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Efficacy of a Combined Application of *Trichoderma* sp. and *Bacillus velezensis* B-27 in Suppressing Moler Disease in True Shallot Seed in Glasshouse

Efektivitas Kombinasi *Trichoderma* sp. dan *Bacillus velezensis* B-27 dalam Menekan Penyakit Moler pada Bawang Merah Asal Biji di Rumah Kaca

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ABSTRACT

Moler disease caused by *Fusarium acutatum* represents a major constraint in shallot production. The use of biological control agents has emerged as an environmental friendly disease management. This study aimed to evaluate the effectiveness of a combined application of *Trichoderma* sp. and *Bacillus velezensis* B-27 in suppressing moler disease and to assess its effects on plant growth and yield of shallots. The study consisted of *in vitro* antagonistic assays and *in vivo* glasshouse experiments using two shallot varieties derived from botanical seeds or true shallot seed (TSS). The observed parameters included pathogen growth inhibition, disease intensity, number of bulbs, bulb diameter, fresh bulb weight, and dry bulb weight. The results demonstrated that the combined biological agents effectively inhibited pathogen growth and significantly reduced moler disease intensity. Although several yield-related parameters did not differ significantly among treatments, the application of biological agents showed considerable potential as an eco-friendly and sustainable approach for managing moler disease in shallot cultivation.

Keywords: antagonistic assay, biological control agents, botanical seeds, disease intensity, *Fusarium* sp.

ABSTRAK

Penyakit moler yang disebabkan oleh *Fusarium acutatum* menjadi kendala utama dalam budi daya bawang merah. Penggunaan agens hayati merupakan alternatif pengendalian yang ramah lingkungan. Penelitian ini bertujuan mengevaluasi keefektifan kombinasi *Trichoderma* sp. dan *Bacillus velezensis* B-27 dalam mengendalikan penyakit moler serta pengaruhnya terhadap pertumbuhan tanaman dan produksi tbawang merah. Penelitian dilakukan melalui uji antagonis *in vitro* dan percobaan *in vivo* di rumah kaca menggunakan dua varietas bawang merah asal biji botani (TSS). Peubah yang diamati meliputi daya hambat terhadap patogen, intensitas penyakit, jumlah umbi, diameter umbi, bobot segar umbi, bobot kering umbi, dan diameter umbi. Hasil penelitian menunjukkan bahwa kombinasi agens hayati mampu menghambat pertumbuhan patogen dan menekan intensitas penyakit moler. Meskipun peubah panen tidak menunjukkan perbedaan nyata, aplikasi agens hayati menunjukkan potensi sebagai alternatif pengendalian penyakit moler bawang merah yang ramah lingkungan dan berkelanjutan.

Kata kunci: agens hayati, biji botani, *Fusarium* sp., intensitas penyakit, uji antagonis

INTRODUCTION

The use of shallot bulbs as planting material poses a considerable risk of transmitting pests and pathogens (Prayudi *et al*. 2020), particularly *Fusarium* spp., which are frequently disseminated through infected bulbs (Sintayehu *et al.* 2014). This constraint has triggered increasing interest in alternative propagation systems and sustainable disease management strategies. Among commercial varieties, 'Lokananta' is well adapted to lowland areas during the dry season, with a yield potential of 17–20 ton ha^{-1} (East West Seed 2025), whereas 'Sanren' F1 exhibits a higher yield potential ranging from 23.23 to 28.14 ton ha^{-1} and is adaptable to elevations of 50–100 m above sea level (East West Seed 2017).

Previous studies have demonstrated the potential of biological control agents in suppressing moler disease in shallots. Pratiwi *et al*. (2024) reported that the application of *Bacillus velezensis* B-27, *B. cereus* RC76, and their combination on five shallot varieties propagated from bulbs extent the incubation period and significantly reduced both disease incidence and severity, particularly in the Tajuk variety. In addition, *B. velezensis* has been shown to enhance plant height, tiller number, and yield in shallot cultivation, highlighting its dual role in disease suppression and plant growth promotion (Rahma *et al*. 2020).

