the mycelium was purified based on the colony color on the PDA. The pure fungal cultures obtained were then morphologically identified based on the colony color, colony shape, spores and hyphae (Baxter et al., 1983).

Molecular identification of *B. thuringiensis Bt* BMKP isolate

The *Bt* BMKP isolates were culture collected from the Laboratory of Plant Pathology, Faculty of Agriculture, Universitas Gadjah Mada identified based on their morphological characteristics and suspected to be *B. thuringiensis* bacteria. Molecular identification of *B. thuringiensis Bt* BMKP isolate was carried out through DNA extraction, PCR amplification, visualization and sequencing analysis. Total genomic bacterial DNA extraction was performed according to the ZymoBIOMICS® DNA Miniprep Kit protocol. Subsequently, the DNA obtained was amplified using the PCR method following the procedure of Rahma et al. (2020) with an amplicon length target of ±1500 bp using the primer pairs *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'). PCR was performed with a total volume of 25 µl, where each reaction included 12.5 µl Redmix 2x MyTaq Polymerase (Bioline), 1 µl forward primer, 1 µl reverse primer, 1 µl DNA and 9.5 µl nuclease-free water. The amplification was carried out on a PCR machine (Bio-Rad T100 Thermal Cyclers) with an initial denaturation program at 95 °C for 3 minutes, followed by 35 cycles including denaturation at 95 °C for 1 minute, annealing at 57.5 °C for 30 seconds, extension at 72 °C for 1 minute and final extension at 72 °C for 10 minutes. The amplified DNA was visualized using electrophoresis through 0.8% agarose gel in 1x TBE buffer with a 1 kb DNA ladder (Promega) as a DNA marker for comparison (Joko et al., 2007).

Nucleotide sequencing was obtained using the Sanger DNA method through capillary electrophoresis by sending DNA amplicon to Genetika Science Indonesia Company. The sequencing results were analyzed using basic local alignment search tool (BLAST), which was available on the website of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/Blast.cgi) to determine the percentage of homology. The *gyrB* database of the closely related *B. thuringiensis* was retrieved from GenBank, with various homology percentages used for comparison. The results were used for phylogenetic analysis to obtain

associations between samples and other *Bacillus* spp. using BioEdit and MEGA 11. Furthermore, the phylogenetic tree was created using the neighbor-joining algorithm method with classification stability through bootstrap analysis with 1000 times replication (Wisanggeni et al., 2023).

In vitro antagonism test

Antagonism test of *B. thuringiensis Bt* BMKP isolate against *C. gloeosporioides* was carried out *in vitro* using the co-culture method (Jayanti and Joko, 2020). Meanwhile, PDA media in a petri dish was prepared beforehand and allowed to solidify. Approximately 10 ml of 6% water agar (WA) at 50 °C, which had been added with 100 µl of bacterial suspension with a density of 10^8 cfu ml^{-1}, was poured over a solid PDA medium. Pathogenic fungal isolates were cut using a cork borer with a diameter of ±0.5 cm and then placed on WA medium. The cultures were incubated for seven days at room temperature until the fungal colonies in control filled the petri dish and carried out with a total of five replications. The inhibition ability of *B. thuringiensis Bt* BMKP isolates was determined by measuring the growth of the fungal and calculated using Equation 1 (Raut et al., 2021).

$$P = \frac{D1 - D2}{D1} \times 100\% \quad (1)$$

Where, P = inhibition percentage, D1 = diameter of the pathogenic fungal colony on control plate, D2 = diameter of the pathogenic fungal colony on the treatment plate.

The application of *R. intraradices*, *B. thuringiensis* and Silica

R. intraradices in zeolite media and *B. thuringiensis* bacteria (*Bt* BMKP isolate) were obtained from the Laboratory of Plant Phytopathology, Faculty of Agriculture, Universitas Gadjah Mada and silica used was a commercial product (BIOMAX®). The greenhouse experiment was arranged in a completely randomized design (CRD) with six treatments and three replications. The treatment consisted of control (P1), spraying silica (P2), spraying *B. thuringiensis* suspension (P3), application of *R. intraradices* (P4), application of *R. intraradices* combined with spraying of *B. thuringiensis* suspension (P5), and application of *R. intraradices* combined with spraying of *B. thuringiensis* and silica (P6).

