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The potential of local *Bacillus* sp. BK7.1, EG6.4, and LSD4.2 as biocontrol agents against the pathogenic fungus *Fusarium oxysporum*

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Abstract. *Nurhariyati T, Salamun, Supriyanto A, Sinurat TBM, Yuliana F, Geraldi A, Pratiwi IA, Nafidiastri FA, Izzuddin M. 2025. The potential of local* Bacillus *sp. BK7.1, EG6.4, and LSD4.2 as biocontrol agents against the pathogenic fungus* Fusarium oxysporum*. Biodiversitas 26: 3273-3280.* Plant-pathogenic fungi can cause horticultural wilt disease. Some *Bacillus* species exhibit antifungal activity against plant pathogens. This study aimed to examine the effects of several indigenous *Bacillus* species on the growth of *Fusarium oxysporum*. The research was conducted experimentally using a 5x6 factorial design. The first factor was isolate variation, consisting of *Bacillus subtilis* BK7.1, *Bacillus mojavensis* EG6.4, *Bacillus velezensis* LSD4.2, a negative control (C-), and a positive control (C+). The second factor was incubation time (contact time) at 1st, 2nd, 3rd, 4th, 5th, and 6th days. This study tested the antifungal activity of *Bacillus* isolates and controls against *F. oxysporum* using the pour plate method. The growth of the pathogenic fungus was measured based on the diameter of the growth area of *F. oxysporum*. Antagonistic testing was conducted to determine whether the three isolates could be combined in a biofungicide consortium. Antifungal activity test results revealed that all isolates (BK7.1, EG6.4, and LSD4.2) effectively inhibited the growth rate of *F. oxysporum*. Variations in isolate type, incubation time, and combinations significantly affected the inhibition of *F. oxysporum* growth. The antagonistic activity test yielded negative results. Therefore, isolates BK7.1, EG6.4, and LSD4.2 will be developed as biofungicide candidates for the biocontrol of plant-pathogenic fungi.

Keywords: *Bacillus*, biofungicide, biocontrol, *Fusarium oxysporum*, plant protection

INTRODUCTION

Indonesia is an agricultural country that relies on the agricultural sector for food and economic development. Most of Indonesia's population lives in rural areas and works as farmers. The agricultural sector significantly contributes to national income and is crucial to the country's employment, food production, and textile industry (Abduh 2023). The presence of pathogenic fungus *Fusarium oxysporum* can reduce horticultural crop yields. Under favorable climatic conditions, fungal growth can escalate, leading to crop failure (Vignesh et al. 2021). Efforts to control wilt disease caused by *F. oxysporum* have traditionally involved chemical pesticides. However, the usage of chemical pesticides can increase resistance of fungi and pest pathogen. Chemical pesticides also have negative impacts on the environment, particularly to soil health, soil biodiversity, wildlife habitats, water quality, and increase health risks for farmers and consumers (Chandrasekaran et al. 2014; Nawaz et al. 2016; Chintagunta et al. 2020; Datta et al. 2020); Amaria et al. 2024. Microbial biocontrol does not leave harmful residues, is environmentally friendly, and can benefit plant health (Fira et al. 2018). *Bacillus* species are known to produce antibiotic enzymatic compounds such as chitinase, which inhibits fungal growth by hydrolyzing fungal cell walls (Khairah et al. 2023). Additionally, protease and siderophore macromolecular degradative enzymes produced by bacteria can inhibit the development of plant-pathogenic fungi (Nurikhsanti et al. 2024).

Previous studies have identified isolate EG6.4 as *Bacillus mojavensis* (Salamun et al. 2022), LSD4.2 as *Bacillus velezensis* (Salamun et al. 2023a), and BK7.1 as *Bacillus subtilis* (Salamun et al. 2023b). These three *Bacillus* species were isolated from breeding sites and larvae of *Aedes aegypti* Linnaeus 1762 mosquitoes. *Bacillus subtilis* BK7.1 exhibits β-hemolytic activity, encodes genes for the surfactin biosynthesis enzymes Srf-AA and Srf-AD, and produces biosurfactants on glucose, glycerol, and molasses substrates (Salamun et al. 2023a). *B. mojavensis* EG6.4 also exhibits β-hemolytic activity, encodes the Srf-AD gene for surfactin biosynthesis, and has larvicidal activity against

A. aegypti larvae (Salamun et al. 2022). Meanwhile, *B. velezensis* LSD4.2 has been screened for its larvicidal activity against *A. aegypti* larvae, morphological and physiological characteristics, and 16S rRNA molecular identification (Salamun et al. 2023b).

