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RESEARCH PAPER

Response of five shallot varieties applied with *Bacillus* spp. against twisted disease

Annisa Hasta Pratiwi, Arif Wibowo, Tri Joko, Ani Widiastuti, & Siti Subandiyah

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ABSTRACT

The use of several shallot varieties applied with the biological agent *Bacillus* spp. is one of the most developed methods of controlling twisted disease, as it is safe and efficient. The large number of shallot varieties released to farmers requires the selection of varieties with the best resistance response to twisted disease. This study aimed to determine the different responses between five local shallot varieties treated with a combination of *Bacillus velezensis* B-27 and *B. cereus* RC76 against the twisted disease. This study was conducted in a greenhouse and on the field using *Tajuk*, *Bima Brebes*, *Bauji*, *Crok Kuning*, and *Manjung* varieties, which were dipped and sprayed with a *B. velezensis* B-27 and *B. cereus* RC76. The treatment of *B. velezensis* B-27 and *B. cereus* RC76 on five varieties showed a good response to suppressing twisted disease. The twisted disease incubation period in five varieties treated with the combination of *B. velezensis* B-27 and *B. cereus* RC76 showed a slower result than the control, the disease incidence and intensity could be reduced by 70–90%. The best resistance response of varieties treated with the combination of *B. velezensis* B-27 and *B. cereus* RC76 was shown by *Tajuk* compared to the other four varieties.

Key words: *Bacillus cereus* RC76, *Bacillus velezensis* B-27, Fusarium disease, Tajuk variety

INTRODUCTION

Twisted disease is one of the crucial diseases in shallots, which causes a decrease in shallot production. It is caused by the infection of the pathogenic fungus *Fusarium* spp., which can result in abnormal plant growth, leaf chlorosis, bulb rot, and plant death (Emeliawati et al., 2022). An early symptom of twisted disease is the curling of pale green and yellowish leaves from the base, along with stunted plant growth. This symptom can progress to rotting of the bulbs and make the plants easily uprooted (Rohma & Wahyuni, 2022).

The three species of *Fusarium* that cause different symptoms of twisted disease are *F. solani*, *F. oxysporum*, and *F. acutatum*. *F. solani* and *F. acutatum* induce symptoms such as yellowing, dryness, twisting, and wilting of leaves in plants. Moreover, *F. solani*, *F. oxysporum*, and *F. acutatum* cause rotting symptoms in shallot bulbs (Lestiyani et al., 2021). *Fusarium* spp. infect shallot plants during the growing season and cause yield loss of up to 50% in the field (Wiyatiningsih, 2021). Additionally, the inoculum of *Fusarium* spp. can survive in the soil or carried by bulbs and causing damage to bulbs in storage, accounting for around 30–40% of losses (Le et al., 2021).

In the cultivation of shallots, the use of resistant varieties is a common practice for controlling twisted disease. Planting shallots with resistant varieties is chosen for its efficiency, simplicity, and environmental friendliness. Local varieties like *Tiron* and *Bauji h*ave been reported independently to exhibit high resistance to twisted disease (Wiyatiningsih et al., 2009; Wibowo et al., 2016). However, a current challenge is the extensive number of varieties available to farmers, necessitating further studies to identify several varieties that are resistant to twisted disease.

The utilization of bacterial biological agents is also carried out as an effort to control twisted disease, alongside the use of resistant varieties. The use of bacterial biological agents for controlling plant diseases has gained widespread development and application. *Bacillus* spp. is a type of bacteria extensively employed due to its role as *Plant Growth Promoting Rhizobacteria* which stimulates plant growth and can suppress plant diseases (Khan et al., 2017). *Bacillus* spp. can enhance plant resistance to disease either directly or indirectly by competing for space or releasing secondary metabolites that are antifungal and supportive of plant growth (Ntushelo et al., 2019; Sudana & Lotrini, 2005).

Bacillus velezensis B-27 and *Bacillus cereus* RC76, employed in this study, had been tested and demonstrated their potential as bacterial biological

Corresponding author:
Arif Wibowo (arif_wibowo@ugm.ac.id)

Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Jln. Flora No. 1, Bulaksumur, Sleman, Yogyakarta, 55281 Indonesia.

control agents. In a previous study, it was reported that *B. velezensis* B-27, using the bulb-dipping technique, could reduce twisted disease and purple blotch by 67% in shallots of *Crok Kuning* variety (Rahma et al., 2020). Bulbs dipping using a combination of *B. velezensis* B-27 and *B. cereus* RC76 could suppress twisted disease by 84% and increase the induction of plant resistance to twisted disease in *Bima Brebes* variety (Wulan et al., 2022). *B. velezensis* B-27 was also reported to increase antioxidant activity, total phenol, and flavonoid compounds, which play a role in metabolic pathways and plant resistance to disease (Ilmiah et al., 2021; Djaenuddin et al., 2018).

