



Influence of Inoculum Concentration on *In Vivo* Incubation Period of *Emmia lacerata*, Pathogenesis and Management of Wilt in Pepper (*Capsicum annuum* L.)

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Abstract

Emerging infectious disease, caused by *Emmia lacerata* (a host-jumping phytopathogen with medical significance), is a potential threat to production and consumption of pepper. Characterizing the pathogenesis of *E. lacerata* and management of *Emmia* Wilt (EW) in pepper is imperative. *In vitro* antifungal property of *Bacillus velezensis* EBs02 and *B. thuringiensis* EBs04 against *E. lacerata* FMIB29 was screened on dual-culture agar. Effect of inoculum dose on incubation period, severity of EW and its biocontrol was investigated. *Bacillus velezensis* EBs02 expressed the highest mycelial control (68.33%) against FMIB29. A 2-week incubation period was observed for *E. lacerata* in pepper and plants infected with 10⁸ spore/mL exhibited the highest EW severity index (45.01%). Highest *in vivo* disease control (72.75%) was observed in pepper treated with EBs02 and differences in inoculum dose of FMIB29 neither influenced wilt severity nor the biocontrol potential of *Bacillus* strains. However, both *Bacillus* strains drastically reduced the manifestation of wilt in pepper.

Keywords: *Bacillus*; Biocontrol; *Emmia* Wilt; Inoculum Dose; Pepper

Introduction

Pepper (*Capsicum* species) fruit is an outstanding source of micro and macronutrients for several households all over the world [1,2]. It is a reservoir of bioactive metabolites, such as carotenoids, phenolics, flavonoids, as well as iron, dietary source of provitamin A, ascorbic acid (vitamin C), and other antioxidants [3]. These metabolites have been reported to improve cardiovascular ailment and possibly, prostate cancer; while carotenoids in pepper produce its characteristic aroma and flavor [2]. This makes pepper a vegetable of interest for farmers and a spice of choice for cuisines, especially, in southwestern States of Nigeria.

Unfortunately, phytopathogens militate against the production of fruits and vegetables globally [4]; consequently, frustrating the concept of global food security and nutrition. If poorly managed, pathogens could cause up to 80 - 100% of pepper loss on the field, while 20 -25% of harvested fruits are damaged by rot-causing microbes [5]. Nevertheless, commercial farmers are recording significant progress in the management of common pathogens in pepper. However, emerging infectious diseases of pepper constitute an impending threat to the production of this economically significant crop.

In their study on the pathogenicity and host range of *Alternaria alternata*, Balamurugan and Kumar described the

intensity and aggressiveness of the pathogen on solanaceous crops. Xhemali B, et al. [6] also documented the first report on *Colletotrichum scovillei* causing anthracnose in pepper fruits. Typical symptoms of *C. scovillei*-associated anthracnose were observed in *Capsicum annum* cv. Somborka cultivated in different fields of Peja and Rahovec, Kosovo. In 2020 *Emmia lacerata* was isolated from a tomato farm along Ile-tuntun/ Idi-ishin area, Ibadan, Oyo State, Nigeria (7°24'N; 3°50'E) [7]. The first report on the pathogenicity (chlorosis, necrosis and wilt) of *E. lacerata* in tomato was thereafter documented [8]. *Emmia lacerata* equally infected pepper plants cultivated at the Horticultural Unit of the Federal College of Agriculture, Ibadan (7°22'N; 3°50'E), both in 2021 and 2022.

In addition to their saprophytic potential, *Emmia lacerata* has been reported as an endophyte and respiratory parasite [9]. In their report on the biostimulation of *Michelia macclurei*, Pan X, et al. [10] reported a clone-specific improvement in nutrient uptake and a consequential growth promotion caused by *E. lacerata* SR5. This strain was thereafter proposed as a potential biofertilizer. Through clinical evidence and molecular sequencing, *E. lacerata* was identified as an emerging invasive mold, responsible for fungal pneumonia and allergies, especially in immunocompromised patients in Korea [9]. Infected patients were presented with occasional manifestation of hemoptysis. *Emmia lacerata* has been associated with lethal wilt, as well as root and stem rot of olive trees, originally caused by *Dematophora necatrix* in Central Italy [11]. Although, *Emmia lacerata* has been investigated as a soil-borne mycopathogen of tomato plant [8], their virulence and management in other solanaceous crops like pepper and eggplants have not been investigated.

