Jurnal Perlindungan Tanaman Indonesia, Vol. 27, No. 2, 2023: 103–109
DOI: 10.22146/jpti.89296
Available online at http://jurnal.ugm.ac.id/jpti
ISSN 1410-1637 (print), ISSN 2548-4788 (online)

Research Article

Utilization of Arbuscular Mycorrhizal Fungi and *Bacillus velezensis* Inoculation to Suppressing Twisted Disease of Shallot

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ABSTRACT

Twisted disease is one of the problems in shallot cultivation. The application of the biological agents *Bacillus velezensis* and arbuscular mycorrhizal fungi is an alternative to overcome disease problems in shallot plantings. The purpose of this study was to determine the effect of the application of *B. velezensis* and *Arbuscular Mycorrhizal Fungi* on the growth and health of shallot. The research was conducted in Gotakan, Panjatan, Kulon Progo, and the Phytopathology Laboratory, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta. Shallot treatments with the application of biological agents were carried out either individually by soaking the bulbs in *B. velezensis* suspension before planting, coating the bulbs using Arbuscular Mycorrhizal Fungus (*Rhizophagus intraradices*) with a carrier medium of kaolin flour before planting, and a combination of spraying the *B. velezensis* suspension on plants at two-week intervals during the growth period and coating the bulbs before planting with *R. intraradices*. The results of the study showed that the shallot-applied single application of *B. velezensis* reduced the intensity and incidence of twisted disease by 2.51% and 37.6%, respectively. The combination treatment of *B. velezensis* and *R. intraradices* was able to increase the resistance of bulbs to postharvest pathogen *Fusarium solani* infection, with infected bulbs and areas of 0.70% and 0.71 cm^2, respectively.

Keywords: Arbuscular Mycorrhizal Fungi (AMF); *Bacillus velezensis*; damage; *Fusarium* sp.; shallots

INTRODUCTION

One of the factors causing low production of shallots (*Allium cepa* L. var. *aggregatum*) in Indonesia is the presence of *Fusarium* spp., the pathogen causing twisted disease in shallots. Twisted disease can cause the bulbs to rot, damage roots, cause leaves to grow longer, twist, become pale green but do not wilt, and reduce size and number of marketable bulbs (Nugroho *et al.*, 2015; Prakoso *et al.*, 2017).

Bacillus velezensis is a Plant Growth Promoting Rhizobacteria (PGPR) that produces secondary metabolites with antibiotic properties, which can increase plant resistance and suppress growth and development of pathogens, thereby also having an impact on plant health (Wisanggeni *et al.*, 2023). *B. velezensis* can also induce plant growth by increasing plants nutrient absorption by, increasing nitrogen fixation and assisting phosphate dissolution, as well as producing the hormone Indole Acetic Acid (IAA), which can increase plant resistance induction (Sevirasari *et al.*, 2022; Zaid *et al.*, 2022). The use of *B. velezensis* by soaking and spraying application by Rahma *et al.* (2020), reported that *B. velezensis* was able to suppress twisted disease and purple spots development on shallot plants by 67% and increase plant growth and protect shallot bulbs postharvest. Wulan *et al.* (2022) reported that the use of *B. velezensis* reduced disease development by 89%, with disease intensity and incidence of 4% and 4%, respectively.

Arbuscular Mycorrhizal Fungi (AMF) are fungi that have a symbiotic relationship with plant roots because they require plant photosynthesis for hyphae growth. AMF hyphae infect plant roots and colonize inside plant roots to help plants absorb nutrients in the rhizosphere zone so that plants can grow

and develop more quickly and induce plant resistance to pathogen infections as well as extend the incubation period (Damanik & Suryanto, 2018; Wibowo *et al*., 2022). There are several species of AMF, namely *Acaulospora* sp., *Glomus etunicatum*, *Glomus* sp., *Glomus maniholtis*, *Gigaspora* sp., and *Rhizophagus intraradices*. The *R. intraradices* application on shallot plants suppressed growth and development of fusarium wilt disease and increase plant growth compared to the control . This was proven by Fitriani *et al.* (2019) showing that a single application of *R. intraradices* resulted in the tallest plants and most number of leaves compared to other endophytic fungi and controls, suppressed pathogen growth with a disease incidence of up to 60% and a latent period of 21 days. Artanti *et al.* (2022) showed that coating treatment of shallot seeds using *R. intraradices* suppressed disease symptoms to appear after inoculation using *F. solani* for four weeks when applications were done either a month before planting or during planting.