Research by Rahma et al. (2020) and Wulan et al. (2022) applied *B. velezensis* B-27 and *B. cereus* RC76 to shallots using only one variety and/or one application technique. Research regarding the application of a combination of *B. velezensis* B-27 and *B. cereus* RC76 through bulb dipping and plant spraying on five varieties, namely *Tajuk*, *Bima Brebes*, *Bauji*, *Crok Kuning*, and *Manjung,* against twisted disease had not been carried out before. Therefore, in this study, five local shallot varieties were tested, treated by bulbs dipping and plants spraying with a combination of *B. velezensis* B-27 and *B. cereus* RC76. This study aimed to determine the increase in resistance response of the five shallot varieties treated with a combination of *B. velezensis* B-27 and *B. cereus* RC76 to twisted disease.

MATERIALS AND METHODS

Research Site. The research was conducted in two locations: in the greenhouse of the Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, and in Samas Beach Sand Field, Sanden District, Bantul Regency, D.I. Yogyakarta. The sandy field is of coastal sandy land with regosol eutric soil type and a sand dunes landscape type. These two experiments used five local shallot varieties that are frequently cultivated by farmers in Java, namely *Tajuk*, *Bima Brebes*, *Bauji*, *Crok Kuning*, and *Manjung* (description in Table 1).

The selection of five varieties in this study was based on local farmers's habits regarding the most planted varieties in shallot cultivation. The selection was also informed by literature that states discusses varieties in terms of production, weather adaptation, and resistance to plant diseases. The *Tajuk* variety demonstrated has good adaptation to seasons (Maharijaya et al., 2016) and is widely cultivated on the island of Java. About 71.43% of Central Java farmers have been planting the *Bima Brebes* variety since its release, according to Pusdatin (2013). *Crok Kuning* is a local variety from Bantul, the location of the research experimental fields, and has a high yield potential. The *Bauji* and *Manjung* varieties hold superior variety status according to information from the Ministry of Agriculture.

Experimental Design. The greenhouse experiment used a completely randomized design with two factors and three replications. The first factor was five shallot varieties, and the second factor was the treatment, which consisted of:

A. (*Bacillus* spp.+ inoculation): Dipping the bulbs before planting and spraying shallot plants using a combination of *B. velezensis* B-27 and *B. cereus* RC76 + inoculation of *Fusarium acutatum* pathogen.

B. (Inoculation): Inoculation of *F. acutatum* and without the application of a combination of *B. velezensis* B-27 and *B. cereus* RC76.

C. (Non-inoculation): Without inoculation of *F. acutatum* and without the application of a combination of *B. velezensis* B-27 and *B. cereus* RC76.

The experimental field used a randomized block design, where the treatment involved five shallot varieties treated by bulb dipping before planting and plant spraying using a combination of *B. velezensis* B-27 and *B. cereus* RC76.

Propagation of *Bacillus* spp. *B. velezensis* B-27 and *B. cereus* RC76 were two bacterial species used in this study as biological control agents for twisted disease. The two bacterial isolates were obtained from the collection of the Laboratory of Plant Diseases, Faculty of Agriculture, UGM. They were cultured in *Yeast Peptone Agar medium* (0.5% yeast, 1% peptone, and 1.5% agar) for 48 hours. After that, the isolates were dissolved in sterile distilled water to obtain the required bacterial

Table 1. Description of five shallot varieties were used

No.	Varieties	Origin	Variety character
1.	*Tajuk*	Nganjuk, East Java	Well adapted at dry and wet season
2.	*Bima Brebes*	Brebes, Central Java	Moderately resistant to bulb rot disease
3.	*Bauji*	Nganjuk, East Java	Tolerant to *Fusarium* sp. and *Spodoptera* sp.
4.	*Crok Kuning*	Bantul, Yogyakarta	High production and well adapted in lowland
5.	*Manjung*	Pamekasan, East Java	Well adapted in lowland, dry and wet season

suspension with a density of 10^8 CFU/mL (Rahma et al., 2020). The suspension of *B. velezensis* B-27 was mixed with a suspension of *B. cereus* RC76 in a ratio of 1:1 as a combined suspension before being applied to shallots.

Treatment of *Bacillus* spp. in the Greenhouse. Prior to inoculation and planting, the bulbs of five shallot varieties (treatment A) were dipped in a combined suspension of *B. velezensis* B-27 and *B. cereus* RC76 at a density of 10^8 CFU/mL, which had previously been obtained, using 10 mL per bulb for 30 min. Then, the bulbs were air-dried (Wulan et al., 2022). Shallot bulbs with treatments B and C were dipped in sterile water and then air-dried.

The next treatment involved spraying the shallot plants in treatment A with a combination of *B. velezensis* B-27 and *B. cereus* RC76 every two weeks. A combined suspension of *B. velezensis* B-27 and *B. cereus* RC76 at a density of 10^8 CFU/mL were applied to shallot plants in treatment A using a 1000 mL hand sprayer. Each shallot plant was sprayed with 10 mL suspension every two weeks starting from seven days after planting (DAP). The time interval for spraying plants were at 7, 21, and 35 DAP, conducted around 7–8 AM in the morning.