The use of true shallot seed (TSS) offers several agronomic advantages, including healthier seed quality, year-round seed availability, lower production costs, reduced seed requirements, and higher productivity compared with conventional bulb-based propagation (Sumarni *et al.* 2011; Rosliani *et al*. 2022). Further improvements in the productivity of TSS-based shallot production can be achieved through the application of combined biological control agents. Noticeably, the combination of *Trichoderma* sp. and *Bacillus* sp. has been reported to effectively reduce disease severity caused by *Fusarium oxysporum* f. sp. *cepae* (Foce) (Poromarto *et al*. 2022), indicating the potential synergistic effects of these microorganisms in sustainable shallot disease management. This study aimed to evaluate the effectiveness of *B. velezensis* B-27 and *T. asperelloides* in enhancing resistance to moler disease and their effects on growth and yield performance of shallot varieties Sanren and Lokananta.

This study refines previous work by integrating laboratory compatibility assays and glasshouse efficacy evaluation of the combined use of *B. velezensis* B-27 and *Trichoderma* sp. in TSS for moler disease suppression. The findings establish a scientific basis for the future development of integrated and sustainable plant health management strategies for shallot cultivation, particularly toward field-scale implementation.

MATERIALS AND METHODS

Compatibility Test and Antagonistic Activity Assay

The *in vitro* compatibility test of *B. velezensis* B-27 and *Trichoderma* sp. was evaluated using the dual culture method on potato dextrose agar (PDA) in petri dishes. The *in vitro* antagonistic activity of *B. velezensis* B-27 and *Trichoderma* sp. against *F. acutatum* was evaluated using the dual culture method on PDA in petri dishes. This assay was conducted to assess the inhibition of *F. acutatum* mycelial growth over a 7-day incubation period with three replicates experiment. The percentage of mycelial growth inhibition was calculated using the following formula:

$$I = \frac{(R_1 - R_2)}{R_2} \times 100\%, \text{ with}$$

I, percentage inhibition; R_1, colony diameter of *F. acutatum* growing away from *B. velezensis* B-27 or *Trichoderma* sp.; and R_2, colony diameter of *F. acutatum* growing toward *B. velezensis* B-27 or *Trichoderma* sp.

Glasshouse Experiment

The experiment was conducted in the glasshouse of the Faculty of Agriculture, Gadjah Mada University using a pot trial arranged in a completely randomized design (CRD). Each treatment consisted of three replicates, with one pot containing one plant considered as one experimental unit. The study involved two shallot varieties and six treatments per variety, resulting in the following treatment combinations (Table 1).

Table 1 Treatment combinations of *Bacillus velezensis* B-27 and *Trichoderma* sp. against *Fusarium acutatum* on Sanren and Lokananta varieties in the glasshouse

Code	Treatment combination
SAI	Sanren + *B. velezensis* B-27 + *Trichoderma* sp. + *F. acutatum*
SFI	Sanren + fungicide + *F. acutatum*
SKI	Sanren + inoculated control + *F. acutatum*
SA	Sanren + *B. velezensis* B-27 + *Trichoderma* sp. without *F. acutatum*
SF	Sanren + fungicide treatment without *F. acutatum*
SK	Sanren + uninoculated control without *F. acutatum*
LAI	Lokananta + *B. velezensis* B-27 + *Trichoderma* sp. + *F. acutatum*
LFI	Lokananta + fungicide + *F. acutatum*
LKI	Lokananta + inoculated control + *F. acutatum*
LA	Lokananta + *B. velezensis* B-27 + *Trichoderma* sp. without *F. acutatum*
LF	Lokananta + fungicide treatment without *F. acutatum*
LK	Lokananta + uninoculated control without *F. acutatum*

Propagation of *Bacillus velezensis* B-27, *Trichoderma* sp., and *Fusarium acutatum*