Bacillus subtilis has been recognized as a biocontrol agent against various plant diseases (Kumbar et al. 2019). It has antibacterial, antifungal, and growth-promoting properties and can induce systemic resistance in plants. It also produces antifungal compounds such as iturin, surfactin, and fengycin lipopeptides (Kourmentza et al. 2021). *B. mojavensis* is similar to *B. subtilis* but differs in fatty acid composition, DNA sequence, and genetic transformation resistance (Mounia et al. 2014). *B. mojavensis* produces surfactin, iturin, and fengycin lipopeptides, which exhibit antimicrobial and antifungal activity (Mounia et al. 2014). It can also produce surfactin on various carbon sources, with optimal production observed in glucose media. According to Myo et al. (2019), *B. velezensis* has demonstrated antifungal activity against fungal pathogens. Additionally, volatile organic compounds produced by *B. velezensis* NKG-2 can influence fungal growth (Myo et al. 2019). It has demonstrated antifungal activity against plant pathogens and has produced biomolecules with potential applications in genetic engineering and agriculture (Adeniji et al. 2019). Thus, these three *Bacillus* isolates can be developed as potential biofungicides for environmentally friendly biocontrol of *F. oxysporum*.

Additionally, *Bacillus* sp. requires sufficient contact time to function as a biofungicide, meaning variations in incubation periods can influence their effectiveness in inhibiting plant-pathogenic fungi. The aim of this research was to know the effect of *Bacillus* sp. isolates BK7.1, LSD4.2, and EG6.4, along with incubation times of 1, 2, 3, 4, 5, and 6 days, and their combinations, inhibit the growth of *F. oxysporum.* Antagonistic activity testing was also performed to determine whether these three species can be combined into a biofungicide consortium for plant pathogen control.

MATERIALS AND METHODS

This study was conducted at the Microbiology Laboratory, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia, to prepare *Bacillus* sp. bacteria and *F. oxysporum* pathogenic fungi, as well as to test the antifungal and antagonistic activities between isolates. The materials used for bacterial, fungal, and antifungal tests included *Bacillus* sp. isolates BK7.1, EG6.4, and LSD4.2; Nutrient Agar (NA) medium; Mueller Hinton Broth (MHB) medium; 70% alcohol; sterile distilled water; *F. oxysporum* culture; Potato Dextrose Agar (PDA) medium; and Nystatin (antifungal).

Preparation of isolates

Isolate rejuvenation was carried out by taking one inoculation loop inoculating loop of each isolate and streaking it onto a slanted NA medium, followed by incubation at 33°C for 24 hours. After rejuvenation, three inoculation loop of each isolate were suspended in separate culture bottles containing 100 mL of MHB medium. The cultures were incubated on a shaker at room temperature for 72 hours. The absorbance was then measured using a spectrophotometer, with an optical density (OD) of 0.5 at a wavelength of 600 nm.

Preparation of the pathogenic fungus

The pathogenic fungus *F. oxysporum* was obtained from the Microbiology Laboratory, Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia. The fungus was rejuvenated onto a Petri dish containing PDA medium, and incubated for six days at room temperature.

Antifungal activity test

The antifungal activity test was conducted by preparing 200 mL of PDA medium, which was poured into Erlenmeyer flasks and sterilized using an autoclave. *Bacillus* sp. isolates BK7.1, EG6.4, and LSD4.2, adjusted to OD600 = 0.5, and positive control (C+) used chemical fungicide and negative control (C-) used pure PDA medium, were inoculated into Petri dishes using the pour plate method and incubated at 33°C for 24 hours. The rejuvenated *F. oxysporum* test fungus was then inoculated using a cork borer and placed in the center of the Petri dish containing solidified PDA medium with the bacterial isolates. The plates were incubated at room temperature for 1, 2, 3, 4, 5, and 6 days. Observations and measurements of fungal growth areas were made daily using a calliper for six days. The antifungal activity test was repeated three times using different Petri dishes.