Shallot used was Tajuk and the variety was planted in a growing medium with a mixture of soil, manure, and roasted husks at a volume ratio of 1:1:1 in a polybag (35 cm x 35 cm). Furthermore, shallot bulbs were planted in polybags filled with planting media and each polybag contained three bulbs in each planting hole. The application of *R. intraradices* was performed when planting shallot at a dose of 20 g per planting hole. Treatment with *B. thuringiensis* was carried out by spraying a 10 ml bacterial suspension (density of 10^8 cfu ml^{-1}) per plant once every week after planting. The bacterial suspension was obtained from *Bt* BMKP isolate grown in yeast peptone agar (YPA) medium (0.5% yeast extract, 1% polypeptone, 1.5% agar) and incubated for 48 hours. The bacteria used were a pure culture suspended in sterile water. Subsequently, the resulting *B. thuringiensis* proliferation was diluted with water to a density of 10^8 cfu ml^{-1}. Silica (10 ml per plant) was also applied at 15, 30 and 45 days after planting. The spraying of *B. thuringiensis* and silica was carried out using a 2 l capacity hand sprayer.

Shallot plants aged four weeks after planting were inoculated with the pathogenic fungal *C. gloeosporioides*, which was isolated from leaves infected with anthracnose, by spraying 10 ml of fungal suspension ($1.25x10^6$ spores ml^{-1}) per plant (Hidayat and Sulastrini, 2016). Before inoculation, cotton moistened with water was placed on the top of the soil in the polybag. The plants were covered using a plastic bag for 24 hours after inoculation. Spraying was performed in the afternoon to keep the spores from growing at high humidity.

Calculation of disease severity, AUDPC and agronomic performance of shallot

The observed variables consisted of anthracnose disease severity, AUDPC, disease reduction and agronomic performance, including plant height, number of leaves, and number of tillers. Observations of disease severity, AUDPC and agronomic performance were carried out every 2 to 6 weeks after planting. Anthracnose symptom scores on shallot were categorized according to Dutta et al. (2022) with the following modifications: 0 = no symptoms, 1 = oval white spots, leaf curl and chlorosis, 2 = spots turn black, acervuli appear on the surface of the spot, 3 = shallow, sunken necrotic spots with masses of orange/salmon-colored conidia, 4 = drooping leaves, dry, elongated pseudo stem, bulbs slender and small, dead shoots, 5 = dead plant, black rotting bulbs. Disease severity was calculated using Equation 2 (Hidayat and Sulastrini, 2016).

$$\text{Disease severity} = \frac{\Sigma(ni \times vi)}{N \times Z} \times 100\% \quad (2)$$

Where, n = the number of plants in the i^{th} category, v = the score of the 1^{st} attack category, N = the total number of plants observed, and Z = the highest attack category score.

The AUDPC value was calculated using Equation 3 according to Simko and Piepho (2012).

$$\text{AUDPC} = \sum_{i=1}^{n-1} \left(\frac{Y_i + Y_{i+1}}{2}\right)(t_{1+1} - t_i) \quad (3)$$

Where, AUDPC = area under disease progression curve; n = total number observed; Y_i = disease intensity in the i^{th} observation; t_i = time at the i^{th}-observation.

Disease reduction was calculated using Equation 4 with modification (Handini and Nawangsih, 2014).

$$\text{Disease reduction} = \frac{(\text{AUDPC of control} - \text{AUDPC of treated})}{\text{AUDPC of control}} \times 100\% \quad (4)$$

Observation of *R. intraradices* infection on shallot roots

R. intraradices infection on shallot roots was observed after the plants were harvested using the root staining method. The root samples were washed under running water to remove any remaining soil, cut (1 to 2 cm) and stained (Janos, 1984). The cut roots were then immersed in a glass bottle containing 10% KOH, heated for 5 minutes and rinsed with running water. The roots were soaked using 1% HCl for 5 minutes, and the 1% HCl solution was removed. Subsequently, the roots were soaked in lactophenol cotton blue for 24 hours. The stained roots (100 pieces) were arranged on a glass object, covered with a glass and observed under a microscope (Nikon® Eclipse Ci-L Plus) at 200x magnification to calculate the percentage of the roots. The appearance of hyphal, arbuscular and vesicle structures indicates that the roots are colonized by *R. intraradices*. The percentage of root infection was calculated using Equation 5.

$$\text{Percentage of infection} = \frac{\text{Number of infected roots}}{\text{Number of roots observed}} \times 100\% \quad (5)$$

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) and differences between treatments were determined using the duncan multiple range test (DMRT) at a level of 5%.