Inoculation of Pathogenic Fungi in the Greenhouse. The inoculation of pathogen was carried out during the shallot planting process using *F. acutatum* isolates from the collection of the Laboratory of Plant Diseases, Faculty of Agriculture UGM. *F. acutatum* was cultured in Potato Dextrose Agar (PDA) medium, which consisted of a broth of 250 g potatoes, 20 g dextrose, and 20 g agar dissolved in 1000 mL of distilled water and sterilized in an autoclave at 120 ºC. The *F. acutatum* isolate from laboratory collection was plugged using a cork borer with a diameter of 1 cm, and then the mycelium pieces were transferred into a new PDA medium to propagate *F. acutatum*. *F. acutatum* was incubated for seven days at a room temperature of 22–25 ºC. The fungal isolate was then dissolved in sterile distilled water to obtain a suspension of *F. acutatum* conidia at a density of 1 x 10^6 conidia/mL (Lestiyani et al., 2021).

Three bulbs were planted in each polybag measuring 35 × 35 cm, consisting of sterile soil with three replicate polybags per treatment. The total number of polybags used was 45, and the bulbs used were 135 shallot bulbs. The conidial suspension of *F. acutatum*, which had previously been obtained, was then inoculated by pouring the conidial suspension into bulbs of five shallot varieties in treatments A (*Bacillus* spp. + inoculation) and B (inoculation) each with 10 mL per shallot bulb (Wijoyo et al., 2020).

Treatment of *Bacillus* spp. on Five Varieties in the Field. Field research was conducted during the dry season from the end of March until May. Shallot planting in the field was carried out in 1 × 10 m beds with three replicate blocks. The total number of beds used were 15, and the total number of shallot plants planted in one bed was 300. The bulbs of five shallot varieties were dipped in a bacterial combined suspension of *B. velezensis* B-27 and *B. cereus* RC76 at a density of 10^8 CFU/mL with a ratio of 1:1. Each variety was dipped in 300 mL of the bacterial combination added to 3000 mL of water for 30 min, then air-dried (Wulan et al., 2022). The five shallot varieties were then planted in beds with a spacing of 20 × 15 cm.

One week after planting, the shallot plants were first sprayed with a combination of *B. velezensis* B-27 and *B. cereus* RC76 at a density of 10^8 CFU/mL. Spraying shallots in the field was done using a 16 L automatic knapsack sprayer. The combination of *B. velezensis* B-27 and *B. cereus* RC76 was applied to each bed with 300 mL of bacterial suspension added to 3000 mL of water, or about 10 mL per plant. The time interval for spraying the plants was once every two weeks, recorded at 7, 21, and 35 DAP, which was done around 7–8 AM in the morning.

Maintenance of Five Shallot Varieties in the Filed. Maintenance for shallot was carried out by watering plants every morning and evening automatically using an irrigation hose spraying method. Furthermore, the plants were fertilized using SP36 at a rate of 150 kg/ha at 7 DAP, 150 kg/ha of urea, 200 kg/ha of ZA and 150 kg/ha of KCl at 2, 4 and 6 weeks after planting. Pest control was also implemented by applying chemical insecticides with the active ingredient Sipermethrin and biological insecticides with the entomopathogenic *Beauveria bassiana* to control armyworm pests and thrips on shallot plants every week.

Observation of Incubation Period, Incidence, Intensity of Twisted Disease and Area Under Disease Progress Curve (AUDPC). The incubation period of twisted disease was observed in the greenhouse experiment every day from the inoculation of *F. acutatum* until the onset of twisted disease symptoms in shallot plants (Prakoso et al., 2016). Symptoms of twisted disease observed in shallots included dry, yellowing, or twisting leaves, and rotting bulbs.

The incidence of twisted disease was observed in the greenhouse and in the field every week after the application of the combination of *B. velezensis* B-27 and *B. cereus* RC76, at 14, 28 and 42 DAP. The incidence of twisted disease was determined by calculating the total number of diseased plants in each polybag or treatment bed and then using the formula (Wiyatiningsih et al., 2009):

$$DI = \frac{n}{N} \times 100\%$$

DI = Disease incidence;
n = Number of diseased plants observed;
N = Number of plant observed.

The intensity of twisted disease was observed in all shallot plants in each treatment in polybags and beds, and then categorized based on symptom scoring and calculated using the formula (Hadiwiyono et al., 2020):

$$DS = \frac{\sum(n \times v)}{Z \times N} \times 100\%$$

DS = Disease severity;
Z = Highest score;
N = Total plants observed;
n = Number of infected plants with a certain score;
v = Disease severity score.

Scoring is based on Rahma et al. (2020) with modifications: a score of 0 = no symptoms, 1= 1–20% leaves turning yellow and twisting, 2= 21–40% leaves turning yellow, dry and twisting, 3= 41–60% leaves turning yellow, dry and twisting, 4= 61–80% leaves turn yellow, dry, twisting and the bulbs rot, 5= dead plants).

