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Full Length Research Paper

In-vitro antifungal activity of rhizospheric *Bacillus* spp. of faba bean (*Vicia faba* L.) in Bale Zone, South-eastern Ethiopia against *Botrytis fabae* and their Plant growth promoting traits

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Abstract

Bacillus species are potentially used to stimulate plant growth and to protect plants from phytopathogens. The antifungal activity of rhizospheric Bacillus isolates of faba bean ((Vicia fabae L.) from the districts of Bale zone, south-eastern Ethiopia against a phytopathogenic fungus Botrytis fabae were evaluated. Soil samples were collected from the rhizosphere of faba bean varieties (Shallo, Mosisa and Wolki) *in the three districts of Bale Zone (Goba, Sinana and Dinsho) and screened for Bacillus spp. Dual culture assay and in-vitro techniques were employed for this study. Descriptive statistics and analysis of variance (ANOVA) with a General Linear Model were used for analysis of data. Difference among means were significant at $P < 0.05$. Sixteen Bacillus isolates were employed for in-vitro test and all of them were able to grow at high temperatures (50 °C and 55°C) and tolerated high NaCl (7–10%). All isolates exhibited in-vitro antifungal activity against B. fabae. The percent inhibition of radial growth significantly varied among Bacillus isolates and faba bean variety ($P < 0.05$), but not among districts ($P > 0.05$). The highest percent inhibition of radial growth was exhibited by Bacillus isolates G3 (96.53 ± 1.14) followed by isolate D12 (91.11 ± 2.94) and S5 (81.48 ± 3.20). Our Bacillus isolates exhibited an appreciable enzymatic activity, phosphate and ZnO solubilization activity. IAA and HCN were produced by most of the isolates. About 68.8% of our Bacillus isolates were positive for at least five of plant growth promoting rhizobacteria traits. The results of this study revealed a promising antifungal activity of Bacillus spp. against chocolate spot disease and for their plant growth promoting traits. Further in-vivo study is recommended for Bacillus isolates G3, D12 and S5 in focus as they could be potential inoculants in suppression of B. fabae and for increasing productivity of faba bean*

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1. Introduction

Faba bean is botanically known as *Vicia fabae* L., with the common names such as broad bean, horse bean, tick bean and field bean. It is a good source of protein (20–35%), fiber (10–15%) and rich in carbohydrates (55–65%); but it contains low fat (1–2%), minerals and vitamins (Badjona et al., 2023). It is a cool season crop that grows all over the globe for food, feed, and ecosystem services. China is the leading producer in both area harvested and production (807.4 thousand hectares, 1.7 million tons) followed by Ethiopia (491.9 thousand hectares, 1.12 million tons) (FAOSTAT, 2024).

Both abiotic and abiotic factors are accountable for reduction in the yield of faba bean (Sahile et al., 2008 a). Among faba bean diseases, chocolate spot disease caused by a fungal pathogen *Botrytis fabae* has been the major fungal diseases devastating faba bean production (Lee et al., 2020), in Ethiopia and elsewhere (Sahile et al., 2008 a, b; Yitayih and Azmeraw, 2018). In Ethiopia, the disease may occur among all the agro-ecological zones, but it is highly prevalent in areas of high rainfall (>900 mm) and high elevation (>2000 m.a.s.l) (Sahile et al., 2008 a). Mean disease incidence of chocolate spot reported to be 70.9 to 93.2% in eight districts of Bale highlands (i.e., Agarfa, Dinsho, Gasera, Goba, Gololcha, Goro, Ginir, and Sinana) during the main cropping season of 2017 while percentage severity index (PSI) ranged from 10.5 to 47.1% (Eshetu et al. 2018). The disease was also reported from different parts of Ethiopia with varied level of disease prevalence, incidence and severity (Wakoya et al., 2021; Legesse, 2025). Yield losses as a result of chocolate spot disease is estimated as 60-80% among susceptible cultivars and up to 34% among tolerant cultivars in African regions, Nile Delta or Morocco (Bouhassan et al., 2004; Sahile et al., 2008 a). Yield losses substantially impact smallholder farmers who principally depend on faba bean for food and income.