RESULTS AND DISCUSSION

Identification of pathogens causing anthracnose disease

Based on the results of isolating the pathogenic fungus from the isolation of infected leaves with anthracnose symptoms, the morphology of the fungal colonies on PDA media was grayish-white to black. The mycelium formed like cotton and grew to fill the petri dish on day 7 (Figure 1a). At the bottom of the petri dish, the fungus was gray to black and formed concentrically (Figure 1b). On the surface of the colony, salmon-orange or black conidiomata containing masses of conidia were formed (Figure 1c). Microscopic observation with 400x magnification showed fungal spores in the form of rods or cylinders with blunt ends, single-celled and not insulated (Figure 1d). Fungal spores in the form of conidia were formed from the tips of hyaline-colored conidiophores, which were found in acervuli with setae resembling stiff black hairs (Figure 1e). The fungal mycelium was hyaline in color, insulated and branched (Figure 1f). Based on these morphological characteristics, the observed isolates were included in *C. gloeosporioides* species.

The characteristics of the isolated fungi are consistent with the description by Baxter et al. (1983), where *C. gloeosporioides* colonies on agar media can reach a diameter of 35 to 75 mm in seven days. Colonies of the fungus form cottony to woolly white, to light gray, grayish brown, grayish white and greenish gray. At the top of the mycelium, orange or salmon-colored conidiomata can be formed. At the bottom of the colony, concentric zones appear in the middle. Conidiogenic cells are cylindrical in clusters on conidiophores, which branch off in conidiomata. However, they are often found singly or in small groups in the aerial mycelium, undifferentiated from sterile hyphae. Conidia are short cylinders with a blunt end tapering slightly to a truncated base and narrowed in the middle. Setae are observed on several isolates, frequently occurring exclusively on the host or under specific agar media conditions, typically exhibiting a relatively short and straight morphology.

Molecular identification of *B. thuringiensis Bt* BMKP isolate

The *gyrB* gene encodes a DNA gyrase protein that plays an essential role in replication within