The *B. velezensis* B-27 isolate was cultured on yeast peptone agar (YPA) medium using the streak plate method and incubated at room temperature (approximately 25 °C) for 48 h (Abdullah *et al*. 2024). Bacterial suspensions were prepared at a concentration of 1×10^8 cfu mL^{-1}, corresponding to an optical density (OD_{600}) of 0.3, which was measured using a spectrophotometer (Genesys 10S UV–Vis, Thermo Scientific) at a wavelength of 600 nm (Rahma *et al*. 2020). The application of *B. velezensis* during the nursery stage was performed once a week using a suspension prepared at a ratio of 100 mL bacterial culture to 900 mL water (1:9). Following transplanting, *B. velezensis* was applied at two-week intervals. The bacterial suspension was sprayed at a dose of 100 mL culture mixed with 900 mL water per 100 plants, equivalent to approximately 10 mL per plant (Pratiwi 2023).

The pathogenic fungus *F. acutatum* was subcultured on PDA medium in petri dishes. The cultures were incubated at room temperature for 7 days until the mycelium fully colonized the petri dishes. The conidial suspension of *F. acutatum* was quantified using a hemocytometer and adjusted to a final concentration of 1×10^6 conidia mL^{-1}. Each plant was inoculated with 10 mL of the suspension (Pratiwi 2023). A commercially formulated powder product of *Trichoderma* sp. obtained from the Laboratorium Agens Hayati Jawa Tengah was used in this study. The product was applied once at planting by soil drenching at 0.23 g $plant^{-1}$.

DNA Extraction

Total DNA was extracted from root samples collected from the biological agent treatments at the bulb initiation and harvest maturity stages. Root DNA extraction was performed using a Genomic DNA Mini Kit (Plant) according to the manufacturer's instructions.

Bacterial and Fungal Confirmation

The presence of *B. velezensis* in plant samples was confirmed by PCR following the method of Abdullah *et al*. (2024) using the primers UGM Bv-F and UGM Bv-R targeting an amplicon of approximately 576 bp. PCR amplification was performed in a 10 µL reaction volume containing 5 µL ready mix, 1 µL DNA template, 1 µL each of forward and reverse primers, and 2 µL nuclease-free water. The thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 34 cycles of 95 °C for 1 min, 57 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

The confirmation of *Trichoderma* sp. was carried out using the TDP-F and TDP-R primers under the following PCR conditions: initial denaturation at 98 °C for 30 s, followed by 35 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR products were separated by electrophoresis on 1% agarose gel in TE buffer at 50 V for 50 min, and the amplified bands were visualized under a UV transilluminator (Lee *et al*. 2020).

Observation Variable

Disease intensity (DI) was assessed at 7 days after transplanting intervals following the method of Pratiwi (2023). Observations were conducted on sample plants from each treatment and replicate using the following formula:

$$DI = \frac{\sum (n_i \times v_i)}{N \times Z} \times 100\%, \text{ with}$$

n_i, represents the number of plants assigned to a given disease severity score; v_i, corresponding severity score; N, the highest disease score; and Z, total number of observed plants. Disease severity was scored on a 0–5 scale, where 0, no symptoms; 1, 1%–20% yellowing and twisting leaves; 2, 21%–40% yellowing, drying, and twisting; 3, 41%–60% severity; 4, 61%–80% severity accompanied by bulb rot; and 5, plant death.

Yield parameters, including bulb number, fresh bulb weight, dry bulb weight, and bulb diameter, were measured at harvest when plants reached 60–70 days after transplanting (East West Seed 2017).

Data Analysis

The observational data were analyzed using analysis of variance (ANOVA) at a 5% significance level. Significant differences among treatments were determined based on the ANOVA results. Treatment(s) with significant difference was determined using Duncan's Multiple Range Test (DMRT) at the 5% significance level.