Palencia *et al.* (2015) showed that the combination of *Glomus intraradices* with *B. velezensis* increased strawberry fruit growth and quality due to the synergistic interaction between *B. velezensis* colonization on *G. intraradices* mycelium that formed a biofilm. *R. intraradices* and *B. velezensis* interaction as a biological agent on shallots has been done and this this study attempts to explore this matter. Therefore, this research aimed to determine the benefits of using *R. intraradices* and *B. velezensis* as biological agents in shallots, either in a single treatment or in combination.

MATERIALS AND METHODS

Study Area

The research was conducted between July–December 2021 in Gotakan, Panjatan, Kulon Progo, and the Phytopathology Laboratory, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta.

Application of *Bacillus velezensis* and *Rhizophagus intraradices* on Shallot Bulbs

B. velezensis B-27 was cultured on Yeast Peptone Agar media (5 g yeast, 10 g peptone, and 20 g agar) and incubated at 25 °C for 24−48 hours. The bacteria cultures were then suspended and measured to obtain population density of 10^8 cfu/mL for shallot bulb application. Shallot bulbs were soaked in *B. velezensis* suspension for 30 minutes before planting (Rahma *et al.,* 2020), and the plants were sprayed with *B. velezensis* suspension during the experiment once every two weeks. *R. intraradices* with a spore density of 12.03 spores/g obtained previously by filtering zeolite flour containing *R. intraradices* spores. *R. intraradices* in zeolite flour and kaolin flour (1:1) were used to coat the bulbs before planting by placing bulbs in mixtures and shaking until bulbs were fully coated.

The seeds used were the 'Siyem' local variety obtained from farmers. This research was arranged as a Randomized Complete Block Design (RCBD) with four treatments, three blocks, and three replications. The treatments consisted of Bv (*B. velezensis* soaking application), Ri (*R. intraradices* coating application), Bv+Ri (combination of *R. intraradices* coating treatment before planting and spraying *B. velezensis* at once every two weeks), and control (without application of *B. velezensis* & *R. intraradices*). Treated seeds were then planted in ± 100 cm × 200 cm beds with distances of 15 cm × 20 cm, and NPK and ZA fertilizers were applied at a dose of 15−200 kg/500 m^2 and 25 kg/500 m^2, respectively.

Disease Incidence, Disease Intensity, and Area Under Disease Progress Curve (AUDPC) Analysis

Disease incidence and intensity were measured every two weeks. Disease incidence was calculated based on the number of diseased plants at each observation, and the percentage was calculated using the formula according to Ismiyatuningsih *et al.* (2016):

$$\text{Incidence of disease} = \frac{\text{Number of diseased plant}}{\text{Total number of plants observed}} \times 100\%$$

The intensity of twisted disease was carried out using a scoring method for symptoms on shallot plants (Table 1) and calculated using the formula according to Widyaningsih *et al.* (2019) as follows:

$$\text{Intensity of disease} = \frac{\Sigma(n \times v)}{N \times Z} \times 100\%$$

Information:
n = number of diseased plants per category; v = score value per category; N = number of plants observed; Z = the highest score value.

Analysis of the Area Under Disease Progress Curve (AUDPC) value was calculated based on the graph of disease intensity development using the formula according to Rahma *et al.* (2020) as follows:

$$AUDPC = \sum_{i}^{n-1} \left(\frac{Y_{i+1} + Y_1}{2} \right) t_{i+1} + t_1$$

Remarks:
Y_{i+1} = Observation data i + 1; Y_i = Observation data i; t_{i+1} = Observations time i +1; t_i = Observation time i; n = Total plants

Table 1. Scoring of twisted disease intensity

Score	Characteristics
0	Healthy/normal leaves
1	1−20% of leaves turn yellow and twist
2	21−40% of leaves turn yellow and twist
3	41−60% of leaves turn yellow and twist
4	61−80% of leaves turn yellow and twist
5	81−100% of leaves turn yellow and twist

Source: (Köycü & Oezer, 1997)

Postharvest Shallot Resistance

Postharvest shallot resistance was tested using bulbs from previous field research. *Fusarium solani* fungus isolates for inoculation were obtained from the Plant Disease Science Laboratory collection, which were cultured on Potato Dextrose Agar medium (200 g Potato extract, 20 g dextrose, and 15 g agar). Before inoculated, the bulbs were washed with running water and dried, then disinfected with 70% ethanol for 2 minutes and rinsed using sterile water for 1 minute. After that, the bulbs were inoculated by cutting the *F. solani* inoculum using a cork drill with a diameter of 0.8 cm. Pieces of *Fusarium solani* isolates mycelium were attached to the bulb disc that had been injured by piercing using a sterile preparation needle. The inoculated bulbs were then placed in a closed container with sterile cotton wool and sterilized water then incubated for seven days at room temperature.