F. oxysporum growth observations were conducted by measuring the fungal growth area, including vertical (d1) and horizontal (d2) diameters, using a calliper. The growth area (G) was calculated using the following formula (Saputra et al. 2022):

$$G = 3.14 \times \left[\left[\frac{d2}{2}\right]^2 - \left[\frac{d1}{2}\right]^2\right] \text{ cm}^2$$

Where:
G : Growth area of the pathogenic fungus (cm²)
d_1 : Initial diameter of the pathogenic fungus (cm)
d_2 : Growth diameter of the pathogenic fungus (cm)

The percentage of fungal growth inhibition was calculated using the following formula (Kamaruzzaman et al. 2021):

$$I = (C\text{-}T)/C \times 100$$

Where:
I : Mycelial growth inhibition
C : Growth in the control plate
T : Growth of mycelium in the inserted plate

Antagonistic activity test

The antagonistic activity test began with the preparation of the NA medium. Isolates were rejuvenated on NA medium by dissolving 1.4 g of NA in 50 mL of distilled water, homogenizing it with a stirrer, heating it on a hot plate, and sterilizing it by autoclaving at 121°C for 15

minutes. The NA medium was then poured into three sterile Petri dishes, with 15 mL of NA per dish, and incubated for 1, 2, and 3 days. Antagonistic testing was conducted three times. A positive test result (+) was indicated by the presence of inhibition zones between isolates, while a negative test result (-) was indicated by the absence of inhibition zones (Li et al. 2020).

Data analysis

Data were analyzed using statistical Package for the Social Sciences (SPSS) software. Quantitative data included the growth area of pathogenic fungi under the influence of antifungal activity from *Bacillus* sp. isolates BK7.1, EG6.4, and LSD4.2, incubation times, and their combinations.

RESULTS AND DISCUSSION

Effect of isolate variation

Figure 1 presents the growth area of *F. oxysporum* after incubation with isolates BK7.1, EG6.4, and LSD4.2. The best inhibition of *F. oxysporum* growth was observed with the BK7.1 isolate. The EG6.4 isolate also inhibited the pathogen, but to a lesser extent compared to LSD4.2. These results indicate that both BK7.1 and EG6.4 isolates can effectively suppress *F. oxysporum* growth. Moreover, their inhibitory effects were superior to those of the positive control treatment (fungicide).

Effect of incubation time

Figure 2 presents data on the effect of incubation time on the growth area of pathogenic fungus. The result shows that BK7.1 and EG6.4 isolates effectively inhibit the growth of *F. oxysporum* up to six days of incubation. The colony diameter of *F. oxysporum* under these treatments was approximately 2 cm², which was significantly smaller than that observed in the positive control treatment. In contrast, LSD4.2 isolate showed an increase in *F. oxysporum* growth with each day of incubation, reaching approximately 14.0 cm² by the final day.

Percentage of inhibition growth for *F. oxysporum* by all bacteria was also measured. As shown in Figure 3, BK7.1 and EG6.4 isolates could inhibit 100% *F. oxysporum* for day-1 incubation. This inhibition reduced time by time around 84.3 % for BK7.1 and 76.1% for EG6.4.

Effect of the combination of isolate variation and incubation time

Data on the combination of isolates with different incubation times and their effect on the growth of the pathogenic fungus are presented in Figures 4 and 5.

The morphological changes in *F. oxysporum* growth after inoculation with *Bacillus* isolates are shown in Figure 5. Observations and measurements were conducted over six days. Noticeable color changes in the fungal morphology were observed, which are likely due to the effects of secondary metabolites produced by *Bacillus*.

Antagonistic activity

Antagonistic test results for the three isolates (BK7.1, EG6.4, and LSD4.2) were negative, as no inhibition zones were observed between different colonies. The results of antagonistic test are presented in Table 1 and Figure 6. Based on the results, it can be shown that three *Bacillus* isolates exhibit synergistic activity and could be candidates for biofungicides.

Discussion

Antifungal activity testing of various *Bacillus* strains and *Fusarium* pathogens has been conducted to evaluate the potential of *Bacillus* as a biological control agent against *Fusarium* infections, which often cause damage to plants. Development efforts have been made to create an environmentally friendly product such as a green biocide derived from a consortium of various *Bacillus* strains. In this study, the primary focus was to identify the effectiveness of *Bacillus* strains in inhibiting *Fusarium* growth through mechanisms, such as the production of antifungal compounds, toxins, lytic enzymes, or competition for space and resources.