RESULTS

Compatibility between Biological Agents

The compatibility assay between *T. asperelloides* and *B. velezensis* B-27 using a dual-culture method revealed a slight inhibition of *T. asperelloides* growth. However, microscopic observations confirmed that *B. velezensis* B-27 remained viable and grew normally (Figure 1a). Similarly, *T. asperelloides* exhibited normal growth without visible hyphal damage, indicating that both biological control agents were largely compatible.

Antagonistic Interaction between Biological Agents

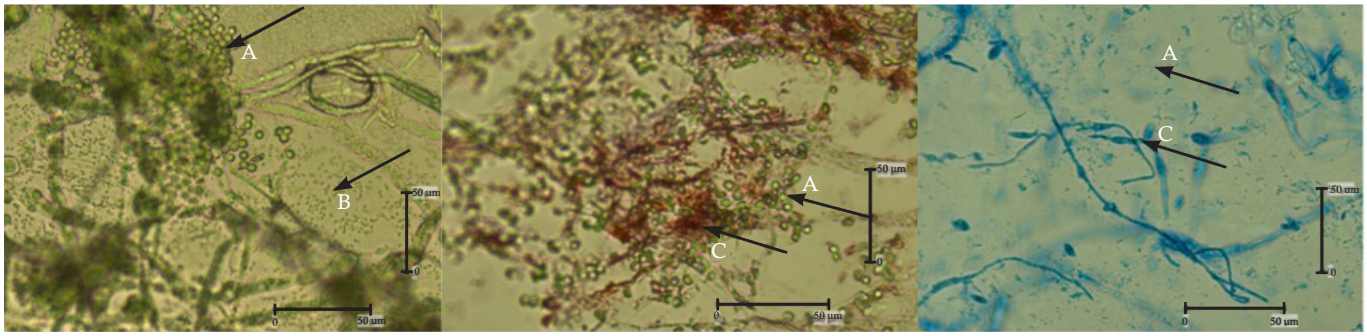
The antagonistic interaction between *T. asperelloides* and *F. acutatum* from day 1 to day 7 showed significantly suppressed mycelial growth of *F. acutatum*, with inhibition rates ranging from 58.78% to 65.87% (Figures 2b and 3). Microscopic examination revealed that the hyphae of *T. asperelloides* caused structural damage to *F. acutatum* hyphae, leading to hyphal rupture and lysis (Figure 1b). Similarly, *B. velezensis* B-27 exhibited antagonistic activity against *F. acutatum*, inhibiting pathogen growth by 30.55% to 43.33% (Figure 3). Microscopic observations showed clear signs of hyphal lysis of *F. acutatum* in the presence of bacterial cells, indicating bacterial-mediated degradation of fungal hyphae (Figure 1c).

Moler Disease Intensity

Moler disease intensity in the two shallot varieties remained relatively low throughout the observation period. The highest disease intensity was recorded in the inoculated control treatment, reaching up to 24% at 49 days after transplanting (DAT). In contrast, treatments involving biological control agents and fungicide applications consistently exhibited lower disease intensity compared with the control (Figures 4 and 5).

The Effect of Biological Agents on Crop Yield

Yield-related parameters included the number of bulbs, bulb diameter, fresh bulb weight, and dry bulb weight. Based on ANOVA results, these parameters



a b c

Figure 1 Microscopic observations of microbial interactions: a, *B. velezensis* B-27 with *T. Asperelloides;* b, *T. asperelloides* with *F. acutatum;* and c, *B. velezensis* B-27 with *F. acutatum*; A, conidia *T. asperelloides;* B, *B. velezensis* B-27; and C, hyphae *F. acutatum.*

a b c d e

Figure 2 Microbial growth in *in vitro* assay a, *B. velezensis* B-27 with *T. asperelloides*; b, *T. asperelloides* with *F. acutatum*; c, *B. velezensis* B-27 with *F. acutatum*; d, *F. acutatum* control; and e, *T. asperelloides* control.

