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Biological Control of Gummy Stem Blight Caused by *Didymella bryoniae* (Auersw.) on Watermelon by *Bacillus* sp. Strains

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ABSTRACT

Background: The gummy stem blight caused by the fungal pathogen *Didymella bryoniae* (Auersw.) is a common and serious disease of watermelon plants in Vietnam. *Bacillus* species have been used to control plant diseases as biological control agents. The objectives of this studies were to determine the fungal inhibition of *D. bryoniae* and disease suppression of gummy stem blight under greenhouse by *Bacillus* sp. strains.

Methods: The study was carried out at University of Agriculture and Forestry, Hue University during 2022-2023. Six potential *Bacillus* strains namely S1A1, S1F3, S13E2, S13E3, S18F11 and S20D12 isolated from stem-base of groundnut in central Vietnam were tested its fungal inhibition of *D. bryoniae* and disease suppression of gummy stem blight.

Result: All tested strains of *Bacillus* sp. showed inhibition of the hyphal growth of *D. bryoniae* strain DB-01 in potato dextrose broth. Among the strains, *Bacillus* sp. S20F12 had highest inhibition of the growth of mycelia of *D. bryoniae* strain DB-01 with an antagonistic efficiency of 65.7% at 10 days after fungal inoculation. By 21 days after fungal inoculation, disease incidences were low in the plants treated with *Bacillus* sp. S20D12 and S1F3 by 46.6 and 50.0% and disease severity by 15,3% and 20.6%, respectively. As results, watermelon plants were prevented from damage of gummy stem blight caused by *D. bryoniae* strain BD-01 by *Bacillus* sp. S20D12 and S1F13. The results would contribute to the knowledge of antagonistic activities of the *Bacillus* to optimize the biological control against *D. bryoniae*.

Key words: *Bacillus*, Biological control, *Didymella bryoniae,* Gummy stem blight, Watermelon.

INTRODUCTION

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai] is a common freshly fruit plant in the world (Gusmini *et al.*, 2017; Rahman *et al.*, 2019). The total area of cultivated watermelon in Vietnam was 63,938 hectares with a quantity of 1,534,613 tons in 2021 that was ranking the 10 largest watermelon producers in the world (FAOSTAT, 2023). Watermelon plants in Vietnam were affected by numerous insect pests and disease caused reduction in fruit yield and quality. Among the disease, gummy stem blight caused by the fungal pathogen *Didymella bryoniae* (Auersw.) is a major and serious disease (Ngo *et al.*, 2023a). The symptom of gummy stem blight on watermelon plants is evident as crown blight, stem necrosis, gummy exudates, defoliation, wilt and eventual death (Basim *et al.*, 2016). The colony surface of the fungal pathogen is rough and undulated, the conidia were round-ended, cylindrical, monoseptate and hyaline (Li *et al.*, 2014). Measures to manage disease caused by *D. bryoniae* were recommended to use resistant cultivars, apply chemicals, crop rotation and antagonistic bacteria (Santos *et al.*, 2016; Gusmini *et al.*, 2017; Dalcin *et al.*, 2017; Rahman *et al.*, 2019; Le *et al.*, 2019; Ngo *et al.*, 2023b).

In this regard, *Bacillus* species have been used to control plant diseases as biological control agents (Gupta *et al.*, 2016; Jangir *et al.*, 2018; Le *et al.*, 2019; Sunkad *et al.*, 2023). *Bacillus* is known to produce a wide range of biocompounds such as bacteriocins and lipopeptides that inhibit fungal growth and limit the diseases (Pradhan *et al.*, 2018; Rodríguez *et al.*, 2018; Le *et al.*, 2018, 2019). Keinath (2016) reported that *Bacillus subtilis* QST713 was protective effect against gummy stem blight on muskmelon seedlings. However, native *Bacillus* more consistently control groundnut stem rot, *Scleoritum rolfsii* Sacc. and the native bacteria may be better adaptive in the natural conditions and more consistent in the control of diseases (Le *et al.*, 2018).