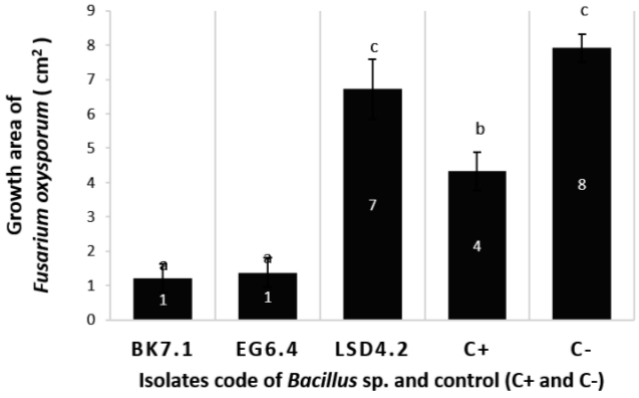

Figure 1. Growth area of pathogenic fungus *F. oxysporum* with different isolates. BK7.1, EG6.4, and LSD4.2. Diagrams with different notations indicate a significant difference

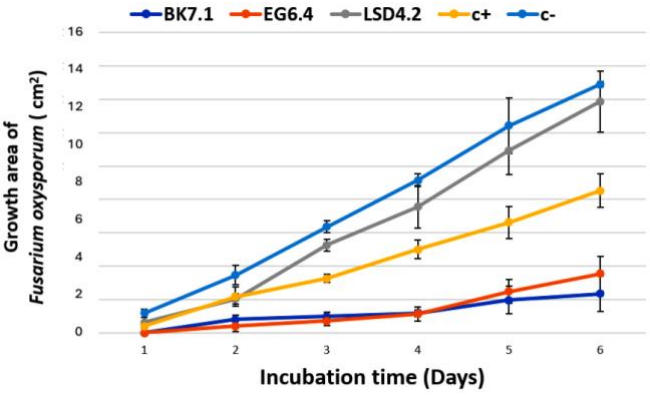

Figure 2. Growth of the pathogenic fungus *F. oxysporum* under different incubation times for *B. subtilis* BK7.1, *B. mojavensis* EG6.4, and *B. velezensis* LSD4.2. (C+): Positive control, (C-): Negative control

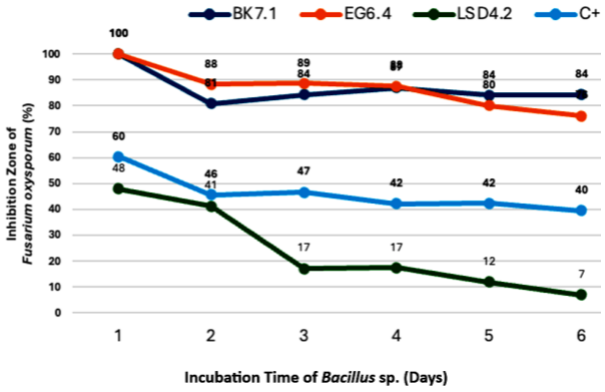

Figure 3. Growth inhibition of the pathogenic fungus *Fusarium oxysporum* under different incubation times for *Bacillus subtilis* BK7.1, *Bacillus mojavensis* EG6.4, and *Bacillus velezensis* LSD4.2. (C+): Positive control

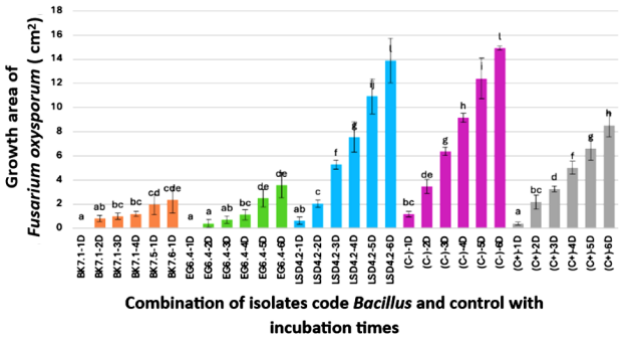

Figure 4. Growth area of the pathogenic fungus *F. oxysporum* with different combinations of isolates (BK7.1, EG6.4, and LSD4.2) and incubation times. (C+): Positive control, (C-): Negative control. A bar chart with different notations indicates a significant difference