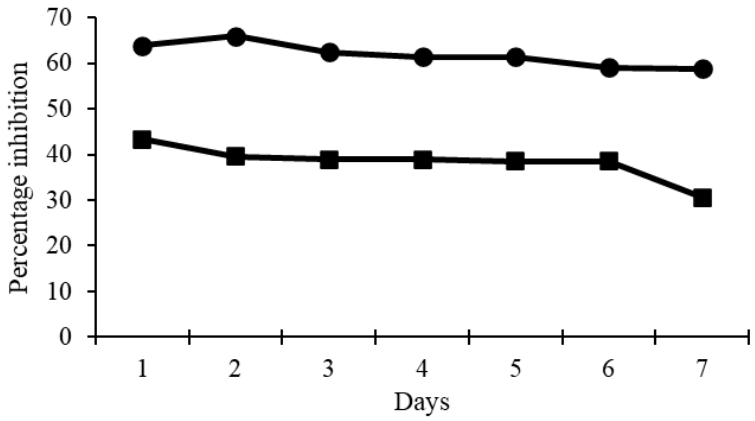


Figure 3 Percentage inhibition of pathogen growth recorded from day 1 to day 7 of observation. ▬●▬, *B. velezensis* B-27 vs *F. acutatum*; and ●▬●, *Trichoderma* sp. vs *F. acutatum.*

did not show significant differences among treatments. Similar trends were observed in both shallot varieties (Tables 2 and 3).

Detection of Biological Control Agents

PCR-based detection confirmed the presence of the applied biological control agents, *B. velezensis* B-27 and *T. asperelloides*, in shallot plant tissues at 28 and 49 days after transplanting. Agarose gel electrophoresis (Figures 6 and 7) revealed distinct DNA bands of approximately 560 bp for *B. velezensis* and approximately 500 bp for *T. asperelloides*, indicating successful colonization of both biological agents within plant tissues.

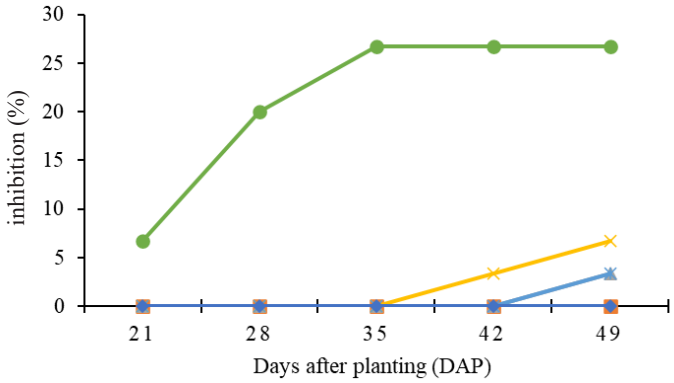


Figure 4 Moler disease severity of the Sanren variety under inoculated treatments at 21–49 days after planting. ◆, SF 'Sanren' fungicide without inoculation; ■, SA 'Sanren' treated with biological agents without inoculation; ▲, SK 'Sanren' control; ×, SFI 'Sanren' fungicide no pathogen inoculation; ✳, SAI 'Sanren' treated with biological agents and pathogen inoculation; and ●, SKI 'Sanren' control and pathogen inoculation.

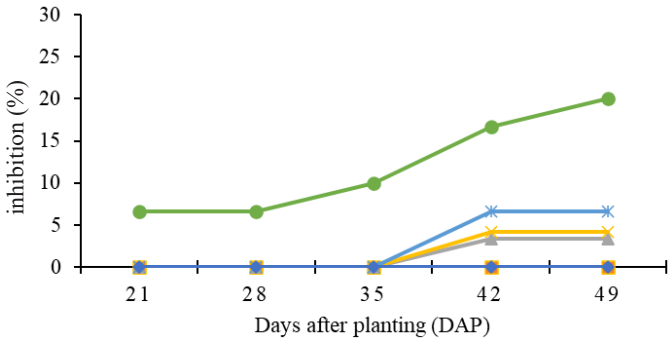


Figure 5 Moler disease severity of the Lokananta variety under inoculated treatments at 21–49 days after planting. ◆, LF 'Lokananta' fungicide without inoculation; ■, LA 'Lokananta' treated with biological agents without inoculation; ▲, LK 'Lokananta' control; ×, LFI 'Lokananta' fungicide and pathogen inoculation; ✳, LAI 'Lokananta' treated with biological agents and pathogen inoculation; and ●, LKI 'Lokananta' control and pathogen inoculation.