On the 7th day after inoculation, the bulbs were cut open, and the area of necrosis symptoms were measured using a millimeter block and calculated using the following formula:

$$\text{Area of bulb infection damage} = \frac{\text{Area symptoms of bulb necrosis}}{\text{Total area of bulb}}$$

$$\% \text{ Infected bulb} = \frac{\sum \text{infected bulb}}{\sum \text{observed bulb}} \times 100\%$$

Data Analysis

The data obtained were analyzed using ANOVA and continued with the Tukey Test with a level of 5%.

RESULTS AND DISCUSSION

Disease Incidence, Disease Intensity, and Area Under Disease Progress Curve (AUDPC) Analysis

Highest disease incidence occurred in the control treatment (65.6%), and lowest was in the *B. velezensis* treatment (37.6%). *R. intraradices* and combination treatments resulted in 44.3% and 41%, respectively. Disease incidence were significantly different in the biological agent treatment compared to the control treatment. Likewise, twisted disease intensity showed significantly different results in the biological agent treatment compared to the control treatment, with the highest disease intensity in the control treatment (7.28%) and the lowest in the *B. velezensis* treatment (2.51%). Meanwhile, the disease intensity value in *R. intraradices* and combination treatment were 2.78% and 2.67%, respectively (Table 2). This research showed that *B. velezensis* and *R. intraradices*, either applied individually or combined, were able to suppress the growth and development of twisted disease on shallots. *B. velezensis* ability to inhibit pathogen growth was reported by Moradi-Pour *et al.* (2021), mentioning that the presence of siderophore compounds affected the utilization of Fe that can inhibit *Rhizoctonia solani* growth.

Research by Jiang *et al.* (2019) showed that *B. velezensis* F21 had antagonistic activity against pathogenic fungi with hydrolase activity such as protease, ferric enzyme, glucanase, and cellulose, but did not show chitinase activity. *B. velezensis* was able to suppress the development of Fusarium wilt disease in watermelon plants by 80.35% in field settings and 65.81% in greenhouse settings. In addition, *B. velezensis* F21 was able to induce resistance in watermelon plants affected by fusarium wilt disease due

Table 2. Effects of *Bacillus velezensis* and *Rhizophagus intraradices* application on the development of incidence, intensity and AUDPC of twisted disease on shallot plants

Treatment	Disease Incidence ((%)/WAP)			Disease Intensity ((%)/WAP)		
	2	4	6	2	4	6
Bv	0	26.7[a]	37.6[a]	0	1.28[a]	2.51[a]
Ri	0	33.3[a]	44.3[a]	0	1.72[a]	2.78[a]
Bv+Ri	0	26.7[a]	41.0[a]	0	1.25[a]	2.67[a]
Control	0	57.8[b]	65.6[b]	0	5.5[b]	7.28[b]

*Remarks: Values followed by the same letters in the same column are not significantly different.
Description: Bv (*B. velezensis* soaking application), Ri (*R. intraradices* coating application), Bv+Ri (combination of *R. intraradices* coating treatment before planting & spraying *B. velezensis* once every two weeks), and control (without application of *B. velezensis* & *R. intraradices*).

to the expression of resistance genes and resistance enzymes such as Cla011143 (gene encoding jasmonic acid) and Cla005426 (gene encoding salicylic acid). Jasmonic acid and salicylic acid compounds induce by *B. velezensis* B-27 was also reported by Wulan *et al.* (2022).

Bacillus velezensis and *R. intraradices* effectiveness in suppressing disease can be determined by calculating the Area Under Disease Progress Curve (AUDPC). The AUDPC value for the control treatment had the highest value (18.28%.days), while the lowest was in the *B. velezensis* treatment (5.16%.days). The combination treatment had an AUDPC value of 5.17%.days and the *R. intraradices* treatment of 6.22%.days (Table 2). Smaller AUDPC values indicate better control (Widyaningsih *et al.,* 2017). Therefore, *B. velezensis*, *R. intraradices*, and combination treatments were able to suppress the twisted disease growth in shallot plants.