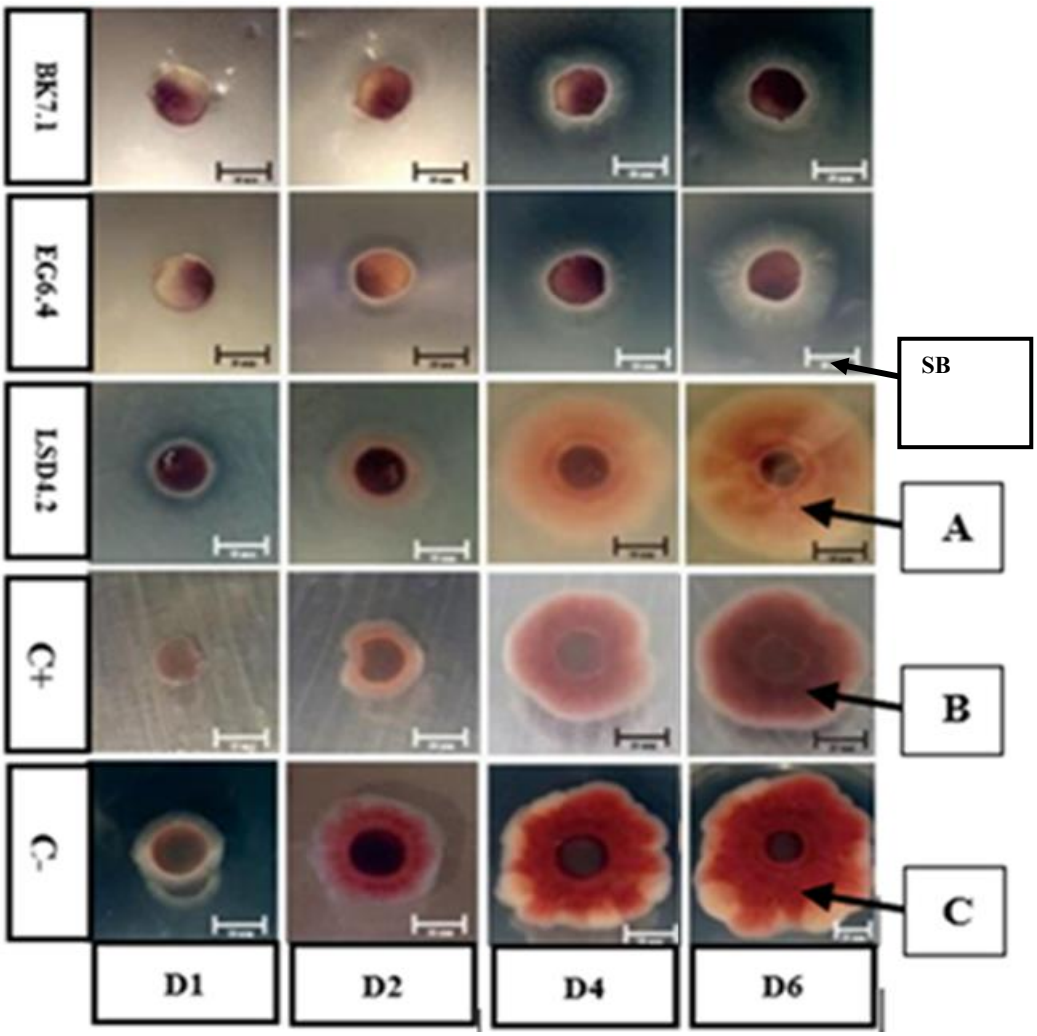

Figure 5. Growth of the pathogenic fungus *F. oxysporum* under different incubation times for isolates BK7.1, EG6.4, and LSD4.2. Notes: Positive control (C+); Negative control (C-); Incubation times (D1: 1st, D2: 2nd, D4: 4th, D6: 6th); A: LSD4.2 mycelial pigment, B: C+ mycelial pigment, C: C- mycelial pigment, SB: scale bars (10 µm)