Table 2 Mean values of bulb number, bulb diameter, fresh bulb weight, and dry bulb weight of the Sanren variety under different disease management treatments in the glasshouse

Treatment[a]	Bulb number	Fresh bulb weight (g)	Dry bulb weight (g)	Bulb diameter (mm)
SF	3.40 ± 0.69 a*	17.71 ± 4.01 a	8.81 ± 3.10 a	19.55 ± 0.91 a
SA	2.67 ± 0.15 a	10.69 ± 1.82 a	7.33 ± 2.28 a	16.39 ± 1.60 a
SK	2.67 ± 0.24 a	13.24 ± 2.37 a	6.64 ± 1.60 a	17.36 ± 1.46 a
SFI	3.60 ± 0.34 a	15.78 ± 4.69 a	10.36 ± 3.79 a	16.38 ± 2.01 a
SAI	3.13 ± 0.20 a	17.47 ± 3.02 a	13.07 ± 2.55 a	19.79 ± 1.57 a
SKI	2.33 ± 0.40 a	10.46 ± 3.31 a	5.97 ± 2.33 a	14.93 ± 2.39 a

Means followed by the same letter are not significantly different according to DMRT at P ≤ 0.05. [a]LF, 'Lokananta' fungicide without inoculation; LA, 'Lokananta' treated with biological agents without inoculation; LK, 'Lokananta' control; LAI, 'Lokananta' treated with biological agents and pathogen inoculatio; SA, 'Sanren' treated with biological agents without inoculation; and SAI, 'Sanren' treated with biological agents and pathogen inoculation.

Table 3 Mean values of bulb number, bulb diameter, fresh bulb weight, and dry bulb weight of the Lokananta variety under different disease management treatments in the glasshouse

Treatment[a]	Bulb number	Fresh bulb weight (g)	Dry bulb weight (g)	Bulb diameter (mm)
LF	2.87 ± 0.49 a*	15.19 ± 4.61 a	8.09 ± 4.23 a	19.16 ± 2.05 a
LA	3.33 ± 0.45 a	14.47 ± 4.55 a	5.44 ± 2.09 a	15.57 ± 2.13 a
LK	2.67 ± 0.35 a	12.47 ± 3.06 a	3.11 ± 2.11 a	17.48 ± 2.92 a
LFI	2.80 ± 0.44 a	18.49 ± 5.27 a	9.89 ± 4.03 a	20.39 ± 2.77 a
LAI	2.87 ± 0,31 a	12.88 ± 3.48 a	8.45 ± 2.78 a	17.69 ± 1.74 a
LKI	2.80 ± 0,31 a	11.30 ± 3.26 a	7.73 ± 3.49 a	15.98 ± 2.31 a

*Means followed by the same letter are not significantly different according to DMRT at P ≤ 0.05. [a]LF, 'Lokananta' fungicide without inoculation; LA, 'Lokananta' treated with biological agents without inoculation; LK, 'Lokananta' control; LAI, 'Lokananta' treated with biological agents and pathogen inoculation; SA, 'Sanren' treated with biological agents without inoculation; and SAI, 'Sanren' treated with biological agents and pathogen inoculation.