Disease resistance criteria are determined based on criteria that have been modified by Herlina et al. (2021). The categories for resistance criteria are based on the percentage of twisted disease intensity in shallots, including: Very resistant (0%); Resistant (1–10%); Moderately resistant (10–30%); Susceptible (30–50%); Very susceptible (50–100%).

Furthermore, with an observation interval of 14 days, the disease intensity is used to calculate AUDPC using the formula (Simko & Piepho, 2012):

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

Yi+1 = Observational data-i + 1;
Yi = Observational data-i;
ti + 1 = Observational time-i + 1;
ti = Observational time-i.

Statistical Analysis. Data were analyzed using analysis of variance and further tested using Duncan Multiple Test Range (DMRT) with a 95% confidence level. Analysis was performed using IBM SPSS Statistics software version 26.

RESULTS AND DISCUSSION

Incubation Period of Twisted Disease. The incubation period of twisted disease in five varieties treated with the combination of *B. velezensis* B-27 and *B. cereus* RC76 showed a slower onset of disease symptoms compared to the inoculation treatment. Symptoms of twisted disease in five shallot varieties treated with the combination of *B. velezensis* B-27 and *B. cereus* RC76 appeared the fastest in the *Crok Kuning* variety at 21 days after inoculation (DAI), while the slowest was shown in the *Tajuk* and *Manjung* varieties at 28 DAI. In the inoculation treatment, the varieties showing the fastest incubation period were the *Bima Brebes* and *Manjung* varieties at 13 DAI, and the slowest at 21 DAI for the Tajuk variety (Figure 1). Study by Cahyaningrum et al. (2020) showed that shallots inoculated by *Fusarium* sp.

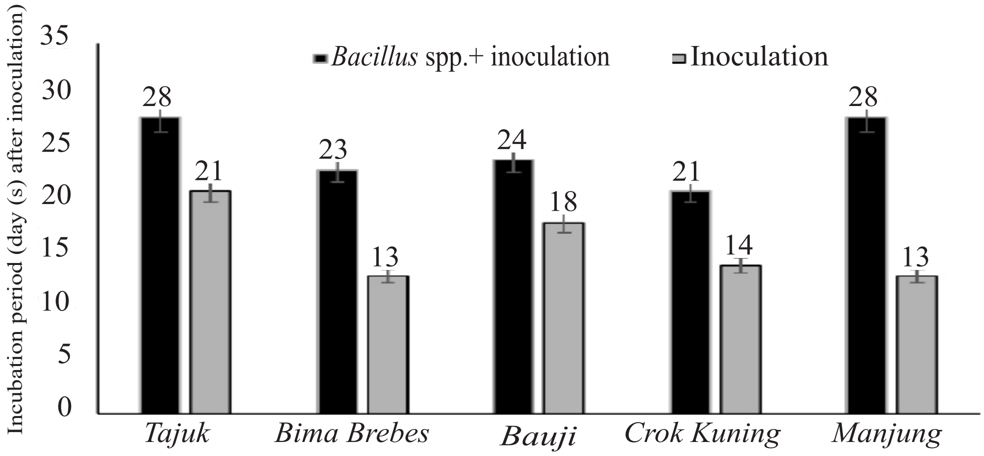

Figure 1. The incubation period of twisted disease on five shallot varieties after applying the combination of *Bacillus velezensis* B-27 and *Bacillus cereus* RC76.

exhibited twisted disease symptoms in 7–12 DAI. The study by Prakoso et al. (2016) demonstrated the twisted disease incubation period for *Bauji Magetan* variety was at 14 days, for *Manjung* at 17 days, and *Bauji Nganjuk* showed the longest period at 20 days. The incubation period for twisted disease in *Crok Kuning* variety is reported at 11 DAI (Wibowo et al., 2016).

Shallots inoculated by *F. acutatum* showed different symptoms of twisted disease in the five shallot varieties tested. *Tajuk* and *Bauji* varieties in the combined treatment of *B. velezensis* B-27 and *B. cereus* RC76, as well as the inoculation treatment, had the same slow incubation period compared to the other four varieties. This result indicated that *Tajuk* and *Bauji* may have potential resistance to twisted disease from the start, and then the resistance increased after being treated with a combination of *B. velezensis* B-27 and *B. cereus* RC76. The difference in the incubation period was due to the resistance response factors of different shallot varieties to twisted disease. The emergence of twisted disease symptoms in all varieties of the inoculation treatment was in the range of 13–21 DAI, which appeared faster than in the combined treatment of *B. velezensis* B-27 and *B. cereus* RC76.