Previous study showed that *Bacillus* sp. strains isolated from stem-base of groundnut plants had inhibition of the mycelia growth of *D. bryoniae* strain DB-03 causing gummy stem blight disease in watermelon in Phu Yen province, South Central Vietnam (Ngo *et al.*, 2023b). While *D. bryoniae* was high genetic diversity in Vietnam (Ngo *et al.*, 2023a), the objectives of this studies were to determine the fungal inhibition of *D. bryoniae* strain DB-01 and disease suppression of gummy stem blight under greenhouse in

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Thua Thien Hue province, North Central Vietnam by *Bacillus* sp. strains which was isolated from stem-base of groundnut in Vietnam. The results would contribute to the knowledge of antagonistic activities of the *Bacillus* to optimize biological control against *D. bryoniae*.

MATERIALS AND METHODS

Six potential *Bacillus* sp. strains namely S1A1, S1F3, S13E2, S13E3, S18F11 and S20D12 which was isolated from stem-base of groundnut in Quang Nam province (15°45′25″ N, 108°25′18″ E) and Thua Thien Hue province (16°26′41″ N, 107°16′48″ E), central Vietnam (Le *et al.*, 2018) were maintained in glycerol stock at -80°C at the Department of Plant Protection, University of Agriculture and Forestry, Hue University, Vietnam. Sclerotia of *D. bryoniae* strain DB-01 collecting from watermelon fields in Thua Thien Hue province, North Central Vietnam on agar plate was maintained in room condition. *Bacillus* strains were freshly cultured on King's B agar while *D. bryoniae* were freshly cultured on potato dextrose agar (PDA; Difco, France).

Inhibition of hyphal growth of *D. bryoniae* strain DB-01 by *Bacillus* sp. strains was investigated in dual culture assays according to the method of Kruijt *et al.* (2009). Brieftly, *Bacillus* sp. strains were spot-inoculated at the edge of a 1/5th- strength potato dextrose agar plate (1/5th PDA, pH 6.5). After incubation for 48 h at 25°C, agar plugs of a 3-day-old culture of *D. bryoniae* strain DB-01 were placed in the centre of the 1/5th PDA plate and incubated at 25°C. After incubation for 6 days and 10 days at 25°C, hyphal growth was measured (in mm) on *D. bryoniae* toward the bacterial colony and the control (no bacterial colony). Hyphal growth inhibition (HGI) was calculated relative to the control with the formula:

$$\text{HGI (\%)} = \frac{\text{Radial hyphal growth in control} - \text{Radial hyphal growth bacterial colony}}{\text{Radial hyphal growth in control}} \times 100$$

Bacillus sp. strains were tested for biological control of the gummy stem blight, *D. bryoniae* strain DB-01 under greenhouse conditions at the Department of Plant Protection, Faculty of Agronomy, University of Agriculture and Forestry, Hue University, Vietnam during 2022-2023. The pre-germinated seeds were subsequently soaked for 30 min in each bacterial suspension of 10^8 cells/mL. For the control, pre-germinated seeds were soaked in sterile water for 30 min. Treated seeds were individually sown in a plastic pot containing 250 g of clay loam soil collected from a watermelon fields. The experiments were arranged as randomized complete block design (RCBD) with three replications and 10 pre-germinated seeds per replication. Two weeks after sowing, a 0.25 cm^2 growing fungal mycelium plug was placed at base of the watermelon stems and covered with soil. The disease incidence, disease severity and mortality rate of the treated plants were recorded at 7, 14 and 21 days after fungal inoculation. The plants were rated on a scale from 0-4 with 0: no disease symptoms, 1: disease symptoms without visible outgrowth of the fungus, 2: disease symptoms with visible outgrowth of the fungus, 3: partial wilting of the plant and 4: complete wilting and plant death (Le *et al.*, 2012). Disease severity was calculated based on the formula:

$$\text{Disease severity} = \frac{[(1 \times \text{No. of pl. rated as scale 1}) + (2 \times \text{No. of pl. rated as scale 3}) + (4 \times \text{No. of pl. rated as scale 4})]}{4 \times \text{Total number of plants}} \times 100$$

Statistical analysis

Disease incidence, disease severity and mortality rates were transformed into arcsine prior to statistical analysis. Statistical differences ($P<0.05$) between treatments were analysed by ANOVA followed by the Duncan multiple range test using statistical software SPSS Statistics, Chicago, IL, USA.