However, emerging infectious diseases could be very difficult to control. Conventional control practices, deployed without adequate characterization of novel pathogens, may not only fail but also predispose host crops to more

devastating microbial attack [12]. Currently, there is a dearth of requisite information on the pathogenesis of *Emmia* wilt in Pepper, the pattern of pathogen (*E. lacerata*) transmission and influence of inoculum concentration on management strategies. This research work was designed to establish the pathogenesis of *E. lacerata* and determine the effect of inoculum dose on the incubation period and management of *Emmia* wilt in pepper plant.

Materials and Methods

Experimental Sites and Sources of Microbial Strains

The *in vitro* and greenhouse experiments were carried out between February - April, 2023 and July - September, 2023, respectively. The experiments were conducted at a Research-Extension Unit of the Federal College of Agriculture, Ibadan (7°25'N; 3°50'E). Molecular identification of microbial strains was done at Bioscience Laboratory of the International Institute of Tropical Agriculture, Ibadan.

Antimycotic *Bacillus* strains (EBs02 and EBs04) were collected from the Microbiology Unit, Federal College of Agriculture, and Ibadan, Nigeria. They were identified as *Bacillus velezensis* and *Bacillus thuringiensis* (Figure 1) and assigned genbank numbers OP975754 and OP975750, respectively. These strains have been reported to express *in vitro* and *in vivo* inhibitory potentials against *Fusarium solani* in maize [13]. *Emmia* species, originally isolated from wilting tomato plants [7], were acquired from the Seed Health Laboratory of National Center for Genetic Resources and Biotechnology (SHL-NACGRAB), Ibadan, Nigeria. The fungal strain was identified as *E. lacerata* (Figure 2), with an assigned genbank number MZ959067. *Bacillus* species were maintained on Nutrient Agar (NA), while *E. lacerata* was cultivated on Potato Dextrose Agar (PDA).

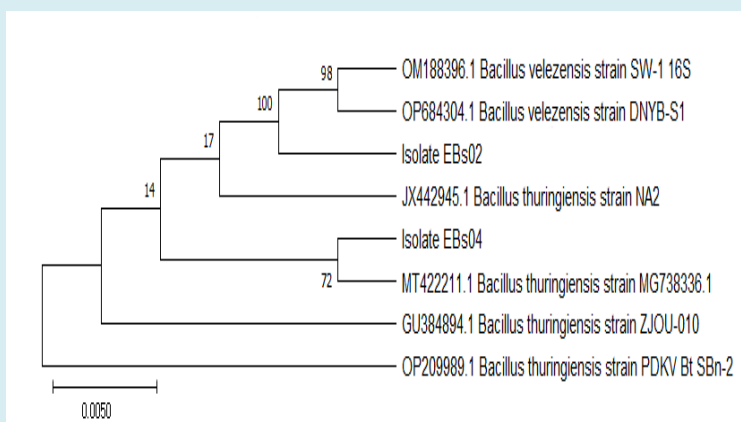


Figure 1: Ancestral relationship of *Bacillus* strains EBs02 (GenBank: OP975754) and EBs04 (GenBank: OP975750) with close relatives (16s rDNA).

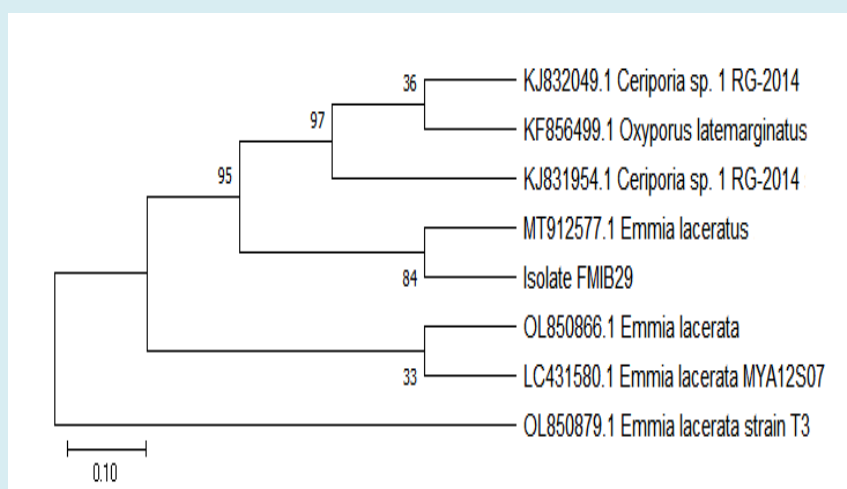


Figure 2: Ancestral relationship of *Emmia* FMIB29 (GenBank: MZ959067) with close relatives (internal transcribed spacer sequences).