Bima Brebes variety showed the fastest incubation period in the inoculation treatment and the second fastest in the combined treatment of *B. velezensis* B-27 and *B. cereus* RC76. The range of the symptoms appearance in *Bima Brebes* was similar to *Crok Kuning,* which showed the fastest incubation period in the combined treatment of *B. velezensis* B-27 and *B. cereus* RC76 and the second fastest in the inoculation treatment. Based on the time span of symptoms appearance, the *Manjung* variety showed the best response, with the greatest period ranged between treatment by *Bacillus* and non-*Bacillus*, namely at 28 DAI and 13 DAI. A comparison of the distance between the disease incubation period in the varieties treated with bacteria and the non-bacterial treated varieties showed the potential of a combination of *B. velezensis* B-27 and *B. cereus* RC76.

The role of the treatment with *B. velezensis* and *B. cereus* is also one of the important factors in the period of the emergence of twisted disease symptoms. The fastest incubation period in the combined treatment of *B. velezensis* B-27 and *B. cereus* RC76 was the same as the slowest incubation period in the inoculation treatment. This showed that the combined treatment of *B. velezensis* B-27 and *B. cereus* RC76 played a role in slowing the infection process of *F. acutatum* and the emergence of twisted disease symptoms. Several studies have shown that *Bacillus* spp. have the potential to inhibit pathogens by producing secondary metabolites. *B. velezensis* can synthesize surfactin, bacillomycin-D, fengycin, siderophore, difficidin, and several other secondary metabolite compounds. Fengycin can inhibit mycelium growth, change the morphology of fungal hyphae and conidia cell walls, and inhibit fungal spore germination (Rabbee et al., 2019). *B. cereus* is capable of producing secondary metabolites and organic components that are antifungal and increase plant growth, for example, indole acetic acid, ACC deaminase, siderophores and phosphates (Zhou et al., 2021).

Incidence and Intensity of Twisted Disease. The twisted disease incidence and intensity were presented in the form of a percentage, which showed the magnitude of the disease incidence caused by pathogens quantitatively. The results of the study in the greenhouse showed that there was no interaction between treatments and varieties on the incidence and intensity of twisted disease. The treatment of *B. velezensis* B-27 and *B. cereus* RC76 was significantly different compared to the inoculation treatment in terms of twised disease incidence and intensity. Five shallot varieties also showed significant differences between varieties to twisted disease incidence and no significant difference in twisted disease intensity. Overall, the percentage of the disease incidence in *Tajuk* variety showed the smallest value of 51.85%, which was significantly different from *Bima Brebes*, 70.37%. *Bauji*, *Crok Kuning* and *Manjung* varieties showed no significant difference with the disease incidence of 66.67%; 62.96% and 66.67%, respectively (Table 2).

The combined treatment of *B. velezensis* B-27 and *B. cereus* RC76 in the greenhouse showed a significant effect compared to inoculation treatment on the disease intensity with a value of 26.67%. The result indicated that the treatment of *B. velezensis* B-27 and *B. cereus* RC76 on five shallot varieties in the greenhouse could reduce the disease intensity by up to 73.33%. Among the five shallot varieties tested, disease intensity results did not show a significant difference in the five varieties tested. The lowest disease intensity was shown by *Tajuk* and *Bauji* varieties but was not significantly different from *Bima Brebes*, *Crok Kuning* and *Manjung*. The suppression of twisted disease based on the disease intensity of five shallot varieties in all treatments ranged from 71.85% to 77.78% (Table 3). The ability of *B. velezensis* B-27 and *B. cereus* RC76 in combination to suppress twisted disease in the greenhouse, based on the study by Wulan et al. (2022) showed that the twisted disease intensity was reduced by up to 90% with the bulb-dipping method.

The field experiment was carried out by applying a combination of *B. velezensis* B-27 and *B. cereus* RC76 in five varieties to compare the resistance response

Table 2. The incidence of twisted disease on five shallot varieties applied with the combination of *Bacillus velezensis* B-27 and *Bacillus cereus* RC76 in the greenhouse

Treatments	Varieties					Average*
	Tajuk	*Bima Brebes*	*Bauji*	*Crok Kuning*	*Manjung*	
Bacillus spp. + inoculation	55.56	88.89	88.89	77.78	88.89	80.00 b
Inoculation	100.00	100.00	100.00	100.00	100.00	100.00 a
Non-inoculation	0.00	22.22	11.11	11.11	11.11	11.11 c
Average*	51.85 b	70.37 a	66.67 ab	62.96 ab	66.67 ab	(-)

The sign (-) indicates that there is no interaction between treatments and varieties. *= Values followed by different letters in the same treatment and column showed significant differences (DMRT; α= 0.05).