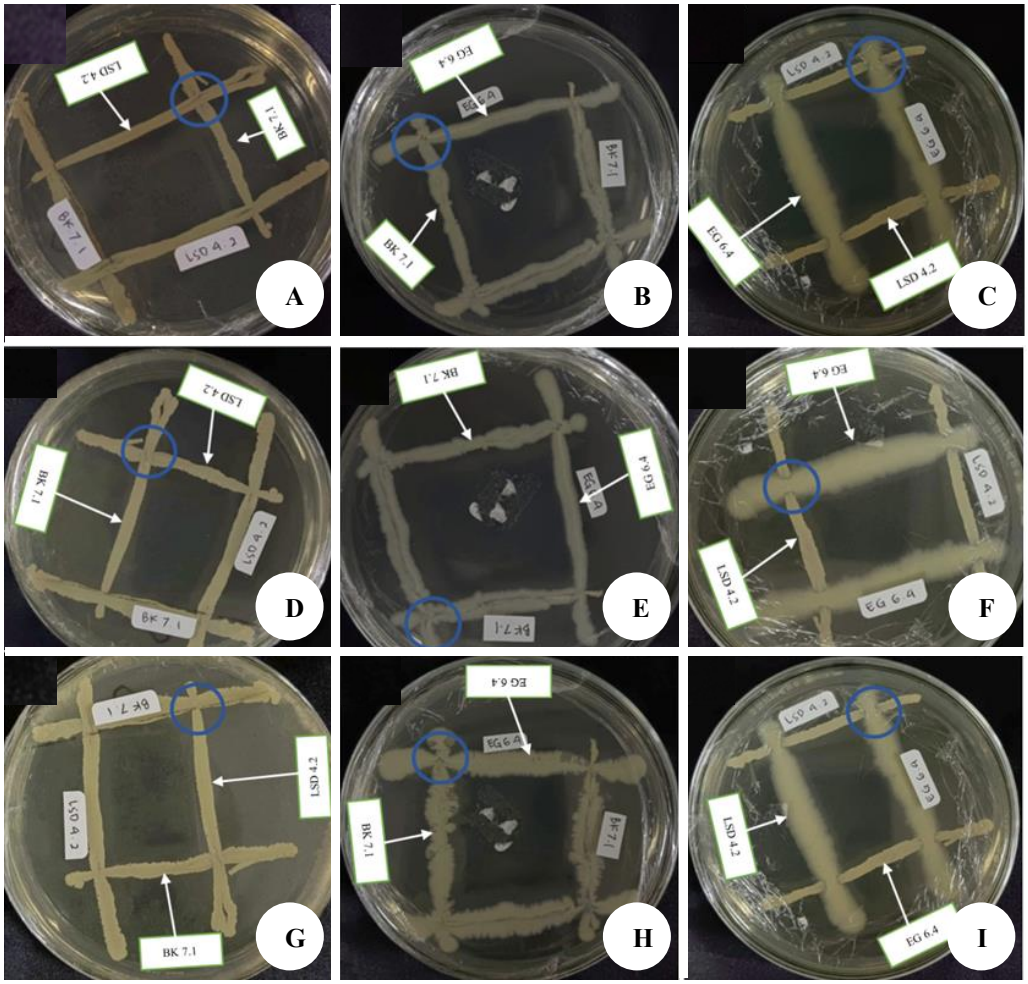

Figure 6. Antagonistic activities between isolates *B. subtilis* BK7.1, *B. mojavensis* EG6.4, and *B. velezensis* LSD4.2. Contact time (incubation) for the 1st day (A, B, C), the 2nd day (D, E, F), and the 3rd day (G, H, I)

Table 1. Antagonistic activities between isolates *B. subtilis* BK7.1, *B. mojavensis* EG6.4, and *B. velezensis* LSD4.2, with different contact times (incubation periods) of 1, 2, and 3 days

Antagonistic activity	Contact time		
	1st day	2nd day	3rd day
BK7.1 vs EG6.4	Negative	Negative	Negative
BK7.1 vs LSD4.2	Negative	Negative	Negative
EG6.4 vs LSD4.2	Negative	Negative	Negative

The results indicate that different *Bacillus* species have varying effects on the growth area of the pathogenic fungus. The lowest fungal growth was observed in isolates BK7.1 and EG6.4, with no significant difference. However, there was a significant difference between isolates BK7.1 and EG6.4 compared to LSD4.2, the negative control (C-), and the positive control (C+). Isolate LSD4.2 exhibited the least effectiveness in inhibiting fungal growth, but its colony color differed from that of the negative control (C-). *B. velezensis* has been reported to produce antifungal compounds in lower quantities and compositions than other isolates. The type and quantity of antifungal compounds produced influence antifungal activity. Additionally, environmental factors such as nutrient availability, pH, temperature, and oxygen levels can affect the antifungal activity of *B. velezensis* (Wu et al. 2019). Non-optimal environmental conditions can inhibit the production of antifungal compounds (Faruqi et al. 2023). *Bacillus* sp. can inhibit pathogenic fungal growth by producing secondary metabolites, such as lipopeptides. Lipopeptides are categorized into three types: fengycin, surfactin, and iturin (Meena and Kanwar 2015). *B. velezensis* has been shown to produce cyclic lipopeptides, including surfactin, iturin, and fengycin. These lipopeptides, composed of amino acids such as glutamic acid, aspartate, lysine, and arginine, and fatty acids, play a significant role in inhibiting fungal growth. Another antifungal mechanism involves disrupting the fungal cell membrane, damaging hyphae, and inhibiting fungal growth. Additionally, antibiotic compounds such as bacillaene, macrolactin, and difficidin inhibit cell wall synthesis, metabolism, and the growth of *F. oxysporum* (Barale et al. 2022).