Effect of Inoculum Dose on Pathogenicity and Incubation of *Emmia lacerata* in Pepper

This was conducted to investigate the influence of spore concentration on disease establishment and severity of wilt in pepper. Such information will be necessary for soil diagnosis before cultivation, as well as subsequent investigation on the impact of soil fungal population on accumulation of *E. lacerata*, especially, in asymptomatic hosts with medical significance.

Sandy-loam soil samples were collected from the Bora Farm of Federal College of Agriculture, Moor Plantation, Ibadan (7°22'32.0"N; 3°50'49.7"E) and subsequently steam-sterilised. Seeds of *C. annuum* (acquired from the Seed Store of the Institute of Agricultural Research and Training, Ibadan) were surface-sterilised with 2% NaOCl, and sown in 7 kg steam-sterilised potted soil. *Emmia lacerata* was cultivated in Potato Dextrose Broth (PDB) for 3 days at 30°C to generate spores. The rhizosphere of 10-day-old pepper seedlings was inoculated with 1 mL of different inoculum doses (106, 107 and 108 spores/mL) of the pathogen [14]. Infected seedlings were observed for 6 weeks after sowing to determine the period between inoculation and manifestation of wilt symptoms (*in vivo* incubation duration). Disease severity rating and percentage severity index were determined using the severity scale described by Bindal S, et al. [15] as 0 = healthy plant to 5 = dead plant stump.

$$\text{Wilt severity index} = \sum \left(\frac{R \times I_p}{T \times Sh} \times 100 \right)$$

Where R = severity score; I_p = number of infected plants; T = total number of plants under consideration, and Sh = highest severity score (5).

In vitro Inhibitory Assay of *Bacillus* Strains Against *Emmia lacerata*

Characterization of antifungal efficacy of biocontrol strains against mycopathogens is an essential step to select inhibitory *Bacillus* species for subsequent *in vivo* studies.

The antimycotic property of selected *Bacillus* strains against *E. lacerata* was determined through a dual-culture confrontation assay, as described by Cao Y, et al. [16]. The growing edge (5 mm) of 5-day-old *E. lacerata* was cut using a cork-borer and inoculated at the centre of PDA in a Petri dish. After 24 hours of incubation at 30°C, a day-old strain of each *Bacillus* species was spot inoculated on the plate, 3.5 cm away from the centre (containing the *E. lacerata*) [16]. Positive control plates (without *Bacillus*) and plates with dual cultures were incubated for 6 days at 30°C. Mycelial control was determined using the formula described by Hammami R, et al. [17]:

$$\text{Mycelial inhibition (\%)} = \frac{MD - Mc}{MD} \times 100$$

Where MD = mycelial diameter on control plate and Mc = mycelial diameter on dual culture plate.

Effect of Inoculum Dose on the Biocontrol of *Emmia lacerata* with *Bacillus* Strains

One of the factors affecting the adoption of commercially available biocontrol materials in crop production is the lack of graduation, targeted towards specific pathogens. The efficacy of whole-cell biocontrol strains could be influenced by population of soil pathogens. Hence, depending on the outcome of initial soil diagnosis, a dose-dependent

management procedure may be necessary. To determine the effect of inoculum dose on *Emmia* wilt management.

The rhizosphere of 10-day-old pepper seedlings was inoculated with 1 mL of different inoculum doses (10^6 , 10^7 and 10^8 spores/mL) of the pathogen [14], as earlier described. Twenty-four hours after seedling inoculation with the pathogen, rhizosphere of hosts was treated with each *Bacillus* strain (10^8 cfu/mL). The experiment was arranged in a completely randomized design containing 3 concentrations of inoculum and two *Bacillus* strains. Positive control plants were uninoculated and negative (infected) control plants were treated with water. The treatments were replicated 4 times and the influence of inoculum size on wilt-biocontrol potential of selected *Bacillus* strains was determined 4 weeks after plant inoculation (6 weeks after sowing). Disease severity was established as illustrated by Bindal S, et al. [15], while percentage wilt control (PWC) was determined as described below.

$$\text{Wilt control} = \frac{\text{DSC} - \text{DST}}{\text{DSC}} \times 100$$

Where DSC = wilt severity index of negative control plant and DST = wilt severity index of *Bacillus*-treated plant [18].