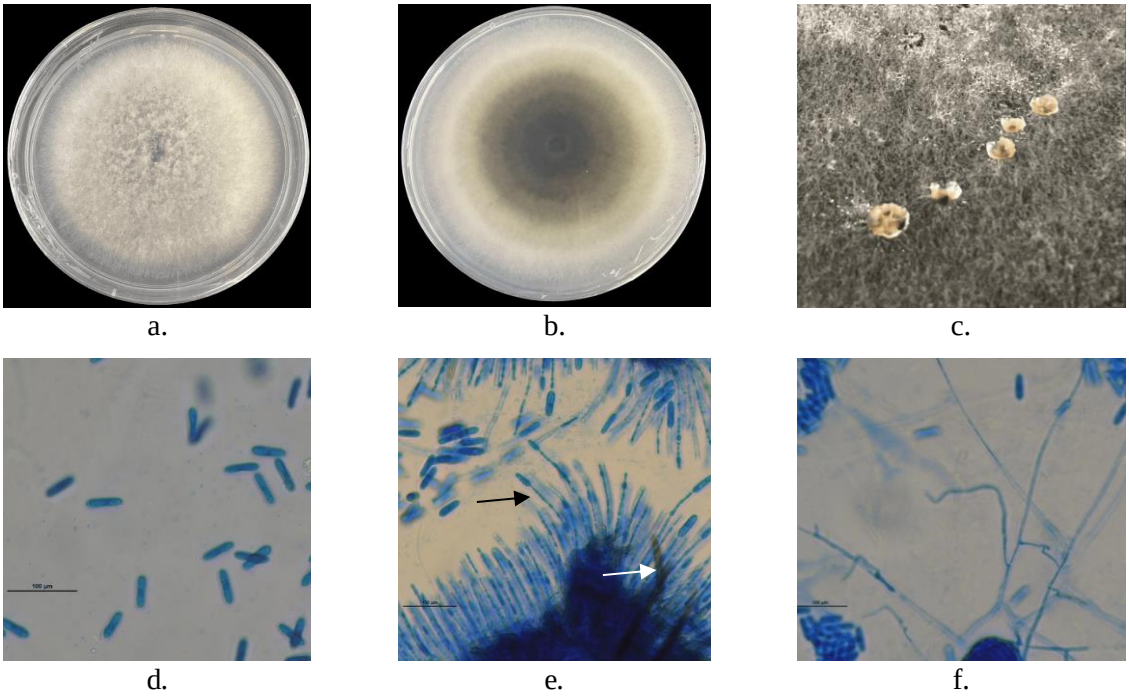


Figure 1. Morphological characteristics of the *C. gloeosporioides* isolated from shallot showing symptoms of anthracnose: (a) top view and (b) bottom view of *C. gloeosporioides* colonies on PDA media aged seven days; (c) conidiomata; (d) conidia; (e) acervuli with black setae (white arrows) and conidiophores (black arrows); (f) hyphae

bacterial species (Wang et al., 2007). Comparison of *gyrB* sequences can also better determine phylogenetic relationships at the species level, specifically in the *Bacillus cereus* sensu lato group, which are difficult to distinguish through 16S rDNA. The visualization of the amplicon of *B. thuringiensis Bt* BMKP isolate showed DNA bands in the range of ±1500 bp (Figure 2). This is in line with Setyawan et al. (2016), where the target DNA amplified using the *gyrB* sequence showed an amplicon length of ±1335 bp. Based on the *gyrB* gene sequence and phylogenetic results (Figure 3), the *Bt* BMKP isolate was similar to the *B. thuringiensis* strain RC9 with 98.44% homology in Genbank data.

Inhibition of *C. gloeosporioides* by *B. thuringiensis in vitro*

The *in vitro* antagonism test using the co-culture method resulted in an inhibition of *C. gloeosporioides* (18.88%) by *B. thuringiensis Bt* BMKP isolate (Figure 4a) compared to control grown to fill the petri dish on day 7 (Figure 4b). The presence of this inhibition indicated that *B. thuringiensis* had the potential to suppress the growth and development of the fungus *C. gloeosporioides*. This was supported by Subbanna et al. (2019), where *B. thuringiensis* was an antifungal agent with the ability to inhibit the mycelium growth of phytopathogenic fungi such as *R. solani*, *F. oxysporum*, *A. porri* and *P. oryzae in vitro*.

The antagonism ability of a biological agent against a pathogen includes one or several ways of inhibition. Mechanisms that can occur are competition, antibiosis, parasitism or induced resistance. *B. thuringiensis* is known to produce antifungal compounds, including cell wall degrading enzymes, fengycin lipopeptides,

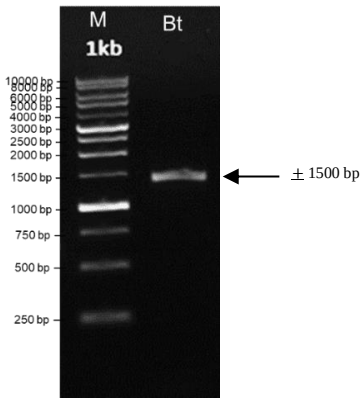


Figure 2. Molecular analysis results of *B. thuringiensis Bt* BMKP isolate: Visualization of DNA amplicon of *Bt* BMKP (Bt) isolate with *gyrB* primer and 1000 bp (M) marker

