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THE EFFECT OF RHIZOBACTERIA OF THE GENERA BACILLUS AND PSEUDOMONAS ON WILT (*Fusarium oxysporum*) IN CHILI PEPPER

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ABSTRACT. *The average productivity of chili pepper (Capsicum annuum L.) is very low in Ethiopia compared to its average productivity in other major producing countries. Wilt disease, caused by the fungus Fusarium oxysporum, is one of the primary causes of reduced productivity. To date, no effective fungicides have been identified for controlling the disease. Moreover, there are significant environmental and human health concerns associated with fungicides. Thus, the objective of this study was to screen and evaluate rhizobacteria possessing a biocontrol trait for the management of chili pepper wilt disease. Combined methods (culture-based, microscopic, biochemical tests, and greenhouse experiment) were used for evaluating the rhizobacteria. The dual culture study revealed that rhizobacteria belonging to the Bacillus and Pseudomonas genera successfully inhibited F. oxysporum (62%) and exhibited biocontrol-associated traits that can be used to combat this disease. The biocontrol-related traits, namely protease, chitinase, hydrogen cyanide, and pyoverdine siderophore production, were detected in these isolates. The evaluated isolates decreased the severity of wilt disease of chili peppers by 86.5% and increased the yield of dried fruits by 35.9% under greenhouse conditions. The analysis of variance revealed that the isolates and crop varieties interacted to significantly influence disease severity and yield of dried fruits ($P < 0.001$). These isolates can be good candidates for biocontrol agents. The study's findings underscore the role of rhizobacteria in organic farming, as well as the importance of local screening in*

areas where the target disease is severe, and highlight their impact on reducing disease and enhancing crop productivity.

***KEYWORDS**: biocontrol, Fusarium oxysporum, management, productivity, rhizobacteria*

INTRODUCTION

In their original tropical settings, the chili pepper (*Capsicum annuum* L.) is a delicate perennial plant. Still, it is frequently farmed as an annual crop in many parts of the world (Kraft et al. 2014, Padilha & Barbieri 2016). It is a commonly cultivated spice crop in tropical and subtropical regions, including Ethiopia (Kim et al. 2014), and dominates the global spice trade (Lin et al. 2013). Ethiopia ranked 4th in the world in chili pepper production with around 0.30 M tons (Food and Agriculture Organization of the U.N. 2021). However, the productivity of the crop is very limited in Ethiopia (1.79 t ha^{-1}) compared to China, which has the highest productivity in the world (6.62 t ha-1) (Food and Agriculture Organization of the U.N. 2021). Diseases are a major cause of low crop productivity in Ethiopia and other sub-Saharan African countries (Bjornlund et al. 2020). Particularly, diseases caused by soil-borne pathogens, namely bacterial (*Ralstonia solanacearum*) or fungal (*Fusarium oxysporum*) wilt, are the major problems constraining chili pepper production (Nordey et al. 2020). *Fusarium* sp. was found in 17% of Ethiopia's wilted hot pepper plants (Assefa et al. 2015). The most harmful vegetable pathogens in the *Fusarium* genus are the oxysporum and solani species (Nordey et al. 2020). *Fusarium* diseases cause yield losses that result in economic losses of billions of dollars annually (Al-Hatmi et al. 2016). Plant pathologists have paid close attention to *F. oxysporum* due to its wide host range, the significant monetary losses it causes, and its enigmatic taxonomy (Lombard et al. 2019). Chili pepper wilt disease, caused by *F. oxysporum*, is severe in Ethiopia, especially in the greater Rift Valley, where it is estimated to affect 65-100% of chili pepper plants (unpublished data). However, there are no reliable reports on the crop yield loss attributed to this disease in Ethiopia. The most common cultural approach for disease management is rotational planting of affected crops with unrelated unaffected crops. Crop rotation prevents

populations of pathogens from growing to dangerous levels. However, one should not expect crop rotation to prevent disease development completely. Similarly, removing the infected plants as soon as they appear is necessary, but this activity is also time-consuming and tedious, especially for large-scale farms. One practical solution for preventing wilt disease in the field is the use of resistant varieties (Pietro et al. 2003). However, due to the limited research facilities, the required expertise, and other related factors, creating wilt disease-resistant chili peppers is a challenging task in Ethiopia. Currently, the use of fungicides for controlling *Fusarium* wilt is ineffective. Fungicide use also poses serious risks to the environment and public health (Pathak et al. 2022). Furthermore, the costs of fungicides are too high and cannot be afforded, particularly by farmers in low-income countries like Ethiopia. Several studies worldwide have indicated the utility of rhizosphere bacteria as biocontrol agents in combating fungus-related plant diseases (Palmieri et al. 2022, Rabbee et al. 2023). For instance, the uses of *Bacillus* sp. against chickpea wilt disease (Mageshwaran et al. 2022), *Burkholderia* sp. against fungal wilt diseases in banana (Wei et al. 2020), *Streptomyces,* and *Pseudomonas* spp. against numerous fungal and bacterial plant diseases for the detection of new bioactive molecules and for developing biofertilizer (Zheng et al. 2021). The use of mycorrhizal fungi as a bio-inoculant has also received considerable attention in organic farming. For example, Soylu et al. (2023) reported that mycorrhizal inoculation increased the morphology of chili pepper roots and shoots, as well as their fresh and dry weight yields. It has also been demonstrated that co-inoculating mycorrhizal fungi and rhizobacteria successfully mitigates the adverse effects of salt stress by enhancing water retention, increasing nutrient uptake, and maintaining chlorophyll levels (Altuntas et al. 2024, Dere 2024). In Ethiopia, an encouraging initiative is underway to utilize rhizosphere bacteria as a biofertilizer by inoculating the bacteria onto legume crops at the Menagesha Biotech Industry, Private Limited Company. However, the potential of rhizosphere bacteria as a biocontrol agent against *F. oxysporum* has not been thoroughly explored. There have been limited research findings in Ethiopia on this topic. Thus, the primary objective of this study was to assess the impact of selected rhizobacteria from the genera *Bacillus* and *Pseudomonas* on the control of *Fusarium* wilt in chili peppers.

MATERIALS AND METHODS

The greenhouse and laboratory experiments for this study were conducted at the Department of Microbial, Cellular, and Molecular Biology (DMCMB), Addis Ababa University, Ethiopia. All laboratory reagents were obtained from the DMCMB's Mycology Laboratory.