Statistical Analysis

Data were analysed using both descriptive and inferential analysis. Replicates were subjected to analysis of variance, while means were separated with Duncan's Multiple Range Test at 5% level of significance, with the statistical package for social sciences (version 25.0).

Results and Discussion

Effect of Inoculum Concentration on Pathogenicity and Incubation Duration of *Emmia lacerata* in Pepper

Emmia lacerata FMIB29 caused wilt disease, through rhizosphere inoculation, in pepper plant. Manifestation of leaf chlorosis (Figure 3) was observed in infected seedlings 2 weeks post-inoculation with different concentrations of *E. lacerata*. The period between plant inoculation and the expression of wilt symptoms (2 weeks) was regarded as the incubation period for the pathogen in infected pepper seedlings. Chlorotic plants gradually became necrotic, and this resulted in crown wilt. Differences in inoculum dose, as used in this experiment, neither affected the incubation period observed for *E. lacerata* in pepper nor the severity of the disease (Table 1). Although, at 6 after sowing, plants inoculated with 10^8 spore/mL expressed the highest wilt disease severity index (45.01%), this was not significantly higher than the severity observed in plant sets inoculated with lower pathogen concentrations (10^7 and 10^6 spore/

mL). Control plants, treated with sterile distilled water, in place of *Emmia* species, expressed no wilt symptom through the course of the test.

As previously observed in tomato plants [8], the phytopathogenicity of *E. lacerata* was demonstrated (in pepper) in this study. However, the medical significance of *E. lacerata*, a fungus previously reported as wood-decaying organism, was established by Lee J, et al. [9]. The organism was described as an emerging human pathogen associated with fungal pneumonia. Supportive clinical pathogenicity assessment included chest consolidation, nodular opacity, air-fluid level, pleural effusions and cavitation. In contrast, the symbiotic potential of *E. lacerata* strains (as biocontrol agents) was investigated against multiple phytopathogenic fungi and oomycetes [19]. *Emmia lacerata* HG2011 was reported to successfully control eggplant stem-blight and cucumber vine-blight in infected plants. The occurrence of different physiological forms of *E. lacerata* might be as a result of horizontal gene transfer between pathogenic and non-pathogenic strains of the species [20].

Mobile pathogenicity chromosomes can be transferred between different morphological forms of the same or different microbial species. A typical example of interspecific virulence gene transfer was demonstrated between *Stagonospora nodorum* and *Pyrenophora tritici-repentis* [21]. However, occurrence of intraspecific, mobile pathogenicity-chromosome transfer was established between non-pathogenic and virulent strains of *Fusarium* species [22]. Such transfer of virulence conferred on the non-pathogenic strains the ability to infect tomato host. Consequently, the awareness of physiological forms with different host specificity is imperative in the application of *E. lacerata* as endosymbiont or as biocontrol agent against phytopathogens. This would guide against the possibility of cross-infection (especially, in immunocompromised human hosts) caused by the consumption of fruits produced by infected or inoculated plants.