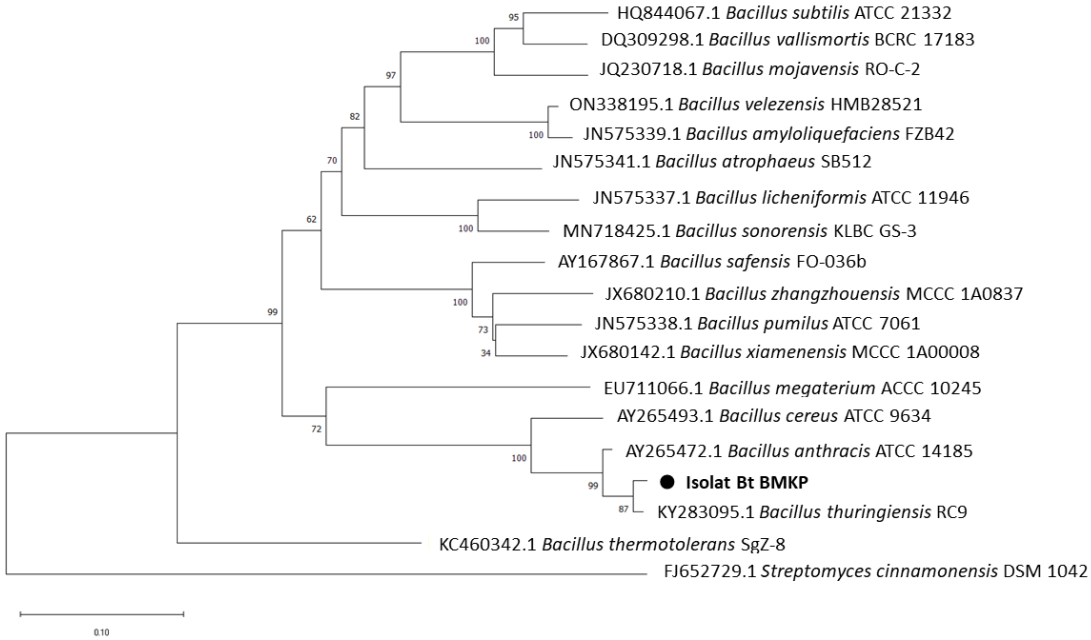
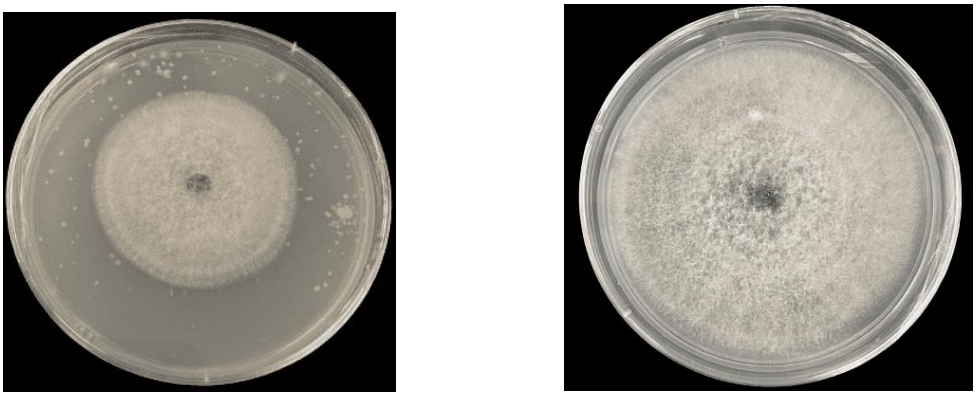


Figure 3. The phylogenetic tree of *Bt* BMKP isolate and its homology to *B. thuringiensis* strain RC9



a. b.

Figure 4. *In vitro* antagonism test results: (a) inhibition of *C. gloeosporioides* by *B. thuringiensis Bt* BMKP isolate suspension using co-culture method on day 7; (b) control on day 7

volatile compounds (VOCs) and signaling molecules inducing systemic resistance (Jouzani et al., 2017). These antifungal compounds can cause malformations in the hyphae, resulting in the underdevelopment of hyphae (Subbanna et al., 2019).