RESULTS AND DISCUSSION

The antagonistic ability of some strains of *Bacillus* sp. was shown by the ability to control the growth of *D. bryoniae* DB-01 mycelium on 1/5 PDA. *Bacillus* sp. showed good inhibitory effect on hyphal growth of *D. bryoniae* strain DB-01. The antifungal efficiency against *D. bryoniae* strain DB-01 of tested *Bacillus* strains was from 18.5 to 28.3% at 6 days after fungal inoculation. By ten days after fungal inoculation, the ability of the tested bacterial strains to inhibit the growth of *D. bryoniae* hyphae was more obvious. The antagonistic performance of *Bacillus* sp. S1F3, S18F11 and S20D12 reached values of more than 50%. *Bacillus* sp. S20F12 was the strongest inhibition of the growth of mycelia of *D. bryoniae* DB-01 with an antagonistic efficiency of 65.7% (Table 1).

Bacillus species are widely used to promote plant growth and control plant diseases (Keinath, 2016; Jangir, 2018; Ngangom *et al.*, 2019; Miljaković *et al.*, 2022). The *Bacillus* genus is known for producing many endogenous biological active ingredients such as bacteriocins and lipopeptides that inhibit pathogens and prevent some harmful

Table 1: Fungal inhibition of *Didymella bryoniae* DB-01 by *Bacillus* sp. at 6 days and 10 days after fugal inoculation on 1/5 strength potato dextose agar (%).

Bacillus strain	Days after fugal inoculation	
	6	10
Bacillus sp. S1A1	18.9±0.18d	45.4±0.73d
Bacillus sp. S1F3	28.7±0.11a	56.6±0.35b
Bacillus sp. S13E2	28.8±0.11a	49.1±0.35c
Bacillus sp. S13E3	22.8±0.52c	47.2±0.55cd
Bacillus sp. S18F11	29.4±0.25a	54.8±0.55b
Bacillus sp. S20D12	27.2±0.10b	65.7±1.07a

Means with the same letters within the same column are not significantly different by one-way ANOVA, $P<0.05$.

diseases on plants (Pradhan *et al*., 2018; Rodriguez *et al*., 2018). Previous studies indicated that the fungal inhibition of *Bacillus* species is influenced by its production of antifungal antibiotics such as bacilysisn, inturin, fengycin, bacillomycin, surfactin, ericin, mersacidin, subtilosin, subtilin and mycosublilin (Mora *et al*., 2011; Rodríguez *et al*., 2018). Based on this mechanism, the ability of antagonistic bacteria to control fungi (or % antagonistic efficiency) is calculated based on the ability to inhibit the growth of fungal mycelium (Le *et al*., 2019). When testing the antagonistic effectiveness of bacteria in *in vitro* conditions, the results showed that all tested *Bacillus* strains demonstrated the ability to inhibit the growth of *D. bryoniae* fungal mycelium on 1/5 PDA. Recently, Ngo *et al.* (2023b) also reported that *Bacillus* sp. S18F11 and S20D12 had inhibitory effect on the hyphal growth of *D. bryoniae* strain DB-03 on 1/5 PDA, the antagonistic performance of *Bacillus* sp. S1F3, S18F11 and S20D12 reached values of more than 50% and *Bacillus* sp. S20F12 had highest inhibition of the growth of mycelia of *D. bryoniae* strain DB-03 with an antagonistic efficiency of 60.7%. Therefore, *Bacillus* sp. S20D12 and S1F3 were high efficacy in antagonistic performance against *D. bryoniae* under *in vitro.*

A bacterial strain capable of limiting pathogens in *in vitro* conditions is a prerequisite for limiting diseases in greenhouse conditions. However, the ability to control the development and growth of fungal mycelium under *in vitro* conditions still does not have enough data to ensure the ability to limit diseases in greenhouses because it still depends on the ability to survive and produce toxic substances, antifungal agents in greenhouse conditions.The results of testing for prevention of *Bacillus* sp. strains to gummy stem blight of watermelon plants inoculated with *D. bryoniae* strain DB-01 under greenhouse conditions were shown in Table 2 and Table 3. Table 2 shown a significant difference in the disease incidence of the plants that were inoculated with *D. bryoniae* strain DB-01 in the treatments treated with all *Bacillus* sp. strains compared to the control. The plants of seeds soaked with bacterial suspension of *Bacillus* strains had a lower disease incidence than that of the control. At 7 days after fungal inoculation, the plants treated with *Bacillus* sp. S18F11 and S1F3 had the lowest disease incidences by 20.0 and 23.3%, respectively. The plants with low disease incidence changed at the following observation times. At 21 days after fungal inoculation, disease incidences were low in the plants treated with *Bacillus* sp. S20D12 and S1F3 by 46.6 and 50.0%, respectively. Table 3 also indicated that all tested *Bacillus*sp. strains were significantly preventing watermelon from disease infection at 7, 14 and 21 days after fungal inoculation. The plants treated with *Bacillus* sp. S20D12 and S1F3 were also low disease severity by 15.3 and 20.6%, respectively, at 21 days after fungal inoculation (Table 3).