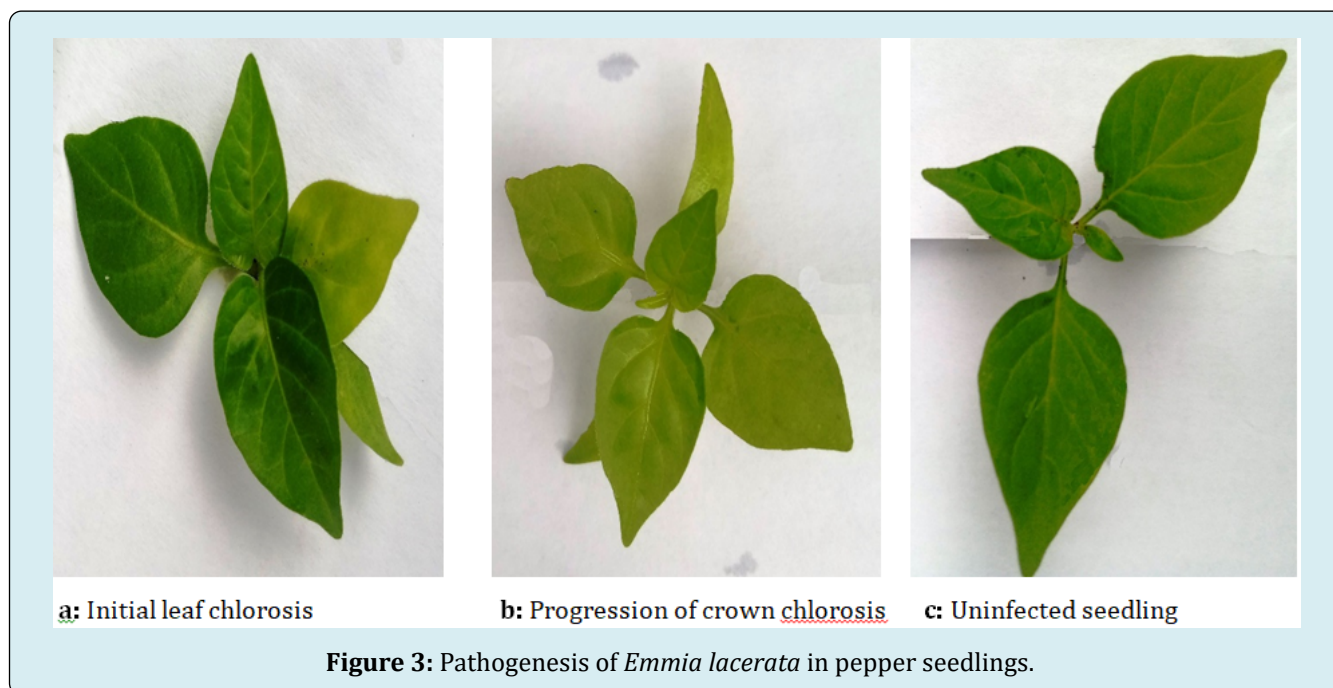
Concentration of *Emmia* inoculum appeared not to significantly affect the incubation period and wilt severity in pepper plant; this could be as a result of inoculum threshold of the pathogen. Chatterton S, et al. [14] investigated the inoculum dose-disease response relationship for pea root-rot pathogen and reported the influence of soil type and microbial interaction on the severity of rot, especially, beyond the inoculum threshold of *Aphanomyces euteiches* in pea. Increase in rhizosphere concentration of phytopathogens (beyond inoculum threshold) could initiate chemical signals, leading to the expression or activation of phytoalexins against the invading pathogen [23]. This will militate against further increment in wilt severity, as observed for 10^7 and 10^8 spore/mL *E. lacerata* in pepper. Although, such expression might

not inhibit pathogen penetration and the consequential occurrence of wilt, it would interfere with the severity of disease expressed by the infected host.

***In vitro* Mycelial Inhibition of *Emmia lacerata* by *Bacillus* Strains**

At 7 days after inoculation on PDA, *E. lacerata* FMIB29 (control) had a mycelial diameter of 66.00 mm (Table 2).

Bacillus velezensis EBS02 expressed the highest percentage mycelial control (68.33) against *E. lacerata* FMIB29; however, this was not significantly different from the average mycelial control (39.56%) observed for *Bacillus thuringiensis* EBS04. Antimycotic *Bacillus* species have potential to enhance crop productivity. Conventionally, *Bacillus* strains are screened for their ability to inhibit potential phytopathogens, usually, through the production of metabolites.



Concentration of <i>Emmia</i> species	Disease severity index (%)			
	1WAI	2WAI	3WAI	2WAI
10 ⁶ spore/mL	0±0.00a	15.00±5.77ab	21.66±1.67a	26.66±4.41a
10 ⁷ spore/mL	0±0.00a	22.33±6.74a	26.66±9.28a	33.33±8.82a
10 ⁸ spore/mL	0±0.00a	22.33±6.74a	28.33±10.14a	45.01±5.77a
Control	0±0.00a	0±0.00c	0±0.00b	0±0.00b

WAI: Weeks after inoculation

Mean values (± standard error).

Means followed by the same letter(s) within a column are not significantly different at 5% probability level according to Duncan's Multiple Range Test.

Table 1: Pathogenicity of *Emmia lacerata* in pepper.