Isolation of rhizobacteria: Soil and chili pepper plant samples were collected from Ethiopia's major chili pepper growing areas (East Shoa, Halaba, and Gurage zones) by carefully uprooting the plants to avoid injuring the root system. The sample is gently shaken to eliminate any extra soil before being transferred to the laboratory in a sterile plastic bag. Bacteria were isolated according to the method described by Pérez-Rodriguez et al. (2020). Purified single colonies were preserved on nutrient agar slants for subsequent research (Admassie et al. 2022).

Source of pathogen (*Fusarium oxysporum*): The most virulent pathogen (*Fusarium oxysporum*) that causes chili pepper wilt disease was obtained from the Mycology Laboratory. These pathogens were previously identified from major chili pepper growing regions of Ethiopia, “Halaba Zone,” and we used them to evaluate the antagonistic activity of isolated rhizobacteria against them.

Screening rhizobacteria for antagonistic test: The potential of the rhizobacterial isolates to produce antifungal metabolites against *Fusarium oxysporum* was then assessed according to Kumar et al. (2012). On potato dextrose agar (PDA), a dual culture in vitro assay was used for this test. From an overnight culture, bacterial suspensions were first made. Exactly 50 µL of each culture was correspondingly inoculated 3 cm away from an agar plug containing mycelium, which was placed in the center of the PDA plate. As a control treatment, PDA plates were infected just with agar plugs containing the mycelium of each *F. oxysporum*. After seven days of incubation at 30°C, percent reduction was computed compared to a control (Skidmore & Dickinson 1976, Syed-Ab-Rahman et al. 2018). Hyphae morphologies in the surrounding area of bacterial colonies were observed under a compound light microscope (CETI MAX BINO, Belgium), where each experiment was triplicated and repeated.

Bacteria identification

Gram staining: Gram staining is typically considered the first essential test in the bacterial identification procedure. Isolated colonies were analyzed for colony morphology, Gram reaction, and cell morphology. A loop of culture was collected, and smears were produced on a clean glass slide. After drying, the smear was stained according to the protocols provided in Gram's staining, and the morphological characteristics were documented.

Culturing in various media: Bacterial cells were streaked on MacConkey Agar and Nutrient Agar, then incubated at 28℃ for 72 hours. The colony characteristics were evaluated after adequate growth and examined by the method described by Wang et al. (2019).

Isolation of fluorescent pseudomonads: Fluorescent pseudomonads are among the rhizobacteria renowned for producing a variety of microbial metabolites that can be utilized as antibiotics. Furthermore, it contains various plant growth-promoting characteristics, making this bacterium one of the well-known biocontrol and bio-fertilizer inocula for sustainable agricultural production (Haas & Défago 2005, Mishra & Arora 2018, Suresh et al. 2021). Hence, we were also interested in investigating our samples for potential fluorescent pseudomonads. To this end, chili pepper rhizosphere samples were collected from various locations in Ethiopia's East Shoa, Halaba, and Gurage zones and brought to the laboratory. Ten grams of chili pepper rhizosphere samples were dissolved in 90 mL of sterilized water and shaken at 120 rpm for 30 minutes (Shanmugaiah et al. 2006). The soil suspension was serially diluted and placed on King's B agar (King et al. 1954). Plates were incubated for 48 hours at 37℃ and examined at 365 nm using a UV illuminator. The purified single colonies were stored in 30% glycerol.

Biochemical tests on rhizobacteria for their traits associated with biocontrol of plant disease

Biocontrol associated traits: Several reports have previously verified that good biocontrol agents produce different enzymes like protease and chitinase (which they use it to degrade the cell wall of the fungal pathogen), and compounds like hydrogen cyanide, siderophore, and others, to deter pathogens from their vicinity (Sehrawat et al. 2022, Sriwati et al. 2023, Deb & Tatung 2024). Accordingly, we have evaluated our isolates of rhizobacteria to determine if they exhibit the biocontrol-associated traits listed below.

Test of protease production: On a modified skim milk agar (pH 7.0) containing (L^{-1}): skim milk powder (28.0 g), tryptone (5.0 g), yeast extract (2.5 g), agar (15.0 g) and dextrose (1.0 g), protease producing ability of the bacterial isolates was assessed (Adinarayana et al. 2003). Rhizobacterial cultures that had been cultivated overnight on YEP (yeast extract peptone) broth were streaked with cotton swabs on agar plates and kept at 28°C for 48 hours. Clear zones showed the presence of protease around the bacterial colony.

Test for HCN (Hydrogen cyanide) production: Using Alström and Burns' techniques, it was possible to qualitatively determine which bacterial isolates produced hydrogen cyanide (HCN) (Alström & Burns 1989, Sehrawat et al. 2022). On King's B medium that had 4.4 gL−1 of glycine added as a supplement, an overnight-grown bacterium in yeast extract peptone (YEP) was streaked with cotton swabs. Whatman no.

42 filter paper was cut to half the size of a Petri dish and sterilized before being soaked in a picric acid (0.5%) and sodium carbonate (2.0%) solution. The soaked filter paper was put in the lid of the petri dishes and sealed with the mother plates containing the inoculant using parafilm to prevent volatilization. These sealed plates were incubated at 28°C for 3-5 days, and the generation of HCN is indicated by a shift in the color of the filter paper from yellow to orange-red.

Test of chitinase production: By inoculating the bacterial isolates on colloidal chitin agar made up of the following (L−1): agar (20.0 g), colloidal chitin (20.0 g), K_2HPO_4 (0.7 g), KH_2PO_4 (0.3 g), $MgSO_4.5H_2O$ (0.5 g), $FeSO_4.7H_2O$ (1 mg), $ZnSO_4$ (1 mg), and $MnCl_2$ (1 mg), chitinase production ability was evaluated (Murthy & Bleakley 2012). Colloid chitin agar was inoculated with a total of 10 mL of overnight bacterial cultures grown in YEP (Yeast extract peptone) broth before being incubated at 28°C in the dark for 5 days. Clarification zones surrounding the bacterial colony on the agar were considered evidence of chitinase production.

Pyoverdine siderophore production test: In liquid succinate minimum media, the production of the siderophore pyoverdine was studied (Meyer & Abdallah 1978). At intervals of 24, 48, and 72 hours, the formation of the typical green-yellow fluorescence in culture supernatants was investigated under UV light at 360 nm, and the absorbance was measured at wavelengths ranging from 360 to 480 nm using a spectrophotometer (Dimkpa 2016). For liquid microbial cultures, SIM (Siderophore-inducing media) was prepared according to Alexander & Zuberer (1991) and Dimkpa (2016). The fluorescent green color of siderophore pyoverdine was further enhanced by the addition of a small amount of ZnO (Zinc oxide) coupled with aluminum (Al) in the Fe-deficient siderophore-inducing media (Fang et al. 2013, Dimkpa et al. 2015).