The mechanism of antibiosis by *B. thuringiensis* in inhibiting the growth of *C. gloeosporioides* is closely related to its ability to produce chitinase enzymes. The enzyme is capable of degrading chitin, which is the main component of cell walls in pathogenic fungi. This is consistent with the statement of Öztopuz et al. (2018) that the influence of chitinase can inhibit the growth of the fungal mycelium and spore germination as well as change the morphology of the fungal cell wall. Chitinase activity against the fungus *C. gloeosporioides* was reported by de la Fuente-Salcido et al. (2016), where *B. thuringiensis* reduced radial growth and increased the hyphal density of anthracnose-causing fungal. The fengycin lipopeptide molecule is also reported to disrupt or inhibit the function of the cell membrane of pathogenic fungi by deforming the membrane or forming pores that cause cell death (Tran et al., 2022).

Effects of *R. intraradices*, *B. thuringiensis* and silica on the development of anthracnose

Observation of the intensity of anthracnose in shallot plants six weeks after planting showed that the combination treatment of *R. intraradices*,

B. thuringiensis and silica resulted in the lowest disease severity value of 68.89% compared to controls and other treatments, reaching disease severity of more than 80% (Table 1). Therefore, the combination of the three treatments inhibited the development of more severe anthracnose disease. The incubation period of the pathogen occurred slower with the application of this combination of treatments. Marlitasari et al. (2016) stated that the incubation period was related to the severity of disease. The intensity of disease is also thought to be related to the resistance of shallot variety used. Hekmawati et al. (2020) reported that shallot cv. Tajuk was considered highly susceptible to anthracnose. This variety had a relatively faster incubation period for anthracnose disease, with an intensity of 92%.

The AUDPC was calculated using disease severity data over time to estimate plant resistance to disease (Widyaningsih et al., 2019). Control and silica had the highest AUDPC value of 128.89 (Table 1). Meanwhile, the lowest value was in the treatment using *R. intraradices* combined with *B. thuringiensis* (108.88) and *R. intraradices* combined with *B. thuringiensis* and silica (108.89). This was followed by the treatment of *R. intraradices* (111.11) and *B. thuringiensis* (124.44). A low AUDPC value indicated a high inhibitory effect on shallot anthracnose, while a high value showed a low inhibitory effect on disease.

In this study, the treatment of *R. intraradices*, *B. thuringiensis* and silica reduced infection from anthracnose by 15.52%. This combination treatment showed a greater potential for disease reduction compared to single applications. Therefore, this combination worked synergistically, even though each had a different mechanism applied to the parts of the plant. The application of AMF in the soil during planting supported the nutritional needs to maintain plant health against pathogen infections. Meanwhile, *B. thuringiensis* applied to the upper part of the plant had a direct mechanism by producing antibiotics.

The association of AMF in plants provided benefits including increased disease tolerance. The mechanism of *R. intraradices* in helping plants to meet their nutritional needs indirectly improved health and resistance to pathogenic infections. Other studies that reported the superiority of AMF in suppressing aerial borne disease through systemic resistance induction mechanisms included tomato plants infected with gray mold disease by the fungus *Botrytis cinerea* (Fiorilli et al., 2011) and anthracnose by *Colletotrichum orbiculare* on cucumber (Saldajeno and Hyakumachi, 2011).

The ability of *R. intraradices* to suppress disease development with various mechanisms was supported by *B. thuringiensis* bacteria to inhibit pathogen infection. This is conducted by producing several antifungal compounds, such as chitinase and fengycin (Jouzani et al., 2017) and silica, which has a protective mechanism. Silica sprayed on shallot plants was stored under the leaf cuticle and associated with cellulose or plant tissue surfaces as a physical barrier and mechanically inhibited the penetration of fungi to reduce or slow down the rate of infection (Zargar et al., 2019).

Effects of *R. intraradices*, *B. thuringiensis* and silica on shallot agronomic performance

The variables of agronomic performance observed included plant height, number of leaves and tillers (Table 2). The application of *R. intraradices* combined with spraying *B. thuringiensis* and silica showed the most significant effect on plant height (30.45 cm), number of leaves (6.33) and number of tillers (2.11) compared to control and other treatments. The combination of the three improved the

Table 1. Effects of *R. intraradices*, *B. thuringiensis* and silica on disease severity at 6 WAP and AUDPC