The results also indicated that the disease incidence of plants treated with *Bacillus* sp. S1F3 and S20D12 at 14 and 21 days after fungal inoculation had no change (46.6% and

Table 2: Effect of *Bacillus* sp. treating on disease incidence (%) of watermelon inoculated with *Didymella bryoniae* DB-01 under greenhouse condition.

Bacillus strain	Days after fugal inoculation		
	7	14	21
Control	46.6±3.34a	73.3±3.34a	100±0.00a
Bacillus sp. S1A1	36.6±6.67ab	60.0±0.00b	70.0±0.00b
Bacillus sp. S1F3	23.3±3.33cd	50.0±0.00cd	50.0±0.00d
Bacillus sp. S13E2	30.0±0.00bcd	56.6±3.34bc	63.3±3.34c
Bacillus sp. S13E3	30.0±0.00bcd	53.3±3.34bcd	60.0±0.00c
Bacillus sp. S18F11	20.0±0.00d	53.3±3.33bcd	60.0±0.00c
Bacillus sp. S20D12	33.3±3.34bc	46.6±3.33d	46.6±3.34d

Means with the same letters within the same column are not significantly different by one-way ANOVA, $P<0.05$.

Table 3: Effect of *Bacillus* sp. treating on disease severity (%) of watermelon inoculated with *Didymella bryoniae* DB-01 under greenhouse condition.

Bacillus strain	Days after fugal inoculation		
	7	14	21
Control	9.3±0.66a	40.0±2.30a	80.0±0.00a
Bacillus sp. S1A1	7.3±1.34ab	30.0±0.00b	49.3±0.67b
Bacillus sp. S1F3	4.6±0.67cd	17.3±0.67d	20.6±1.34d
Bacillus sp. S13E2	6.0±0.00bcd	21.3±1.34c	32.6±1.76c
Bacillus sp. S13E3	6.0±0.00bcd	18.0±0.00d	27.3±1.34c
Bacillus sp. S18F11	4.0±0.00d	14.6±0.67de	25.3±0.67c
Bacillus sp. S20D12	6.6±0.67bc	12.6±0.67e	15.3±0.67d

Means with the same letters within the same column are not significantly different by one-way ANOVA, $P<0.05$.

Table 4: Effect of *Bacillus* sp. treating on mortality rate (%) of watermelon inoculated with *Didymella bryoniae* DB-01 under greenhouse condition.

Bacillus strain	Days after fugal inoculation		
	7	14	21
Control	0.0	13.3±3.34a	100±0.00a
Bacillus sp. S1A1	0.0	10.0±0.00a	30.0±0.00b
Bacillus sp. S1F3	0.0	0.0±0.00b	6.6±3.34d
Bacillus sp. S13E2	0.0	0.0±0.00b	16.6±3.34c
Bacillus sp. S13E3	0.0	0.0±0.00b	10.0±0.00d
Bacillus sp. S18F11	0.0	0.0±0.00b	10.0±3.34d
Bacillus sp. S20D12	0.0	0.0±0.00b	0.0±0.00e

Means with the same letters within the same column are not significantly different by one-way ANOVA, $P<0.05$.

50.0%) (Table 2), but the disease severity had differences after 14 days after fungal inoculation (17.3% and 12.6%) and 21 days after fungal inoculation (15.4% and 13.3%) (Table 3). This demonstrates that the plants treated with *Bacillus* sp. S1F3 and S20D12 were prevented from the disease infection over time. The ability to control of the disease damage by *Bacillus* sp. S20D12 and S1F3 has a slower progression but was more effective than other *Bacillus* sp. strains in this study.