Evaluations of rhizobacteria for their disease-suppressing potential and improvement of yield and yield-related components under greenhouse conditions

Plant materials: Three varieties of chili pepper [‘Mareko Fana’ (local), ‘Melka Awaze’ (PBC600), and ‘Melka Zala’ (PBC972)] were used for the study as the plant materials. The seeds were obtained from the Melkassa Agricultural Research Center (MARC), Ethiopia. These three varieties were released by MARC in 1984, 2006, and 2004, respectively, and they are open-pollinated varieties.

Rhizobacteria: These seven rhizobacteria, provisionally coded as EBa_Bi23_1, EBa_Bi23_2, EBa_Bi23_3, EBa_Bi23_4, EBa_Bi23_5, EBa_Bi23_6, and EBa_Bi23_7, were selected based on their performance on the dual plate assay in the mycology laboratory, Addis Ababa University (AAU). Note: Once they are further evaluated and screened under field conditions (an ongoing activity), their codes will receive the simple prefix “AAU” and will be coded permanently as AAU (EBa_Bi23_X), where “X”

represents the seven Arabic numerals (1-7). This naming will remain the same for the final deposition and distribution to the scientific community and/or appropriate stakeholders. For instance, if someone or an institute is interested in the isolate "EBa_Bi23_6", they can request it as AAU (EBa_Bi23_6).

Pots: Plastic pots with a 18 cm × 18 cm size were obtained from the market. The pots were filled with a sterile topsoil (5 kg pot^{-1}).

Treatments and experimental design: The treatments consisted of three varieties of chili pepper ['Mareko Fana' (local), 'Melka Awaze' (PBC600), and 'Melka Zala' (PBC972)], seven rhizobacteria (EBa_Bi23_1 to EBa_Bi23_7), and one negative control. The experiment was laid out as a completely randomized design (CRD) with five replications per treatment.

Experimental procedures: To evaluate the effectiveness of rhizobacteria in preventing *Fusarium oxysporum*, a pathogen of wilt disease on chili peppers (*Capsicum annuum* L.), a greenhouse experiment was conducted using pots. We used a heated greenhouse with temperatures ranging from 27 to 31°C and an average soil pH of 5.8. Before sowing the chili pepper seeds into the pots, they were surface-sterilized for five minutes with 70% (v/v) ethanol, followed by another five minutes with 5% sodium hypochlorite, and then three rinses with distilled water. According to Lotfy & Moustafa (2021), and Syed-Ab-Rahman et al. (2018) sterilized seeds were submerged in a bacterial suspension containing 10^8CFU mL−1(OD600nm of 0.1) for one hour. The bio-primed chili pepper seeds were then sown in the plastic pots (18 cm × 18 cm in size) filled with a sterile soil (5 kg pot^{-1}). Five seeds were sown in each pot at a soil depth of 1.5–2.0 cm. One plant was kept in each pot after the seeds had fully sprouted, and the rest were thinned out by uprooting. For the control treatment, only plants that had been exposed to the pathogen (negative control) were used. As soon as the bio-primed seeds were sown, 15 mL of the *F. oxysporum* inoculum was introduced to the pot at a rate of 1 × 105 spores/mL to create a fungal-infested soil (Zhang et al. 2022). To maintain the soil moisture level in the pots at field capacity, the pots were watered at one-week intervals.

We measured the plants' degree of wilting (when the plant showed disease symptoms), yield i.e. dried fruits weight (when the crop's fruit becomes completely red and dry) and yield related components such as shoot length, root length, root fresh weight, root dry weight and fruit number (when the plant reached on its physiological maturity). Koch's postulates were fulfilled by re-isolating the pathogen from the infected plants with characteristic symptoms and morphologically identifying it. The modified scale (Horsfall 1945) was used to rate the disease severity (0 up to 6 scales) with 0 denoting no disease observed, 1 denoting slight stunting, 2 denoting slight stunting and chlorosis of leaves, 3 denoting 10% or less of the plant showing symptoms of wilt, 4

denoting 11–25% of the plant showing symptoms, 5 denoting 26–50% of the plant, and 6 denoting 51–100% of the plant exhibiting symptoms or plant death. According to McKinney (1923), plant disease severity and infection rate are expressed in percentage as follows:

$$Plant\ Disease\ Severity\ (\%) = \frac{\sum(n \times v)\ \times 100}{N \times V}$$

where n is the number of assessed plants in each score of disease symptoms, v is the specific symptoms scored, N is the sum of all plants examined, and V is the highest value of symptoms category.

Data analysis

A two-way analysis of variance (ANOVA) was conducted to determine the effects of rhizobacteria and varieties on the variables studied (Plant disease severity, yield of dried fruits, seed germination percentage, and yield-related components). However, before proceeding, the assumptions of the ANOVA were verified using the Kolmogorov-Smirnov test for the normality of the data distribution and Levene's test for homogeneity of variances. Results that showed significant statistical differences were separated with Tukey's HSD (Honestly Significant Differences), and values of probability ≤0.05 at the α level were considered statistically significant in the analysis. We used R Statistical software (v4.1.1; R Core Team 2021), running in R Studio (version 1.4.1717, Public Benefit Corporation), and Microsoft Excel 2016.

RESULTS

Antagonistic activity test

Bacteria isolated from the rhizosphere of chili pepper were tested for their antagonistic effect on one of the most virulent pathogens that causes the wilt disease on the crop. This virulent fungal pathogen is *Fusarium oxysporum*. The bacterial isolates inhibited the growth of the pathogen on the plates by about 62%. We identified promising isolates having from high to moderate inhibition effects on *F. oxysporum*. Approximately 39% of the putative antagonistic rhizobacteria obtained belong to the genus *Bacillus*, and 26% to the genus *Pseudomonas*, with the remainder unidentified at this time. *Bacillus* (EBa_Bi23_6, EBa_Bi23_5) and *Pseudomonas* species (EBa_Bi23_4) were identified as the most promising rhizobacterial biocontrol agents in our studies.

All the candidate isolates were maintained for further evaluation and screening.