These extracellular bioactive components could serve as safer alternatives to chemical pesticides. Jan F, et al. [24] investigated the *in vitro* properties of *Bacillus subtilis* FJ3 and established the biocontrol and plant growth promoting potential of the strain. Mycelial inhibition potential of *B. subtilis* FJ3 was attributed to the production of hydrolytic

enzymes, iturin, fengycin, biofilm and surfactin. In a similar study on the inhibitory potentials of *B. velezensis*, Sicuia OA, et al. [25] reported a broad spectrum of antifungal activity against phytopathogens such as *Fusarium oxysporum*, *F. solani*, *F. equiseti*, *F. proliferatum*, *Penicillium expansum* and strains of *Aspergillus* species. The antifungal potential of *B. velezensis* was unaffected by the features of *in vitro*

screening (whole-cell, cell-free or volatile organic compound assay). *Bacillus thuringiensis* CHGP12 was also reported to produce *in vitro* antifungal lipopeptides and exhibited a high inhibitory potential (3.45 cm) against the mycelial growth of wilt pathogen in chickpea [26]. In addition to its *in vitro* properties, *B. thuringiensis* CHGP12 also induced a 40% reduction in wilt severity and improved the performance of infected chickpeas.

Treatment	Mycelial growth (mm) (7DAI)	Mycelial inhibition (7DAI)	Control (%) (7DAI)
<i>B. velezensis</i> EBs02	20.33±0.33c	0.68±0.02a	68.33±1.67a
<i>B. thuringiensis</i> EBs04	46.33±1.86b	0.39±0.17a	39.56±16.13a
Control	66.00±0.58a	0.00±0.00b	0.00±0.00b

DAI: Days after inoculation

Mean values (± standard error).

Means followed by the same letter(s) within a column are not significantly different at 5% probability level according to Duncan's Multiple Range Test.

Table 2: *In vitro* mycelial inhibition of *Emmia lacerata* by *Bacillus* strains.

Effect of *Bacillus* Treatment on the Severity of Pepper Wilt

Bacillus strains (*B. velezensis* EBs02, *B. thuringiensis* EBs04), used as a single treatment or in combined application, significantly reduced wilt severity in infected pepper plant (Table 3). Lowest wilt severity (0.75) was observed in *Emmia*-inoculated plants (at 10⁶ cfu/mL) treated with *B. velezensis* EBs02; however, this was not significantly higher than other inoculum treatments. Consequently, both *Bacillus* strains have not potentiated the wilt-biocontrol activities of one another. At the inoculum dose of 10⁸ cfu/mL, *Bacillus* EBs02 also expressed the highest wilt control, with a wilt severity score of 1.13; however, this was not significantly different from the severity scores observed for other treatments. Similar to the effect of inoculum dose on pathogenicity of *E. lacerata* in pepper plant, concentrations of the pathogen did not significantly influence the biocontrol potential of selected *Bacillus* strains. Highest wilt control (72.75%, 54.50% and 64.00%) determined for *Bacillus* EBs02, *Bacillus* EBs04 and the combination of both strains, respectively was expressed in pepper plants inoculated with the lowest concentration of *E. lacerata* (10⁶ spore/mL) (Table 4). However, wilt biocontrol was not significantly different with higher inoculum concentrations (10⁷ and 10⁸ spore/mL) of the pathogen.

The rhizosphere of healthy plants has been established to harbor richer and more diverse microbial communities than soil associated with the root of diseased plants [27]. Consequently, biocontrol application (such as the addition of *B. velezensis* and *B. thuringiensis*) could further increase the microbial diversity in pepper rhizosphere with beneficial strains and shift rhizosphere microbial structure for a more sustainable crop production. The assembly (structure) of microbial community is determined by differential root-exudate production [28]. Rhizosphere microorganisms exist in a complex community, regulated by limiting factors such as space, nutrients and interference-competition mediated by the production of inhibitory metabolites [29]. However, exudates that selectively improve proliferation of pathogen within the rhizosphere will expose host plants to microbial attack [28]. Therefore, introduction of pathogen-suppressive bacterial species (such as antifungal *B. velezensis* and *B. thuringiensis*) could check the competitive edge accorded the pathogen by root exudation. In addition to production of bioactive antifungal metabolites, such *Bacillus* strains could increase microbial diversity in the rhizosphere and promote competition for space and resources (in favour of beneficial microbes).