Table 3. The intensity of twisted disease on five shallot varieties in the greenhouse

Treatments	Varieties					Average*
	Tajuk	*Bima Brebes*	*Bauji*	*Crok Kuning*	*Manjung*	
Bacillus spp. + inoculation	13.33	40.00	22.22	33.33	24.45	26.67 b
Inoculation	53.33	40.00	42.22	37.78	42.22	43.11 a
Non-inoculation	0.00	4.45	2.22	2.22	2.22	2.22 c
Average^{ns}	22.22	28.15	22.22	24.44	22.96	(-)

The sign (-) indicates that there is no interaction between treatments and varieties. *= Values followed by different letters in the same treatment and column showed significant differences (DMRT; α = 0.05). ^{ns}= non significantly different (ANOVA; α = 0.05).

between varieties against twisted disease incidence and intensity. The twisted disease incidence and intensity in the field showed significantly different results among the five shallot varieties. Observations made from 14 to 42 DAP showed a different progression of disease incidence. At the 42nd day of observation, the lowest disease incidence in the field was shown by Tajuk of 16.57%, which was significantly different from *Bima Brebe*s, *Bauji*, *Crok Kuning* and *Manjung*. The result of the disease intensity showed that the percentage was directly proportional to the disease incidence, with the lowest disease intensity shown by *Tajuk* at 6.32%, which was significantly different from *Bima Brebes*, *Bauji*, *Crok Kuning* and *Manjung* (Table 4).

Observations made from 14 to 42 DAP showed an increase and differences between the five varieties in disease intensity. The highest development of twisted disease was shown by the *Bauji* variety, and the lowest was shown by *Tajuk* variety. The difference in disease intensity among the five shallot varieties resulted in variations in the resistance levels of the different varieties. Based on the disease intensity of field experiment, five varieties were classified into resistance criteria according to Herlina et al. (2021) with slight modifications. The results showed that *Tajuk* and *Bima Brebes* were classified as resistant, while *Bauji*, *Crok Kuning* and *Manjung* were classified as moderately resistant (Table 5). Differences in resistance responses by each variety can be caused by differences of the variety, treatments and the environment used for the research.

The treatment of a combination of *B. velezensis* B-27 and *B. cereus* RC76 affected the twisted disease incidence and intensity. Overall, the combined treatment of *B. velezensis* B-27 and *B. cereus* RC76 on the *Tajuk* variety had the lowest disease intensity, namely 13.33% in the greenhouse and 6.32% in the field. The low incidence and intensity were influenced by the ability of *B. velezensis* B-27 and *B. cereus* RC76 to suppress twisted disease directly and indirectly. Wulan et al. (2022) stated that shallots dipped in *B. cereus* RC76 and *B. velezensis* B-27 had an intensity of twisted disease of 10%, resulting in a 72.2% suppression of twisted disease. In the study by Rahma et al. (2020), *B. velezensis* B-27 could inhibit twisted disease in shallots by the bulb-dipping technique, where the intensity of twisted disease was shown to be the lowest, at 0.15%.

In previous studies, it has been known that *Bacillus* spp. could produce secondary metabolites that can cause a low intensity of disease due to pathogens (Wulan et al., 2022). Compounds produced by *Bacillus* spp. play a role in inhibiting the growth of pathogens, assisting the development of bacteria, and inducing plant resistance to disease. *B. cereus* is able to produce hydrolytic enzymes and volatile components that can damage cell walls and inhibit the growth of the mycelium of *Fusarium oxysporum* (Ramírez et al., 2022). *B.*

Table 4. The twisted disease incidence and intensity on five shallot varieties applied with the combination of *Bacillus velezensis* B-27 and *Bacillus cereus* RC76 in sandy field

Varieties	Disease incidence (%)			Disease intensity (%)		
	14 DAP*	28 DAP^{ns}	42 DAP*	14 DAP*	28 DAP^{ns}	42 DAP*
Tajuk	8.87 ab	9.45	16.57 b	1.85 ab	3.20	6.32 c
Bima Brebes	8.93 ab	12.35	22.59 ab	1.90 ab	4.12	9.43 b
Bauji	6.70 b	13.57	25.82 ab	1.38 b	5.17	12.71 a
Crok Kuning	12.18 a	12.73	28.19 a	2.53 a	4.34	11.21 ab
Manjung	9.97 ab	11.22	28.46 a	2.08 ab	4.26	10.95 ab
Significance	0.094	0.558	0.097	0.068	0.589	0.004
CV	0.21	0.13	0.20	0.21	0.17	0.24

*= Values followed by different letters in the same treatment and column showed significant differences (DMRT;α = 0.05). ^{ns}= non significantly different (ANOVA; α = 0.05).

Table 5. Classification of resistance criteria of five shallot varieties treated with combination of *Bacillus velezensis* B-27 and *Bacillus cereus* RC76 in sandy field

No.	Varieties	Disease intensity (%)	Resistance criteria
1.	*Tajuk*	6.32	Resistant
2.	*Bima Brebes*	9.43	Resistant
3.	*Bauji*	12.71	Moderately resistant
4.	*Crok Kuning*	11.21	Moderately resistant
5.	*Manjung*	10.95	Moderately resistant

Determination of the resistance category of varieties based on twisted disease intensity in the field at 42 DAP.

velezensis can degrade fungal cell walls by producing glucanase and peptidoglycan. A study on *B. velezensis* FKM10 by Wang et al. (2020) showed damage to the cell walls and mycelium of *F. verticillioides* due to high glucanase production. *B. velezensis* also forms peptidoglycan which replaces the cell wall, resulting in barriers to fungal nutritional needs and fungal growth.