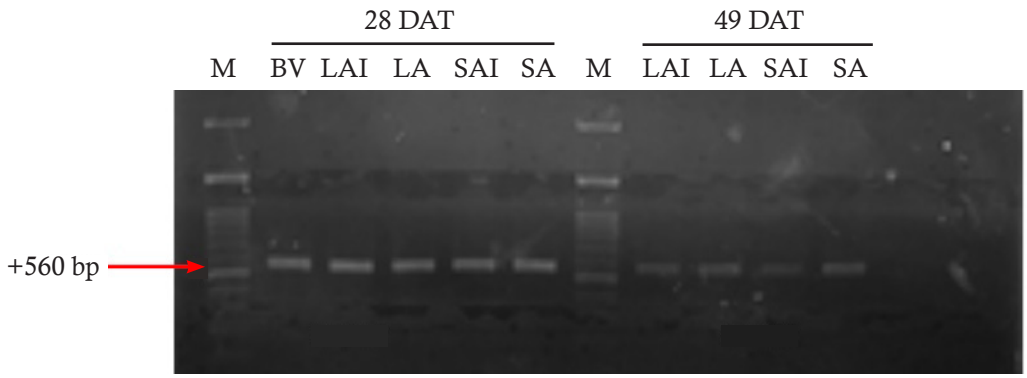


Figure 6 PCR amplification of *B. velezensis* B-27 using Bv-F and Bv-R primers at 28 and 49 days after planting (DAT). M, DNA marker; BV, *Bacillus velezensis* B-27 DNA; LAI, 'Lokananta' treated with biological agents and pathogen inoculation; LA, 'Lokananta' treated with biological agents without inoculation; SAI, 'Sanren' treated with biological agents and pathogen inoculation; and SA, 'Sanren' treated with biological agents without inoculation.

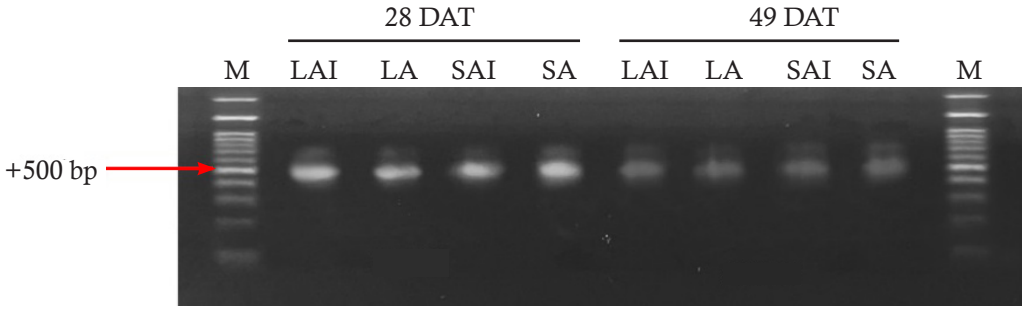


Figure 7 PCR amplification of *T. asperelloides* using TDP-F and TDP-R primers at 28 and 49 days after planting (DAT). M, DNA marker; LAI, 'Lokananta' treated with biological agents and pathogen inoculation; LA, 'Lokananta' treated with biological agents without inoculation; SA, 'Sanren' treated with biological agents without inoculation; and SAI, 'Sanren' treated with biological agents and pathogen inoculation.

DISCUSSION

The compatibility assay between *B. velezensis* B-27 and *T. asperelloides* demonstrated that the hyphae of *T. asperelloides* remained intact, exhibited normal branching patterns, and showed no signs of lysis or morphological abnormalities during interaction with *B. velezensis* B-27. The presence of bacterial cells surrounding the fungal hyphae did not induce damage to the fungal cell wall, indicating a compatible interaction between the two microorganisms. Similar observations were reported by Izquierdo-García *et al.* (2020), who showed that microscopic examination on solid media revealed successful germination of GI006 conidia and directional hyphal growth toward the interaction zone with Bs006. In their study, Bs006 cells were observed adhering to GI006 hyphae in a biofilm-like structure without causing any structural damage to the fungus.