Figure 1. Inhibition of mycelial growth of *Fusarium oxysporum* by *Bacillus* sp. on dual culture on PDA. A 10-day-old culture of *Fusarium* was picked by a cork borer and placed at the center of the plate, while 50µl of an overnight culture of biocontrol agent (*Bacillus* sp.) was inoculated at a 3cm distance from *F. oxysporum* (B). As a negative control (A), potato dextrose agar (PDA) plates were infected with the mycelium of *F. oxysporum* without the biocontrol agent. (C) is colony of *Bacillus* sp.on NA (Nutrient Agar).

Biochemical tests on rhizobacteria for the traits associated with biocontrol of plant disease

Based on dual culture performance, seven rhizobacteria (EBa_Bi23_1 to EBa_Bi23_7) were selected and evaluated for traits associated with biocontrol agents, yielding promising results (Figure 2 and Table 1).

Test of protease production

The isolates of rhizobacteria were evaluated for their potential to produce one of the cell wall-degrading enzymes (Protease). The isolates produced clear zones around the colony. In the milk agar medium where the isolates were not inoculated, no clear zones were observed. This indicates that the clear zones produced on the experimental plates were from the test isolates (Figure 2A).

Test for HCN (Hydrogen cyanide) production

The hydrogen cyanide production by bacterial isolates was assessed qualitatively, as indicated by a change in the color of the filter paper. When the filter paper turned from yellow to a reddish-brown color, it was inferred that the isolates were producing hydrogen cyanide. Accordingly, three out of the seven isolates evaluated produced hydrogen cyanide in a strong manner (Table 1 and Figure 2B).

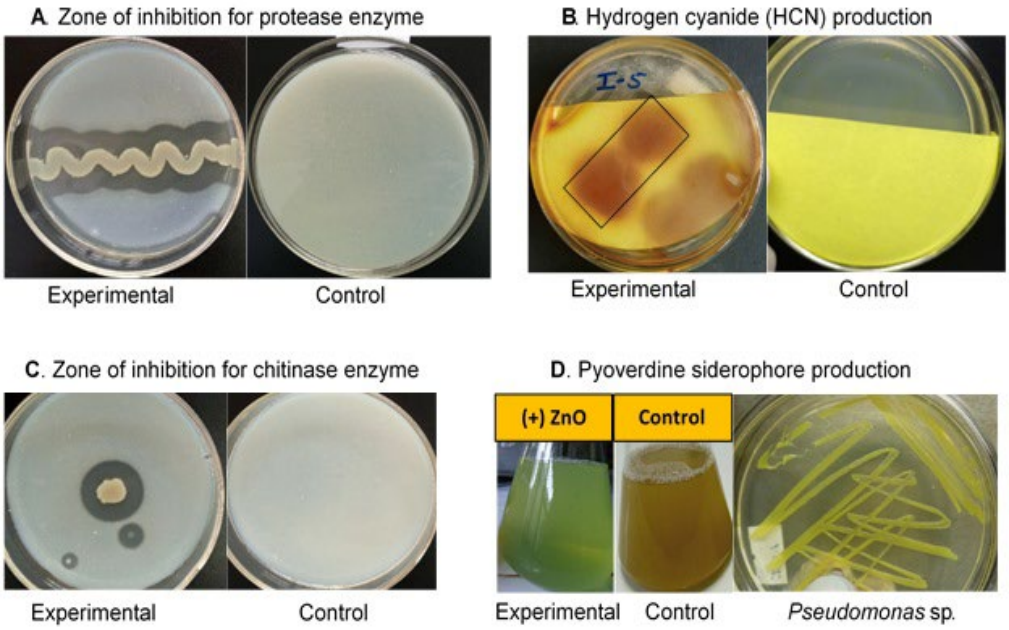

Figure 2. The qualitative biochemical tests on rhizobacteria for the traits associated with biocontrol of plant disease. (A) production of protease enzyme, (B), hydrogen cyanide production, (C) chitinase production, and (D) pyoverdine siderophore production.

Test of chitinase production

The rhizobacteria were evaluated for their potential to produce one of the cell wall-degrading enzymes (Chitinase). The ability to produce chitinase was determined by culturing the isolates on colloidal chitin agar. The presence of

clarification zones surrounding the bacterial colony on agar was thought to indicate the synthesis of chitinase. The colloidal chitin agar that was not inoculated with the test isolate did not produce any clear zone (Control), suggesting that the clear zones observed were from the test rhizobacterial isolate (Figure 2C).

Test of pyoverdine siderophore production in a liquid medium

In the qualitative-based evaluation of the isolates for their potential to produce pyoverdine siderophore using siderophore-inducing medium, it was found that *Pseudomonas* sp. produced an intense green color. This siderophore, released into the rhizosphere by the biocontrol agent, is known to chelate iron during competition with the pathogen in the soil. Thus, it can be considered as one of the essential traits of a biocontrol agent. In the control treatment, where iron was sufficiently available, the isolate did not produce the green color (Figure 2D).

Table 1. Summary of biochemical traits in rhizobacterial isolates (EBa_Bi23_1 to EBa_Bi23_7) and control across selective media.

Rhizobacteria	Protease	HCN	Chitinase	Pyoverdine siderophores
EBa_Bi23_1	+++	++	++	++
EBa_Bi23_2	++	+++	+++	+++
EBa_Bi23_3	+++	+++	+++	+++
EBa_Bi23_4	++	+++	+++	+++
EBa_Bi23_5	++	++	++	++
EBa_Bi23_6	+	++	++	+
EBa_Bi23_7	++	+	+++	+++
Negative control	−	−	−	−

Ratings: (−) negative, (+) weak, (++) moderate, (+++) strong. Protease (Protease enzyme production test), HCN (Hydrogen cyanide production test), Chitinase (Chitinase enzyme production test), pyoverdine siderophore (Pyoverdine siderophore production test).

Evaluations of rhizobacteria for their disease-suppressing potential and improvement of chili pepper yield and yield-related components under greenhouse conditions

The rhizobacteria assessed in pot experiments successfully decreased the disease severity by 86.5% and increased the yield of dried fruits by 35.9%

regardless of the rhizobacterial and varietal differences. Root and shoot growth were also increased by 34% and 35% respectively, for the ‘Mareko Fana’ (The most susceptible variety), regardless of the rhizobacterial differences.