Bacillus velezensis can produce several secondary metabolites with potential biocontrol activity, namely antibiotic properties by inducing antibacterial metabolites and lipopeptides that activate plant resistance in the presence of surfactin toxins and lipopeptin and later triggering plant growth hormones regarding to nutrient absorption and competition against Bacillus with pathogens (Ye *et al.*, 2018). Secondary metabolites (fengycin, iturin, and surfactin) produced by *B. velezensis* are *Bacillus cyclic lipopeptides* (CLPs) or antifungal cycles, which contribute to fungal phytopathogenic activity to penetrate cell membrane, forming ion channels in the cell membrane pores, causing osmotic pressure imbalance in the membrane, damaging membrane and destroying pathogenic cells (Chen *et al.*, 2018). Secondary metabolites of *B. velezensis* can damage fungal cell walls and cell membranes by releasing proteases, for example extracellular protease (aprE), bacillus peptidase (bpr), and β-glucanase that can damage fungal mycelium (Wang *et al.*, 2020).

Resistance of Shallot Bulbs to *Fusarium solani* Infection in the Postharvest Period

Results showed the percentage of infected bulbs in treated with only *B. velezensis* (0.77) and with combination treatment (0.70) were significantly different from the control treatment (1.12). However, the single treatment *R. intraradices* (0.84) was not significantly different compared to all treatments. Meanwhile, the extent of bulb infection in the single treatment of *B. velezensis*, *R. intraradices* or the combination of both were not significantly different compared to the control (Table 3). These results indicate that the combination treatment of *B. velezensis* and *R. intraradices* can increase the resistance of post-harvest bulbs to *F. solani* infection.

R. intraradices root infection will expand the reach of the roots due to the external hyphae produced from mycorrhizal roots. *R. intraradices* infection is also able to increase the availability and supply of phosphate to plants, and around 80% of phosphorus requirements in plants are supplied by *R. intraradices* (Nadeem *et al.*, 2014). Jeffries *et al.* (2003) reported mutualistic interaction between PGPR and mycorrhizal fungi. PGPR will stimulate the growth of mycorrhizal fungal hyphae by increasing cell permeability, making it easier for mycorrhizal fungal to infect roots. Meanwhile, *R. intraradices* will increase the activity of nitrogen-fixing and phosphate-solubilizing bacteria. Phosphate absorbed by plants plays a role in structuring and stabilizing cell walls by strengthening and increasing cell density so that cell walls are not easily damaged (Wibawa *et al.*, 2023).

Table 3. Infection of shallot bulbs on *Fusarium solani* inoculation

Treatment	Infected bulbs (%)	Infected area (cm^2)
Bv	0.77[a]	0.73[a]
Ri	0.84[ab]	0.82[a]
Bv+Ri	0.70[a]	0.71[a]
Control	1.12[b]	0.90[a]

*Remarks: Values followed by the same letters in the same column are not significantly different.
Description: Bv (*B. velezensis* soaking application), Ri (*R. intraradices* coating application), Bv+Ri (combination of *R. intraradices* coating treatment before planting & spraying *B. velezensis* once every two-week intervals), and control (without application of *B. velezensis* & *R. intraradices*).

The hardness of shallot bulbs can be influenced by the change in water-insoluble peptin (protopectin) into water-soluble pectin (Wibawa *et al.*, 2023). Changes in these components can help maintain the durability of the bulbs. Besides, the ability of bacteria and fungi to produce hormones and enzymes, which cause the epidermal cells of shallot bulbs to become strong and thick, can resist and make it difficult for pathogens to penetrate directly. Research conducted by Ilmiah *et al.* (2021) showed that the application of *B. velezensis* with goat manure could increase the content of total phenolic compounds and antioxidant compounds in snake fruit. In addition, Al-Askar and Rashad (2010) reported that the application of *R. intraradice*s was able to stimulate the production of plant resistance-related enzymes, such as polyphenol oxidase and peroxidase.

CONCLUSION

Based on this research, *B. velezensis* and *R. intraradices* application either individually or combined to shallot was able to suppress the growth and development of twisted disease. The combination application of *B. velezensis* with *R. intraradices* was also able to increase postharvest bulb resistance.

ACKNOWLEDGEMENT

The authors would like to thank the Faculty of Agriculture, Universitas Gadjah Mada, through the Flagship Research Grant Program (Number: 2174/PN/PT/2021) for facilitating the implementation of this research. This manuscript is part of thesis by JTA under the guidance of TJ and Sy.

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