Isolate BK7.1 exhibited the lowest fungal growth area, with an average of 1.22 cm². By the 4th day of incubation,

isolate inhibited *F. oxysporum* growth by 87.0%. These findings align with those reported by Jassim et al. (2022) and Cao et al. (2011), who found that *B. subtilis* can inhibit the mycelial growth of *F. oxysporum* by up to 86.6%. They also found that *B. subtilis* SQR9 produces multiple antibiotic compounds, including fengycin, iturin, and bacillomycin, which inhibit mycelial growth and spore germination of *F. oxysporum*. Additionally, hydrolytic enzymes such as chitinase, β-1,3-glucanase, and protease degrade the *Fusarium* cell wall, thereby preventing infection and pathogen development (Win et al. 2021).

The average fungal growth area for isolate EG6.4 was 1.38 cm², and by the 4th day of incubation, isolate inhibited *F. oxysporum* growth by 87.7%. These findings are consistent with those reported by Ze et al. (2024), who found that *B. mojavensis* strain MTC-8 can inhibit the mycelial growth of *F. oxysporum* by 82.3%. The intense antifungal activity of *B. mojavensis* B0621A was attributed to a novel iturinic lipopeptide, Mojavensin-A (Ma and Hu 2015). Similarly, surfactin (Leu7-surfactin) produced by *B. mojavensis* strain RRC 101 has been reported as mycotoxic against *Fusarium verticillioides* (Snook et al. 2009).

Bacillus sp. isolates not only inhibit pathogenic fungi's growth but also suppress *Fusarium*'s pigment production. Differences in pigment color were observed among the tested samples, including a distinct color change in LSD4.2 compared to the negative control (C-). Harish et al. (2023) reported that *Fusarium* sp. colonies typically range in color from red to dark purple, indicating a high level of pathogenicity. In contrast, lighter-colored colonies suggest lower pathogenicity. The red coloration of *Fusarium* sp. colonies is generally associated with the production of mycotoxins, toxic compounds produced by fungi that can cause diseases in plants and animals (Harish et al. 2023).

The differences in fungal growth area across incubation times for each isolate may be attributed to several factors, including variations in *Bacillus* isolates' ability to produce antifungal compounds and *Fusarium*'s capacity to develop resistance mechanisms to certain antifungal compounds over time (Khan et al. 2018). *Fusarium* employs various resistance mechanisms to counteract antifungal compounds produced by *B. subtilis*, *B. mojavensis*, and *B. velezensis*. These mechanisms include changes in cell permeability, modulation of gene expression, cell wall modification, activation of efflux pumps, and adaptation to oxidative stress. The ability of *Fusarium* to develop cross-resistance to antifungal compounds from different *Bacillus* species makes its pathogenicity challenging to control (Fatma et al. 2021).

Bacillus sp. undergoes four growth phases: lag, exponential, stationary, and death (Bruslind 2024). The lag phase occurs at the beginning of bacterial growth, during which bacteria adapt to new environmental conditions without significant growth. In this phase, bacteria synthesize enzymes, proteins, and other cellular components in preparation for exponential growth. The exponential phase (log phase) is characterized by a rapid increase in bacterial growth rate, with metabolic activity and cell division reaching an optimal level. In the stationary phase, the growth rate slows and reaches equilibrium, as the number of newly formed cells equals the number of dying cells. Growth ceases due to limiting factors such as nutrient depletion, oxygen availability, or the accumulation of metabolic byproducts. Finally, the death phase occurs when environmental conditions become increasingly unfavorable for bacterial survival. The rate of cell death surpasses that of cell growth, leading to a decline in bacterial population viability.

The average differences in fungal growth inhibition when combining isolates with different incubation times. The combination of isolates BK7.1 and EG6.4 within one day of incubation exhibited the strongest inhibition, with an average fungal growth area of 0 cm², corresponding to 100% inhibition. In contrast, the combination of isolate LSD4.2 within six days of incubation exhibited the weakest inhibition, with an average fungal growth area of 13.8 cm² (Figure 2), or 6.9% inhibition.