The Tukey’s HSD test results (Table 3) show significant ($P < 0.001$) differences among rhizobacteria in their capabilities to suppress wilt disease across all experimented varieties compared to the negative control. For instance, if we consider the ‘Mareko Fana’ variety, there were wilt disease suppression potential differences among the rhizobacterial isolates designated as EBa_Bi23_2 and EBa_Bi23_5, EBa_Bi23_2 and EBa_Bi23_6; EBa_Bi23_3 and EBa_Bi23_5, EBa_Bi23_3 and EBa_Bi23_6; EBa_Bi23_5 and EBa_Bi23_7, and EBa_Bi23_6 and EBa_Bi23_7. A maximum of 90.3% and 88.8% disease suppression was observed for ‘Mareko Fana’ and ‘Melka Awaze’ varieties, respectively, when inoculated with isolate EBa_Bi23_6. ‘Mareko Fana’ was found to be the most susceptible variety to the disease as it exhibited the highest wilt disease severity of 86.4% (Table 3). Excluding the differences among the rhizobacteria and varieties, the disease was reduced by 86.5% in the experimental treatments compared to the negative control.

The inoculation of rhizobacteria onto the crop resulted in a positive interaction effect on the yield of dried fruits and yield-related components across the experimental crop varieties. Tukey’s HSD test results showed significant differences in the isolates’ performances, except for fruit number, compared to the negative control. For instance, if we consider the most susceptible variety alone (‘Mareko Fana’), the DFW was increased by 35% ($P < 0.001$), RDW by 16% ($P <0.001$,) RFW by 25% ($P < 0.001$), RL by 34% ($P < 0.05$), SL by 35% ($P <0.001$) and SG by 14% ($P <0.01$). Significant ($P <0.001$) differences were also observed in root dry weight enhancement for the same variety between EBa_Bi23_3 and EBa_Bi23_5. Overall, an improvement of about 35.9% was observed due to the application of the rhizobacteria on one of the most essential response variables (Dried fruits weight (DFW)).

DISCUSSION

Dual culture plate assays enabled us to screen isolates with the most

antagonistic activity against the deadly soil-borne pathogens causing the wilt disease of chili peppers (*Fusarium oxysporum*). This finding confirms the availability of potential biocontrol agents for further evaluation (Figure 1). The observed moderate (≥51.4% inhibition zone) to strong (≥62% inhibition zone) inhibitory activities within the individual isolates against *F. oxysporum* indicate the presence of promising biocontrol-associated traits in the isolates, which can be used to characterize the mechanism of the antagonism. Numerous biocontrol agents have been investigated and reported from rhizobacteria for the management of plant diseases (Dukare & Paul 2021, Admassie et al. 2022, El-Saadony et al. 2022), which corroborate the findings of this study.

The biochemical tests (Table 1) revealed that the mechanism of antagonism in the biocontrol agents that deter the pathogen from approaching their vicinity is attributable to the production of enzymes that degrade the pathogen's fungal cell walls, such as protease and chitinase [Figure 2 (A and C)], respectively. Some of the isolates also produced hydrogen cyanide (Figure 2B), a highly volatile, colorless, and extremely toxic substance, as well as pyoverdine siderophore (Figure 2D), which is a chemical that scavenges iron from the environment (Sehrawat et al. 2022, Roca-Couso et al. 2021). This suggests that biocontrol agents may tactically release different enzymes or molecules, either one at a time or all at once, as a multi-pronged defense to deter the pathogen from their vicinity, primarily to compete for growth resources (Pandit et al. 2022). Until now, rhizosphere bacteria, such as *Bacillus* sp., have received considerable attention for their role in controlling various soil-borne diseases, including *Fusarium* wilt (Meng et al. 2016). This laboratory and greenhouse evaluation result confirmed that *Bacillus* sp. significantly inhibited the pathogen and minimized the wilt disease, thus complementing the importance of considering biocontrol-associated traits during the screening of effective biocontrol agents from rhizobacteria. This might be attributed to the defensive mechanism of biocontrol agents against pathogens.

The analysis of variance revealed that disease severity was significantly ($P<0.05$) affected by the main effects of varieties, rhizobacteria, and their interaction (rhizobacteria by varieties) ($P<0.001$) (Table 2). Most notably, the combined effect analysis (Table 2) of varieties and rhizobacteria from the pot experiment showed statistical significance for DS (Disease severity) at $P<0.001$,

and the disease was decreased by 86.5% (Figure 3A and Table 3). The lowest disease severity score (9.1%) across the three varieties was associated with rhizobacterial isolate designated as EBa_Bi23_6, followed by 9.3% with EBa_Bi23_5 and 10.7% with EBa_Bi23_4 (Table 3).

Table 2. Mean square values for the effects of 7 bioinoculants and 3 chili pepper varieties on wilt severity and yield traits (Greenhouse experiment).

Source of variations	DF	DS	DFW	RFW	RDW	RL	SL	FN	SG
Varieties	2	10.0*	284.02***	36.93***	4.06ns	11.91*	149.2***	0.13ns	107.63**
BI	7	9818.9***	1517.33***	810.45***	180.36***	196.7***	3041.4***	2.67*	358.81***
Varieties*BI	14	11.8***	127.48***	14.34***	28.04***	6.23*	64.9***	2.87**	44.84**
Error	96	2.5	12.45	4.01	4.59	3.44	19.91	1.18	17.99
CV (%)		7.79	4.73	2.37	5.53	6.66	3.94	21.12	4.63

Chili pepper varieties: 'Ma. Fana' (Local), 'M. Awaze' (PBC600), and 'M. Zala' (PBC972). Bioinoculants: EBa_Bi23_1 to EBa_Bi23_7 and control. Two-way ANOVA: * P < 0.05, ** P < 0.01, *** P < 0.001. BI=Bioinoculants (Rhizobacteria); CV=coefficient of variation (%); DF=Degrees of freedom; DS=Disease severity; DFW= Dried fruits weight (g); RFW=Root fresh weight (g); RDW=Root dry weight (g); RL=Root length (cm); SL=Shoot length (cm); FN=Fruit number; SG=Seed germinations (%).

The pathogen's severity on the chili pepper was reduced, and the yield and yield-related components were enhanced upon the administration of rhizobacteria. This suggests that rhizobacteria can play a crucial role in defending plants against pathogens on a systemic level (Kai et al. 2016, Meena et al. 2020). This finding is consistent with the results of a recent study conducted by Liu et al. (2018), which found that isolates of rhizobacteria promoted plant growth and acted as biological control agents against several plant diseases through mechanisms of antagonistic relationships and systemic resistance. Several research findings have been reported on the effectiveness of rhizobacteria, such as *Bacillus* species, as biocontrol agents and biofertilizers (Wang et al. 2013). This suggests that the application of these bacteria in organic farming may not only suppress pathogenic microbes but also create favorable conditions in the rhizosphere, thereby facilitating the mobilization and uptake of

nutrients by plants (Bach et al. 2016).