Bacillus spp. can also affect the physiological response of plants by inducing plant resistance to disease. Secondary metabolite compounds produced by bacteria can recognize and activate the signaling pathways of plant resistance induction. *B. cereus* RC76 can induce jasmonic acid in shallots which act as a signaling pathway for plant resistance to twisted disease (Wulan et al., 2022). *B. velezensis* produces surfactin, iturin, L-hydroanticapsin, Oxydifficidin, and Bacillibactin, which can increase plant growth and induce plant resistance to pathogens (Ye et al., 2018; Nifakos et al., 2021). Chen et al. (2022) stated that the gene of SIMPK3 in *B. velezensis* regulates the accumulation of reactive oxygen species by regulating genes related to pathogen pressure and can produce indole acetic acid and surfactin, which prevent and inhibit disease.

Area Under the Disease Progression Curve (AUDPC). The AUDPC value from the results of the study indicated the disease progression of twisted disease. The AUDPC value was obtained based on the twisted disease intensity used to describe the type of plant resistance to pathogens quantitatively. Observations in the greenhouse showed that the AUDPC of shallots treated with combination of *B. velezensis* B-27 and *B. cereus* RC76 was significantly different from the inoculation and non-inoculation treatments (Table 6). The five shallot varieties did not show a significant difference between varieties in the AUDPC value. The smallest AUDPC was shown by Bauji with AUDPC of 248.88, which was not significantly different from *Tajuk*, *Bima Brebes*, *Crok Kuning* and *Manjung*. The AUDPC of *Bauji* with inoculation treatment showed that the disease development in *Bauji* was slow, and the AUDPC of *Bauji* after applied with the combination of *B. velezensis* B-27 and *B. cereus* RC76 also showed a lower number than some other varieties.

The treatment with a combination of *B. velezensis* B-27 and *B. cereus* RC76 had significant effect in AUDPC or disease progression on five shallot varieties. *Tajuk* variety treated with a combination of *B. velezensis* B-27 and *B. cereus* RC76 had the lowest AUDPC value, and the largest was shown by *Crok Kuning*. The results indicated that the development of twisted disease on *Tajuk* was the lowest compared to the other four shallot varieties. This was consistent with the twisted disease intensity on *Tajuk* treated with the combination of *B. velezensis* B-27 and *B. cereus* RC76 in the greenhouse, which had the smallest number. Based on AUDPC overall, *Bauji* and *Tajuk* showed the best resistance response among the five shallot varieties against twisted disease, and there was an increase in resistance response after the treatment with combination of *B. velezensis* B-27 and *B. cereus* RC76.

The greater the AUDPC value, the lower the level of plant resistance to disease. Disease progression, as calculated by AUDPC, is frequently used because the phenology and growth of the plant host also affect disease development. The resistance of plant hosts must be measured by quantitative parameters in the form of disease rates when the disease emerges in plants during the growing season, and pathogen infection is not sustainable but depends on the environment (Jeger & Viljanen-Rollinson, 2001).

The results of the field study revealed that the varieties treated with the combination of *B. velezensis* B-27 and *B. cereus* RC76 had a significant effect on the AUDPC value. AUDPC describes the progression of twisted disease based on the disease intensity from 14 to 42 DAP. The disease progression in the field showed variations among the five shallot varieties. The lowest AUDPC was found at *Tajuk* variety of 114.92, which was significantly different from cv. *Crok Kuning* (174.77) and Bauji, which had the highest AUDPC value (Figure 2). The AUDPC in the *Tajuk* variety in the field mirrored the results from the greenhouse, where the AUDPC of *Tajuk* was the lowest compared to the other varieties. The results indicated that the *Tajuk* variety had better resistance than *Bima Brebes*, *Bauji*, *Crok Kuning* and *Manjung* varieties because it exhibited a slower disease progression.

A comparison of the AUDPC values of shallot varieties in the field showed lower results than in the greenhouse because the disease intensity in the field was also lower than in the greenhouse. The slower disease development in the field could be attributed to environmental factors that were not conducive to the development of pathogens, the number and virulence levels of pathogens, and the resistance level of shallot

Table 6. AUDPC of twisted disease on five shallot varieties applied with the combination of *Bacillus velezensis* B-27 and *Bacillus cereus* RC76 in the greenhouse

Treatments	Varieties					Average*
	Tajuk	*Bima Brebes*	*Bauji*	*Crok Kuning*	*Manjung*	
Bacillus spp. + inoculation	217.75	435.54	248.90	451.13	233.38	317.4 b
Inoculation	622.25	497.82	482.18	482.28	575.56	532.02 a
Non-inoculation	0.00	62.25	15.56	15.56	46.69	28.01 c
Average^{ns}	280.00	331.87	248.88	316.32 a	285.21	(-)

The sign (-) indicates that there is no interaction between treatments and varieties. *= Values followed by different letters in the same treatment and column showed significant differences (DMRT; α= 0.05). ^{ns}= non significantly different (ANOVA; α= 0.05).