Treatments	Disease severity (%)	AUDPC	Disease reduction (%)
No treatment	88.89±4.44^{c}	128.89±5.88^{b}	0.00
Silica (Si)	80.00±0.00^{bc}	128.89±4.44^{b}	0.00
B. thuringiensis (Bt)	84.44±4.44^{bc}	124.44±4.44^{ab}	3.45
R. intraradices (Ri)	80.00±0.00^{bc}	111.11±13.88^{a}	13.79
Bt + Ri	77.78±4.45^{ab}	108.88±4.45^{a}	15.52
Si + Bt + Ri	68.89±2.22^{a}	108.89±2.22^{a}	15.52
Sig. (p)	0.019	0.032	
CV	9.90	10.13	

Note: means followed by the same letters in the same column are not significantly different based on the DMRT test at the 5% level. WAP = week after planting

Table 2. Effects of *R. intraradices*, *B. thuringiensis* and silica on plant height, number of leaves and number of tillers six weeks after planting

Treatments	Plant height (cm)	Number of leaves	Number of tillers
No treatment	10.16±2.86[a]	2.15±0.71[a]	1.11±0.11[a]
Silica (Si)	15.42±0.00[a]	3.46±0.00[a]	1.33±0.00[a]
B. thuringiensis (Bt)	9.03±3.51[a]	1.82±0.82[a]	1.00±0.19[a]
R. intraradices (Ri)	11.89±2.80[a]	3.33±0.88[a]	1.33±0.00[a]
Bt + Ri	15.60±5.43[a]	3.67±0.88[a]	1.11±0.22[a]
Si + Bt + Ri	30.45±2.35[b]	6.33±0.33[b]	2.11±0.40[b]
Sig. (p)	0.006	0.008	0.027
CV	56.71	52.08	36.40

Note: means followed by the same letters in the same column are not significantly different based on the DMRT test at the 5% level

agronomic performance of shallot up to six weeks after planting.

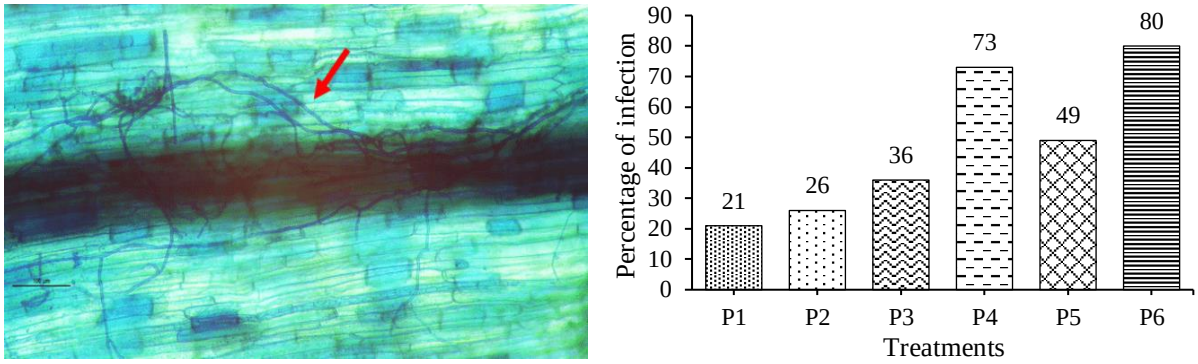
The ability of *R. intraradices* to promote plant growth has been widely reported, among others, in increasing plant height and biomass of tomato plants (Chitarra et al., 2016), plant height and number of leaves of soybean plants (Spagnoletti et al., 2020), and number of leaves, changes in dry weight of plants and dry weight of shallot bulbs (Rahman et al., 2019). The species compete with pathogens by forming colonization with plant roots to increase the uptake of water and nutrients to plants (Dowarah et al., 2022). *R. intraradices* can expand the root surface by having a network of external hyphae, smaller than root hairs allowing the plant to penetrate soil pores (Nadeem et al., 2017). The treatment encourages the plants to remain in prime condition after being infected by the pathogen. Rahman et al. (2019) reported that the application of *R. intraradices* increased nutrient P uptake in shallot plants, specifically under conditions of low P availability. In conditions of sufficient P availability, the additional P increased the dissolved P content in the soil.