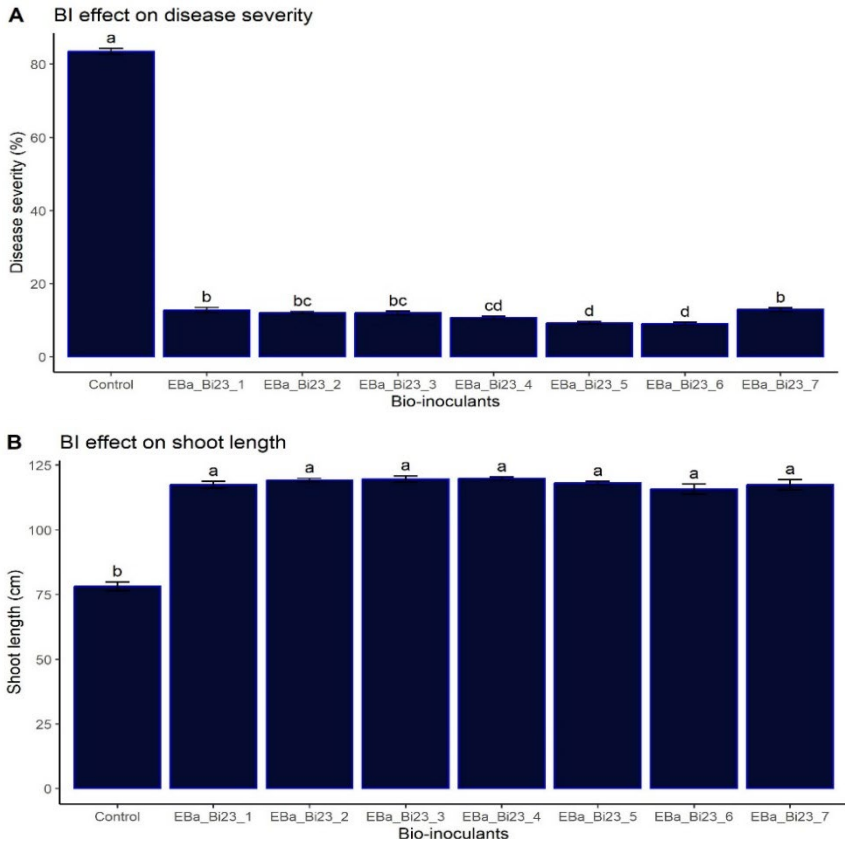

Figure 3 A-B. Effects of 7 bioinoculants (Rhizobacteria) and control on disease severity, yield, root, and shoot length of chili pepper (Greenhouse experiment). (A) is disease severity (%), (B) is shoot length (cm). Note: The error bars correspond to standard error. For each panel on “A”, the letters “b, bc, cd, and d” represent data that are significantly different from the control (a) at P<0.001. For each panel on “B the letter “a” represents data that are significantly different from the control (b) at P<0.001. Control is seed that has not been treated with inoculants.

Rhizobacteria are selectively attracted to and maintained by the rhizosphere soil and plant roots for the mutualistic benefit (Kumar et al. 2012). The present results of the greenhouse evaluation have confirmed that the studied variables were significantly influenced by rhizobacteria. This may

validate the need to evaluate multiple rhizobacteria against virulent pathogens to identify potential biocontrol agents. The analysis of variance revealed that all the test isolates equally significantly (P<0.001) enhanced shoot length of the chili pepper by about 34.03% (Table 4) when compared to the negative control (Figure 3B).

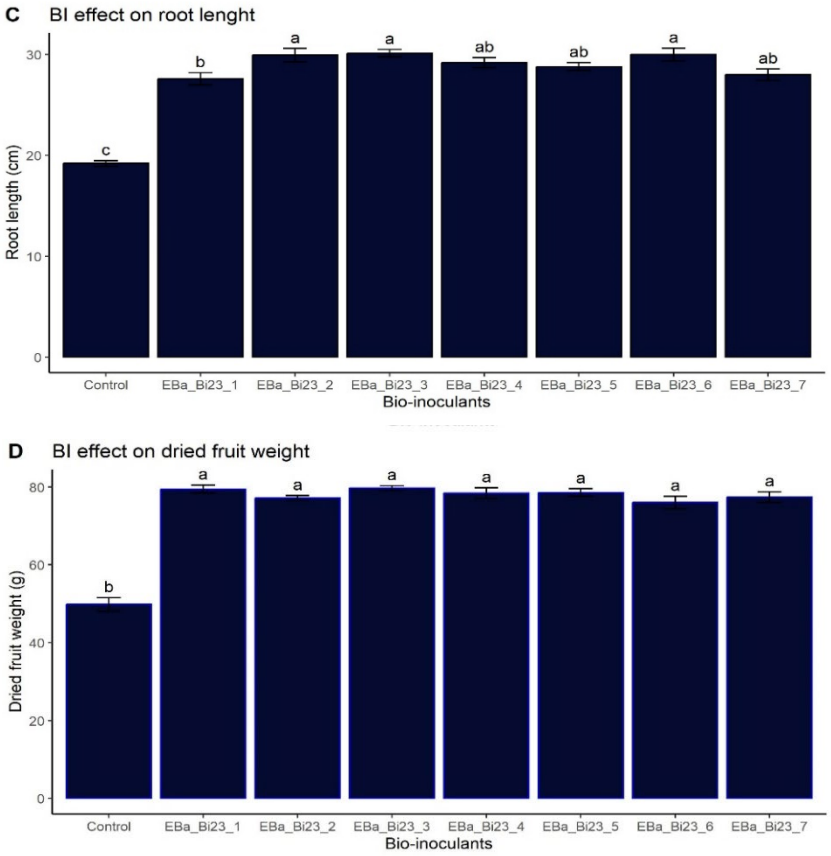

Figure 3 C-D. Effects of 7 bioinoculants (Rhizobacteria) and control on disease severity, yield, root, and shoot length of chili pepper (Greenhouse experiment). (C) is root length (cm), and (D) is dried fruit weight (g), of chili pepper. Note: The error bars correspond to standard error. For each panel on “C”, the letters “a, b, and ab” represent data that are significantly different from the control (c) at P<0.05. For each panel on “D”, the letter “a” represents data that are significantly different from the control (b) at P<0.001. Control is seed that has not been treated with inoculants.