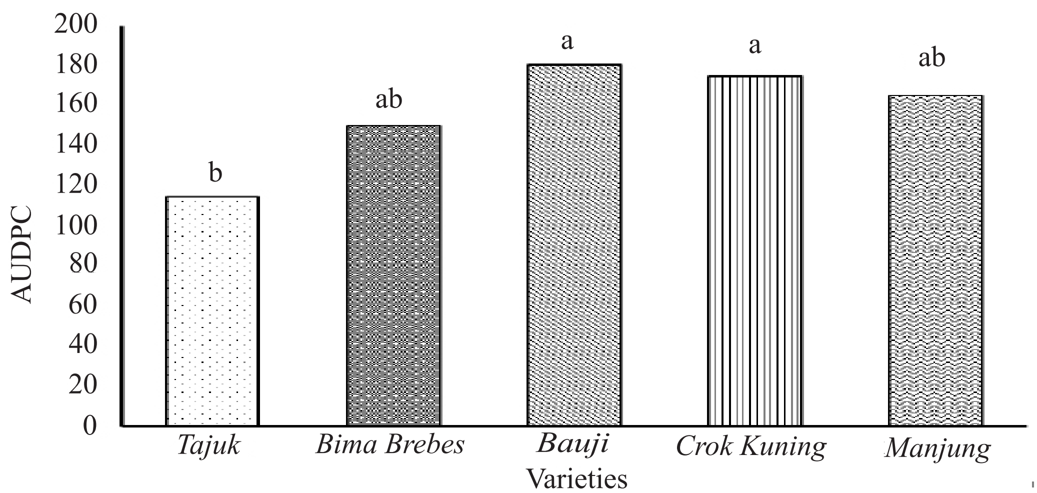

Figure 2. Area under the disease progress curve on five shallot varieties applied with a combination of *Bacillus velezensis* B-27 and *Bacillus cereus* RC76 in the field. *= Values followed by different letters in the same treatment and column showed significant differences (DMRT; α= 0.05).

varieties induced by the combination of B. velezensis B-27 and B. cereus RC76.

AUDPC serves as a standard for classifying varieties in terms of disease resistance. Jeger & Viljanen-Rollinson (2001) state that AUDPC is the basis for quantitatively determining the resistance of barley varieties to leaf rust disease. Various measurements are involved in the classification of plant resistance, including assessment of the growth phase, calculation of disease development, and the computation of the area under the disease progress curve (AUDPC).

Aprilia et al. (2020) analyzed 19 shallot genotypes with characteristics of resistance to twisted disease based on disease incidence, incubation period and disease infection rate. The dendrogram resulted in three groups of resistance categories. *Bima Brebes*, *Bauji*, and *Manjung* belonged to the susceptible group, which tended to have the highest disease incidence and the fastest disease incubation period. *Tajuk* variety was included in the moderately resistant group because it showed lower disease incidence than the susceptible group but higher than the resistant group and had a slower incubation period than the susceptible group.

Based on the results of field studies, Tajuk and Bima Brebes varieties showed an increase in resistance criteria to a resistant category, an increase in the category level compared to the research by Aprilia et al. (2020). The varieties *Bauji* and *Manjung* increased to moderately resistant criteria, while *Crok Kuning* was not mentioned in the literature and was included in the moderately resistant criteria. The differing results of the studies could be caused by variations in time, treatment, and environment used for research, resulting in different levels of resistance shown.

CONCLUSIONS

The application of the combination of *B. velezensis* B-27 and *B. cereus* RC76 had a significant effect on the incubation period, incidence, and intensity of twisted disease in five shallot varieties. The combined treatment of *B. velezensis* B-27 and *B. cereus* RC76 reduced the incidence of twisted disease by up to 70% and the disease intensity by up to 87%. The five shallot varieties showed different and increased resistance responses to twisted disease, with *Tajuk* and *Bima Brebes* classified as resistant, while *Bauji*, *Crok Kuning*, and *Manjung* were classified as moderately resistant. Overall, *Tajuk* variety treated with the combination of *B. velezensis* B-27 and *B. cereus* RC76 showed the best results in suppressing twisted disease.

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AUTHORS' CONTRIBUTIONS

AHP is the first author for the contribution to performing the research and writing this manuscript. AW as the corresponding author considered the experimental design, as a proofreader and revised this manuscript. TJ is providing the bacteria isolates and revised this manuscript. AW revised the methods and data analysis. SS led and supervised the project. The authors provided responses and comments on the research flow, data analysis, and interpretation as well as the shape of the manuscript. All the authors have read and approved the final manuscript.

COMPETING INTEREST

The authors declared that there is no potential conflict of interest.

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