Contrary to previous reports on improved antimicrobial efficacy associated with the combination of biocontrol agents [30], biocontrol efficacy of antimycotic *Bacillus* strains EBs02 and EBs04 was not additive. Combining both antifungal strains did not significantly improve wilt severity in infected pepper plants, especially, compared with the biocontrol efficacy of each strain. In a similar report, a collection of *Pseudomonas* strains was investigated by Vrieze M, et al. [31] for their ability to control *Phytophthora infestans*, the causal agent of late blight in many economically significant crops. *Pseudomonas fluorescens* S35 was observed to confer significant protection on three cultivars of potato when applied alone, while its biocontrol potential declined when combined with other strains of *Pseudomonas* species. Vrieze M, et al. [31] attributed the poor performance of *P. fluorescens* S35, in dual combination with other strains, to its contrasting mode of action, as well as its inability to compete for space and nutrients.

Conclusion

Emerging infectious diseases of economically significant crops like pepper could cause significant loss to farmers. Wilt caused by *E. lacerata* has been investigated in tomatoes, while the organism's pathogenicity in pepper is reported in this study. Previously reported host spectrum of *E. lacerata* and occurrence of physiological specializations with both medical and agricultural significance make the potential outbreak of *Emmia* infection threatening. Effect of inoculum dose on the incubation period, disease severity index and biocontrol of wilt caused *E. lacerata* (in pepper) were investigated.

<i>Bacillus</i> strain	Inoculum concentration of <i>Emmia</i> species/Wilt severity		
	10 ⁶ cfu/mL	10 ⁷ cfu/mL	10 ⁸ cfu/mL
<i>Bacillus velezensis</i>	0.75±0.48bc	1.50±0.65b	1.13±0.31bc
<i>Bacillus thuringiensis</i>	1.25±0.48b	1.05±0.65b	2.25±0.75b
<i>B. velezensis</i> + <i>B. thuringiensis</i>	1.02±0.01bc	2.00±0.41ab	1.75±0.48b
Negative control	2.75±0.18a	2.75±0.42a	3.25±0.25a
Positive control	0.00c	0.00c	0.00c

Negative Control: Infected, untreated plants; **Positive control:** Uninfected, healthy plants

Mean values (± standard error).

Means followed by the same letter(s) within a column are not significantly different at 5% probability level according to Duncan's Multiple Range Test.

Table 3: Effect of *Bacillus* treatment on the severity of pepper wilt.

Concentration of <i>Emmia</i> species	Biocontrol treatment/Wilt control (%)		
	<i>Bacillus velezensis</i>	<i>Bacillus thuringiensis</i>	<i>B. velezensis</i> + <i>B. thuringiensis</i>
10 ⁶ spore/mL	72.75±17.45a	54.50±17.49a	64.00±1.02a
10 ⁷ spore/mL	47.75±21.80ab	47.75±21.80a	29.50±13.14abc
10 ⁸ spore/mL	65.25±9.84a	36.43±18.87a	45.93±14.69ab

Mean values (± standard error).

Means followed by the same letter(s) within a column are not significantly different at 5% probability level according to Duncan's Multiple Range Test.

Table 4: Control of pepper wilt with *Bacillus* strains.

Increasing the concentration of wilt pathogen to 10⁸ spore/mL did not influence the duration of incubation and wilt severity in infected plants. *Bacillus velezensis* EBs02 and *B. thuringiensis* EBs04 inhibited the mycelial growth of *E. lacerata* and significantly improved wilt severity in infected pepper. However, combining both antifungal *Bacillus* strains did not potentiate their biocontrol efficacy against *Emmia* wilt. The susceptibility of solanaceous crops like pepper, consumed as raw or partially cooked diet, to phytopathogenic forms of *E. lacerata* might be an evolving health concern. Consequently, awareness on the pathogenic and non-pathogenic forms of *E. lacerata*, as well as early development of sustainable biocontrol measure, such as the application of *B. velezensis* or *B. thuringiensis*, is imperative. Also, these strains have exhibited prospects towards management of *Emmia* wilt and should be investigated for the biocontrol of other wilt-associated plant pathogens, such as *Ralstonia solanacearum* and *Fusarium* species. This would significantly improve the yield and quality of susceptible varieties, and equally prompt pepper cultivation, especially in areas endemic to these phytopathogens.

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