Table 3. Mean values for the effects of 7 rhizobacteria and 3 chili pepper varieties on wilt disease severity (greenhouse experiment).

Rhizobacteria	Mean values (%) of wilt disease severity			
	Mareko Fana ($\bar{X}$±sd)	Melka Awaze ($\bar{X}$±sd)	Melka Zala ($\bar{X}$±sd)	Mean disease severity (%) for each rhizobacterium
EBa_Bi23_1	$10.8±2.2^{abcd}$	$12.8±2.4^{bcde}$	$14.8±1.6^{e}$	12.8
EBa_Bi23_2	$12.6±1.1^{bcde}$	$11.6±1.3^{abcde}$	$12.0±1.2^{abcde}$	12.1
EBa_Bi23_3	$12.6±0.9^{bcde}$	$13.2±2.4^{cde}$	$10.2±0.5^{abc}$	12.0
EBa_Bi23_4	$10.0±1.2^{abc}$	$11.8±1.6^{abcde}$	$10.4±0.6^{abcd}$	10.7
EBa_Bi23_5	$8.8±0.8^{a}$	$10.0±0.0^{abc}$	$9.2±1.8^{ab}$	9.3
EBa_Bi23_6	$8.4±0.6^{a}$	$9.4±0.9^{ab}$	$9.6±2.2^{abc}$	9.1
EBa_Bi23_7	$12.8±1.6^{bcde}$	$14.0±2.8^{de}$	$12.0±1.0^{abcde}$	12.9
Negative control	$86.4±2.5^{g}$	$83.8±1.8^{fg}$	$80.4±0.6^{f}$	83.5
Disease severity (%) on each variety	20.3	20.8	19.8	
	SEM(±) = 0.71	CV(%) = 7.8		

Tukey's HSD post hoc test at 5% significance level applied to greenhouse data on wilt severity (%). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means sharing the same letter are not significantly different ($p < 0.05$, Tukey's HSD). SEM = Standard error of the mean; CV = Coefficient of variation; SD = Standard deviation; $\bar{X}$ = Sample mean. Negative control: Plants inoculated with pathogens only (No rhizobacteria). Chili pepper varieties: 'Mareko Fana', 'Melka Awaze', and 'Melka Zala'. Rhizobacteria: EBa_Bi23_1 to EBa_Bi23_7 and control. Wilt severity is recorded post-germination, upon the appearance of symptoms.

This indicates that the rhizobacteria might have common traits that boosted the quantity of easily accessible mineral nutrients, controlled the amounts of phytohormones, and phytopathogens as suggested by Hernández-León et al. (2015). Similarly, the same isolates significantly ($P < 0.05$) increased root growth by 33.99% (Table 4) compared to the negative control (Figure 3C). The HSD post hoc test analysis revealed that there were statistical differences in performance between EBa_Bi23_1 and EBa_Bi23_2; EBa_Bi23_1 and EBa_Bi23_3; EBa_Bi23_1 and EBa_Bi23_6 (Figure 3C). This suggested that the rhizobacteria may have caused inhibition by inducing various biochemical activities against the pathogen, which may have differed in quality, quantity, and

type during the interaction (Lee et al. 2023).

Table 4. Mean values for the effects of 7 bioinoculants +control and 3 chili pepper varieties on yield and yield related components at 5% Tukey's HSD test. Unit of measurements: Fruit and root weight (g), root and shoot length (cm), seed germination (%)