After 7 days of fungal inoculation, the plants only shown symptoms of gummy stem blight, which were small, brown spots on the leaves or, more seriously, large-scale leaf lesions or long-lasting cracks on the stems. Then, some plants shown more severe disease, causing the plants to wilt or part of the leaves to wither completely. However, no dead watermelon plant had been recorded yet. After 14 days of fungal inoculation, the control and *Bacillus* sp. S1A1 treatment recorded a number of severely infected plants, parts gradually withered and completely withered, leading to death of plants by 13.3 and 10.0%, respectively. By 21 days after fungal inoculation, severely infected plants increased, causing plant death. Treatment with *Bacillus* sp. S20D12 had no dead plants (0.0%) and with *Bacillus* sp. S1F3 the plant mortality rate was only 6.6%. In the remaining treatments, the plant mortality rate ranged from 10.0 to 30.0%, while all plants in the control was dead (100%) (Table 4). The results indicated that watermelon plants were prevented from damage of gummy stem blight caused by *D. bryoniae* strain BD-01 by *Bacillus* sp. S20D12 and S1F13.

The results of research on the infection of *D. bryoniae* strain DB - 03 was also evaluated similarly to the infection and damage of *D. bryoniae* strain DB-01 (Ngo *et al*., 2023a). Best effectiveness in control of *D. bryoniae* strain DB-03 that caused the damage to plants was *Bacillus* sp. S1F3, S20D12 and S18F11 (Ngo *et al*., 2023b). However, the disease incidences, disease severities and plant mortality rates reached higher values compared to the treatments using *D. bryoniae* DB-01 as the pathogen. This can be understood because the infection rate and pathogenic virulence of *D. bryoniae* strain DB-03 were faster and stronger than strain *D. bryoniae* DB-01 as previously surveyed (Ngo *et al*., 2023b).

Many *Bacillus* species are well known for their antagonistic activities against plant pathogens such as fungi (Ongena and Jacques, 2008; Raaijmakers *et al*., 2010; Le *et al*., 2019). The lipopeptide antibiotic bacillopeptin B1 produced by *Bacillus amyloliquefaciens* SH-B74 could inhibit several fungal pathogens *in vitro* (Ma *et al*., 2014). Song *et al.* (2013) also reported that lipopeptides produced by *B. amyloliquefaciens* anti-CA (*Candida albicans*) limit fungal growth of *C. albicans*. *Bacillus amyloliquefaciens* isolated from groundnut rhizosphere enhances the activities of defense enzymes through salicylic acid induced systemic resistance and several enzymes such as chitinase, peroxidase, catalase and polyphenol oxidase have strong negative correlation with disease severity index (Rajyaguru *et al*., 2017). Two antagonistic bacterial strains of *Bacillus* sp. S13F1 and S20D12 were originating from the stem-base of the peanut plant. At the species level, strains S1F3 and S20D12 belong to the same clade as the reference strains *B. amyloliquefaciens* (Le *et al*., 2018). Based on the phylogenetic tree of *Bacillus* strains, *Bacillus* sp. S20D12 and S1F3 has a close relationship with *B. amyloliquefaciens* (Le *et al*., 2018), which can partly explain the similarity in the ability to control the growth of *D. bryoniae* fungus in *in vitro* and greenhouse conditions conducted in this study.

CONCLUSION

It is concluded that *Bacillus* sp. tested strains were inhibition of the hyphal growth of *D. bryoniae* strain DB-01 in potato dextrose medium. Among the tested strains, *Bacillus* sp. S20F12 had highest inhibition of the growth of mycelia of *D. bryoniae* strain DB-01 with an antagonistic efficiency of 65.7% at 10 days after fungal inoculation. *Bacillus* sp. reduced gummy stem blight under greenhouse conditions. By 21 days after fungal inoculation, disease incidences were low in the plants treated with *Bacillus* sp. S20D12 and S1F3 by 46.6 and 50.0% and disease severity by 15.3% and 20.6%, respectively. As a result, watermelon plants were prevented from damage of gummy stem blight caused by *D. bryoniae* strain BD-01 by *Bacillus* sp. S20D12 and S1F13. The results would contribute to the knowledge of antagonistic activities of the *Bacillus* to optimize the biological control program against *D. bryoniae.*

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Conflict of interest

On behalf of all authors of the manuscript confirm that all author have no conflicts of interest to declare. All authors have seen and agree with the content of the manuscript and there is no financial interest to report.

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