The combination of isolate BK7.1 within one day of incubation resulted in an average growth area of 0 cm² or 100% inhibition, indicating that *B. subtilis* BK7.1 has strong antifungal activity against *F. oxysporum*. A study by Aydi et al. (2016) similarly reported that *B. subtilis* inhibited the growth of *Fusarium graminearum* within one day of incubation, with minimal to no fungal growth. The combination of isolate BK7.1 within two days of incubation resulted in an average fungal growth area of 0.81 cm² or 80.6% inhibition. This decrease in inhibition is likely due to *F. oxysporum* developing resistance mechanisms, such as producing detoxifying enzymes or modifying the target sites of antifungal compounds (Liao et al. 2023). During the early growth phase, *Bacillus* produces antifungal compounds in sufficient quantities to inhibit *Fusarium*, but over time, this production decreases (Rafanomezantsoa et al. 2022). *B. subtilis* varies in antifungal compound production depending on the growth phase, with a significant decline occurring during the stationary phase at the end of the incubation period (Mora et al. 2011).

The combination of isolate EG6.4 within one day of incubation resulted in a mean fungal growth area of 0 cm² or 100% inhibition, demonstrating that *B. mojavensis* EG6.4 effectively inhibits *F. oxysporum* growth within one day of incubation. This growth inhibition is attributed to the production of antifungal compounds such as lipopeptides (Rais et al. 2018). When combined with an incubation time of two days, isolate EG6.4 resulted in an average fungal growth area of 0.4 cm² or 88.2% inhibition. This variation can be attributed to differences in the production of antibiotic compounds such as surfactin, iturin, and fengycin, which fluctuate during bacterial growth and are influenced by environmental factors. Antifungal compound production tends to decline during the stationary phase, reducing its effectiveness against pathogens (Songwattana et al. 2023). The combination of isolate EG6.4 within three, four, five, and six days of incubation increased fungal growth, indicating a decrease in inhibition. This can be attributed to reduced antifungal compound production over time, as well as the adaptation of *F. oxysporum*, which can develop resistance mechanisms to certain antifungal compounds and can cause resistance to antifungal compounds produced

by *B. mojavensis* at longer incubation times (Ma et al. 2021).

The combination of isolate LSD4.2 within one day of incubation resulted in a mean fungal growth area of 0.61 cm² or 47.9% inhibition. The lower inhibition rate suggests that LSD4.2 has a weaker antagonistic mechanism or requires a more extended incubation period to produce metabolites that can inhibit *F. oxysporum*. When combined with an incubation time of two days, isolate LSD4.2 resulted in a mean fungal growth area of 2 cm², indicating improved inhibition as the bacterial culture entered the stationary growth phase. During this phase, LSD4.2 optimally produces secondary metabolites, such as surfactin, iturin, and fengycin, which have antifungal properties (Kim et al. 2022).

The antagonistic test results demonstrated that three isolates did not exhibit antagonistic interactions. This indicates that these isolates can coexist without inhibiting each other and may have synergistic potential in their natural habitat (Amer et al. 2021). One factor contributing to this synergy is the ability of certain bacterial species to supply essential nutrients that other species cannot synthesize. Additionally, the metabolic byproducts of one species can be utilized by another, enabling a more efficient interaction between multiple species (Deng and Wang 2016).

In conclusion, there were significant differences in the growth inhibition effects on the pathogenic fungus *Fusarium oxysporum* among the isolates BK7.1, EG6.4, LSD4.2, as well as the positive and negative controls (C+ and C-). These differences were attributed to the distinct metabolites produced by the three isolates. Additionally, significant variations were observed in the effects of incubation time among isolates BK7.1, EG6.4, and LSD4.2 on *F. oxysporum* growth, which can be explained by differences in metabolite production dynamics over time. Moreover, the interaction between isolate variation and incubation time significantly influenced the inhibition of *F. oxysporum* growth, reflecting the temporal changes in metabolite synthesis and the adaptive resistance mechanisms developed by the pathogen.

A novel finding of this study was that isolate LSD4.2 can reduce the pathogenicity of *F. oxysporum* by altering its mycelial pigmentation. Furthermore, the lack of antagonistic interactions among the three isolates suggests their potential to function synergistically as a microbial consortium, offering promising prospects as biofungicides for plant pathogen control. Future research should focus on identifying the primary and secondary metabolites responsible for these inhibitory effects, with particular emphasis on the compound responsible for pigment reduction in *F. oxysporum* when exposed to isolate LSD4.2.

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