Microscopic observations revealed that *T. asperelloides* caused significant damage to the hyphae of *F. acutatum*, leading to hyphal rupture and collapse (Figure 1b). Similar morphological alterations were reported in *Colletotrichum* spp. following antagonistic interaction with *T. harzianum*, where hyphae exhibited swelling and lysis due to antibiosis mediated by secondary metabolites such as trichodermin, trichodermol, and trichotoxins (Ainy *et al*. 2015).

Furthermore, microscopic examination of *F. acutatum* exposed to *B. velezensis* B-27 showed pronounced hyphal deformation, characterized by rupture, irregular morphology, distortion, and hyphal shortening. Application of *B. velezensis* CE 100 induced hyphal deformation in several *Colletotrichum* species. These effects may be associated with the production of antifungal compounds, as previously reported (Kim *et al*. 2022).

Bacillus velezensis B-27 exhibited moderate antagonistic activity against *F. acutatum*. These findings are consistent with previous studies reporting that *B. velezensis* effectively suppressed *C. gloeosporioides*, the causal agent of anthracnose in shallot, with inhibition rates of 17.8%–62.8% in dual culture assays (Abdullah *et al*. 2024). Moreover, *B. velezensis* has been shown to inhibit *Rhizoctonia solani*, the causal agent of rice sheath blight, with suppression levels reaching 50%–68.9% further confirming its broad-spectrum antifungal potential (Nubatonis 2025).

In contrast, *Trichoderma* sp. consistently exhibited higher and more stable antagonistic activity against *F. acutatum* across all observation periods. The antagonistic activity of *Trichoderma* sp. against the

pathogenic fungus *F. oxysporum* resulted in inhibition percentages ranging from 25% to 58.33% (Ihkwanisa *et al.* 2023).

Disease management using biological control agents and fungicide treatments resulted in significantly lower disease intensity compared to the untreated control. *Bacillus* B-27 isolate is closely related to *B. velezensis* and is capable of inducing systemic resistance (ISR), resulting in up to a 67% reduction of moler disease and purple blotch in shallot (Rahma *et al.* 2020). *Trichoderma* sp. are able to invade, adhere to, and coil around pathogen hyphae, leading to extensive hyphal degradation and eventual pathogen death (Yao *et al.* 2023). In addition, *T. harzianum* produces a range of antibiotic compounds, including alamectin, paracelsin, glioviridin, and trichotoxin, which disrupt the cell membranes of pathogenic fungi (Vey *et al.* 2001).

The number of bulbs, fresh bulb weight, dry bulb weight, and bulb diameter across all treatments in both Sanren and Lokananta varieties did not differ significantly. Suboptimal environmental conditions, particularly during the dry season, are presumed to have limited the effectiveness of biological control agents as plant growth–promoting organisms. These findings are consistent with Hafri *et al.* (2020), who reported no significant differences in fresh bulb weight, dry bulb weight, bulb diameter, or bulb number among treatments involving five PGPR isolates, *Trichoderma* sp., and untreated controls. Similarly, Pratiwi *et al.* (2024) demonstrated that the combined application of *B. velezensis* B-27 and *B. cereus* RC76 did not significantly affect fresh plant weight, dry plant weight, or dry bulb weight of several shallot varieties, such as 'Tajuk', 'Bima Brebes', 'Bauji', 'Crok Kuning', and 'Manjung'.

Both biological control agents were successfully detected in shallot root tissues at 28 and 49 days after planting. *Trichoderma* sp. are known to colonize root tissues and act synergistically with *Bacillus* sp. to induce plant defense responses against pathogens (Hafiz *et al*. 2022). However, the presence and persistence of microorganisms are also influenced by plant developmental stage, nutrient availability, and environmental conditions.

The combined application of *T. asperelloides* and *B. velezensis* B-27 was compatible and effectively reduced moler disease intensity in TSS-derived shallot varieties Sanren and Lokananta. However, the treatment did not significantly improve yield-related parameters under glasshouse conditions. These findings indicate that the combined biological agents are more promising as a disease-suppression strategy than as a yield-enhancement treatment. Further field experiment to validate this result is required under diverse agroecological conditions and natural disease pressure.

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