Treatments		**Mean values for chili pepper yield and yield-related components**						
Varieties	**Bio-inoculants**	**DFW ($\bar{X}$±sd)**	**RDW ($\bar{X}$± sd)**	**RFW ($\bar{X}$±sd)**	**RL ($\bar{X}$± sd)**	**SG ($\bar{X}$± sd)**	**SL ($\bar{X}$± sd)**	**FN ($\bar{X}$±sd)**
Mareko Fana	EBa_Bi23_1	74.4±2.9^{bcd}	40.2±3.6^{cdef}	84.0±4.4^{b}	28.0±3.67^{bcde}	89.0±3.32^{cd}	113.0±6.8^{d}	4.6±1.3^{ab}
	EBa_Bi23_2	77.8±2.9^{bcdefg}	37.8±1.6^{bcde}	85.0±0.0^{b}	31.2±2.28^{de}	94.8±2.68^{cd}	120.6±2.1^{d}	5.0±1.0^{ab}
	EBa_Bi23_3	81.4±1.9^{defg}	36.0±0.0^{bc}	87.0±3.9^{bcd}	28.8±1.3^{bcde}	96.4±0.55^{d}	118.2±5.8^{d}	6.6±1.3^{b}
	EBa_Bi23_4	78.0±0.0^{bcdefg}	40.0±3.0^{cdef}	87.0±0.0^{bcd}	29.0±0.71^{bcde}	95.0±0^{cd}	118.8±1.9^{d}	4.8±0.4^{ab}
	EBa_Bi23_5	80.8±0.4^{cdefg}	42.2±0.8^{ef}	87.0±1.2^{bcd}	30.2±1.64^{bcde}	89.8±3.49^{cd}	120.6±2.7^{cd}	5.8±0.4^{ab}
	EBa_Bi23_6	77.0±2.7^{bcdef}	40.6±0.5^{cdef}	85.6±0.5^{b}	32.0±1^{e}	87.8±0.84^{bcd}	108.2±9.5^{d}	5.2±1.3^{ab}
	EBa_Bi23_7	75.6±0.5^{bcde}	39.0±1.0^{cdef}	87.8±1.6^{bcd}	28.8±2.49^{bcde}	91.6±3.29^{cd}	116.4±9.7^{d}	4.2±1.8^{ab}
	Control	**50.6±5.3^{a}**	**33.0±5.6^{ab}**	**64.8±3.4^{a}**	**19.6±1.1^{a}**	**78.8±1.3^{ab}**	**76.2±4.6^{b}**	**5.4±1.1^{ab}**
Melka Awaze	EBa_Bi23_1	78.0±0.0^{bcdefg}	41.2±1.8^{def}	87.4±1.3^{bcd}	26.8±1.9^{bc}	93.2±2.17^{cd}	120.6±1.5^{cd}	6.4±1.3^{ab}
	EBa_Bi23_2	79.4±2.6^{bcdefg}	44.0±0.0^{f}	90.8±1.3^{cd}	28.6±3.0^{bcde}	94±2.92^{cd}	119.2±1.6^{d}	4.4±0.9^{ab}
	EBa_Bi23_3	86.0±0.0^{g}	39.8±2.9^{cdef}	91.2±0.4^{d}	31±0.7^{cde}	96.8±0.45^{d}	119±4.6^{cd}	5.2±0.8^{ab}
	EBa_Bi23_4	86.0±1.2^{g}	41.8±2.5^{ef}	87.8±0.4^{bcd}	29.0±1.4^{bcde}	91.8±3.83^{cd}	119.6±1.9^{d}	5.6±0.9^{ab}
	EBa_Bi23_5	83.2±4.4^{efg}	36.2±0.4^{bcd}	87.8±2.2^{bcd}	28.6±0.5^{bcde}	96.2±0.45^{d}	116.8±1.8^{d}	4.6±1.8^{ab}
	EBa_Bi23_6	75.8±7.5^{bcdef}	36.2±0.4^{bcd}	84.4±0.5^{b}	29.8±3.0^{bcde}	96.2±2.59^{d}	121.0±1.2^{c}	5.2±1.6^{ab}
	EBa_Bi23_7	84.0±5.4^{fg}	39.4±2.9^{cdef}	85.2±0.8^{b}	26.4±1.9^{b}	93.4 2.6^{cd}	121.2±2.7^{cd}	4.2±0.4^{ab}
	Control	**46.0±3.7^{a}**	**29.8±1.1^{a}**	**68.8±0.4^{a}**	**18.8±1.5^{a}**	**85.6±8.9^{bc}**	**85.0±5.2^{ab}**	**5.2±2.0^{ab}**
Melka Zala	EBa_Bi23_1	86.0±3.5^{g}	39.8±0.4^{cdef}	85.0±2.2^{b}	28±0.7^{bcde}	95.6±3.3^{cd}	118.6±1.7^{cd}	5.2±0.4^{ab}
	EBa_Bi23_2	74.4±7.1^{bcd}	41.6±0.5^{ef}	86.4±1.1^{bc}	30.0±2.0^{bcde}	95.0±1.2^{cd}	118.0±3.3^{cd}	4.0±0.0^{a}
	EBa_Bi23_3	71.6±0.5^{b}	41.2±0.4^{def}	86.4±1.5^{bc}	30.6±1.1^{bcde}	93.8±3.9^{cd}	121.8±2.6^{d}	4.0±0.0^{a}
	EBa_Bi23_4	71.2±0.8^{b}	41.4±1.3^{ef}	88.2±0.8^{bcd}	29.6±3.1^{bcde}	87.8±7.4^{bcd}	121.4±2.1^{d}	5.6±0.9^{ab}
	EBa_Bi23_5	71.8±0.4^{b}	41.6±0.9^{ef}	88.6±0.9^{bcd}	27.6±0.9^{bcd}	95±2.7^{cd}	117.0±0.7^{cd}	6.0±0.0^{ab}
	EBa_Bi23_6	75.2±5.7^{bcde}	39.8±4.0^{cdef}	85.6±2.3^{b}	28.2±1.1^{bcde}	91.4±9.5^{cd}	117.8±1.8^{cd}	6.0±0.7^{ab}
	EBa_Bi23_7	72.6±1.9^{bc}	39±2.2^{cdef}	88.0±2.5^{bcd}	28.8±1.1^{bcde}	92±8.9^{cd}	114.6±8.3^{cd}	5.0±0.0^{ab}
	Control	**53.0±4.1^{a}**	**28.6±0.5^{a}**	**65.6±3.4^{a}**	**19.2±0.8^{a}**	**75.4±2.2^{a}**	**73.2±3^{a}**	**5.0±1.0^{ab}**
SEM (±)		1.58	0.96	0.89	0.83	1.9	2.00	0.48
CV (%)		4.73	5.53	2.37	6.66	4.63	3.94	21.12

Root length was significantly (P<0.001) affected by the main effects of rhizobacteria, while the effects of varieties and the interaction between varieties and rhizobacteria were statistically significant (P<0.05) (Table 2).

The rhizobacteria and varieties interacted to significantly (P<0.001) influence one of the most important variables studied, i.e., dried fruits weight, which is the economic part of the plant. The dried fruits weight (DFW) was increased by 35.9% (Figure 3D and Table 4). In the same way, the yield-related components such as root fresh weight and root dry weight were significantly (P<0.001) affected by the main and interaction effects of varieties and rhizobacteria except by the main effects of varieties on root dry weight. Fruit numbers were not affected by the main effects of varieties; however, they were affected by the main effects of rhizobacteria at p< 0.05 and by the interaction of varieties and rhizobacteria at (P<0.01) (Table 2**)**. This indicates the remarkable impact variety has on the outcome variable (e.g., disease severity, yield, and or yield related components), which could depend on the level of the other factor (e.g., types of rhizobacteria) and vice versa. Generally, nearly every bacterial isolate investigated showed the ability to produce biocontrol-associated traits. Consistent with these findings, Raaijmakers et al. (2010) reported that especially if the bacteria are from the genera *Pseudomonas, Bacillus,* and *Streptomyces*, they are well known to possess numerous interesting traits in the fields of agricultural/industrial/medical biotechnology.

CONCLUSIONS

In this study, isolates from *Bacillus* and *Pseudomonas* genera that displayed moderate to strong antagonistic activities against the chili pepper pathogen (*F. oxysporum*) were identified. These isolates possessed considerable biocontrol-associated traits, which makes them potential candidates for biocontrol agents. Under greenhouse conditions, these isolates successfully reduced the severity of *Fusarium* wilt disease by 86.5% and increased the yield of dried fruits by 35.9% at P < 0.001. In addition to controlling the pathogen, the isolates may have also improved the root and rhizosphere of the chili pepper and enhanced the mobilization and uptake of nutrients from the soil by the plant, thereby

enhancing its growth. The results imply that the identified isolates can play an indispensable role in controlling the *Fusarium* wilt disease of chili pepper and enhance the crop yield, which is an environmentally friendly approach. Since the soil used in our study was sterilized, it probably had a much lower microbial load than field soil. Hence, we suggest that future work should support studies in a controlled environment with an outdoor experiment.

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