This good agronomic performance also benefited from the spraying of *B. thuringiensis*, extensively studied in the mechanism of promoting plant growth and development. This mechanism supports the ability of *B. thuringiensis* as a bioinsecticide and biofertilizer to trigger plant growth (Bandopadhyay, 2020). The ability is a mechanism owned by the *Bacillus* spp. as PGPB in spurring plant growth and development. Several mechanisms of *B. thuringiensis* as PGPB in helping plant growth include producing indole-3-acetic acid (IAA), which affects the root system by increasing the size and weight, number of branches and surface area in contact with the soil, 1-aminocyclopropane-1-carboxylate-deaminase (ACC-deaminase) as an amino acid precursor of natural ethylene compounds. These are essential plant hormones for development and response to stress against biotic and abiotic stresses, PSE and siderophores (SD), which can function as a supply of Fe to plants (Jouzani et al., 2017; Azizoglu, 2019). Rahma et al. (2020), confirmed that *Bacillus* sp. could reduce disease infections and improve vegetative growth and yield components of shallot. Another study by Ilmiah et al. (2021) also reported that the combination treatment of *Bacillus* sp. and goat manure varied the size and shape of the zalacca fruit by increasing phytohormones content.

The effect of *R. intraradices* and *B. thuringiensis* is supported by their interaction with silica in nano and micro formations, which are easily absorbed by plants. Silica is a multi-talented micronutrient that provides several advantages to stressed plants, including biotic stress by the presence of pathogenic infections (Luyckx et al., 2017; Etesami and Jeong, 2018; Zargar et al., 2019). The application can increase the hydraulic conductance of roots by modifying the growth resulting in the absorption and transport of water to maintain a higher rate of photosynthesis and plant resistance to stress (Luyckx et al., 2017; Chen et al., 2018).

R. intraradices infection on shallot roots

R. intraradices infection on plant roots can be characterized by the presence of hyphae, vesicles and arbuscles in the root cortex (Smith and Read, 2008). The microscopic observations on shallot roots showed that all treatments were infected by the species in the form of internal hyphae (Figure 5a). The percentage of infection on shallot roots showed a high value compared to the treatment without *R. intraradices* (Figure 5b). This high value also indicated that the inoculum used was infective and associated with shallot plant roots. Successful colonization of *R. intraradices* relies



a. b.

Figure 5. *R. intraradices* infection on shallot roots characterized by the presence of internal hyphae (arrows) (a) and the percentage of infection on shallot roots (b)

Note: P1 = control; P2 = spraying silica; P3 = spraying *B. thuringiensis* suspension; P4 = application of *R. intraradices*; P5 = application of *R. intraradices* combined with spraying of *B. thuringiensis* suspension; P6 = application of *R. intraradices* combined with spraying of *B. thuringiensis* and silica

on a pre-symbiotic signal exchange that allows specific reciprocal recognition and subsequent cellular reorganization to internalize hyphae into roots. In AMF, hyphae originating from the germination of spores in the soil will be in contact and firmly attached to the epidermal cells of the lateral roots through hypopodium. Hyphae spread longitudinally within the root and form arbuscles, and the branching structures of AMF are considered the main sites for nutrient exchange (Genre et al., 2020).

This study indicates that the treatment used has the potential to control anthracnose in shallot. Combination treatment using *R. intraradices*, *B. thuringiensis* and silica can be applied to reduce or delay disease development. However, it is necessary to optimize the method and application time which is more appropriate, hence the treatment application becomes more effective. Field studies can be carried out to determine the impact on disease development and shallot crop production under different environmental conditions.

CONCLUSIONS

In conclusion, *B. thuringiensis* was able to inhibit the growth of *C. gloeosporioides in vitro* by 18.88%. The combination application of *R. intraradices*, *B. thuringiensis* and silica reduced infection from anthracnose by 15.52% and increased the growth of plant height, number of leaves and number of tillers up to six weeks after planting. Further study was conducted to determine the effectiveness of *R. intraradices*, *B. thuringiensis* and silica on shallot in the field. In addition, molecular methods to understand the mechanisms underlying the interactions between biological agents and pathogens, such as changes in gene expression or plant defense signaling pathways contributed to a deeper understanding of how these agents work to benefit plants.

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