For the control of chocolate spot of faba bean, integrated disease management strategies such as selection of more resistant varieties, use of clean seed, crop rotation, reduced planting density and chemical fungicides (Sahile et al., 2008 b; Sahile et al., 2018; Zeleke and Legesse, 2023). Chemical fungicides have been implicated to have deleterious effects on the human health and on useful crops and organisms; they cause environmental pollution; and leave toxic residues in certain crops, and they are also responsible for development of pathogenic resistant strains (Walia et al., 2014). In Ethiopia, screening of resistant faba bean varieties was made as a strategy to mitigate the disease (Zeleke and Legesse, 2023). Yet, there is a limitation in provision of resistant varieties that are adapted to a range of growing regions to farmers (Sillero et al., 2010).

Biological control using antagonistic microorganisms is a feasible alternative to the use of artificial chemicals, and is currently becoming the crucial component for disease management (Lahlali et al., 2022). The bacteria that live in the rhizosphere are known as plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980), offer an option to reduce or replace chemical fertilizers and improve soil fertility in agriculture (Ramakrishna et al., 2019). Among PGPR, *Bacillus* spp. have been identified as the major genus that are used as biofertilizer, biopesticide and/ or biocontrol agents (BCAs) for sustainable crop production (Shafi et al., 2017; Yadav et al., 2021). A study revealed that biotic and abiotic factors that have a harmful effect on a plant are alleviated by *Bacillus* spp. induced physiological changes in plants (Radhakrishnan et al., 2017). *Bacillus* spp. have the ability to resist pesticides and survive under unfavorable environmental conditions such as a high salinity, extreme pH, and high temperatures in the field owing to the production of endospores (De Vos et al., 2009; Jiao et al., 2021). Biocontrol *Bacillus* strains have an incredible potential in reshaping other plant microbiomes such as *Pseudomonas* spp. through

biofilm formation for possible biocontrol activity in suppressing plant pathogens and cooperative performance with other beneficial microbes (Zhang et al., 2023).

Studies have documented the inhibitory effect of bacterial strains against phytopathogen causing chocolate spot, *Botrytis fabae* (Sahile et al., 2009; Adal et al., 2022; Firdu et al., 2022; Mengstie et al., 2024). Especially, studies on the use of *Bacillus* species in the control of plant pathogens in general and chocolate spot of faba bean are scanty In addition, such studies were conducted under different zones of Ethiopia and revealed a comparably low to moderate level of percentage of inhibition against *Botrytis fabae*. Yet, chocolate spot disease is the major hazard to the production of faba bean in Ethiopia and at global level (Yitayih and Azmeraw, 2018; Lee et al., 2020; Zeleke and Legesse, 2023). Therefore, it is important to consider microbial biocontrol strategies as an additional alternative to screening for resistant varieties. Studies on biological control of *B. fabae* provides further insight on the mechanisms of host-pathogen interactions for the management of the pathogen. Thus, the aim of this research was to evaluate the antifungal activity of strains of *Bacillus* spp. isolated from faba bean rhizosphere against *B. fabae* and the expression of PGPR traits under in-vitro conditions.

2. Materials and Methods

a. Description of study areas

The study was conducted on soil samples collected from three districts (Goba, Dinsho and Sinana) of Bale Zone, Oromia Regional State, south-eastern Ethiopia. The geographic location and altitude of the study areas are presented (Figure 1, Table 1). The Goba district receives an annual range of rainfall that varies from 900 mm in lowlands and 1400 mm in highlands. It has bimodal rainfall occurring from March to April (a short rainy season) and from July through October (long rainy season).

b. Description of study areas

The study was conducted on soil samples collected from three districts (Goba, Dinsho and Sinana) of Bale Zone, Oromia Regional State, south-eastern Ethiopia. The geographic location and altitude of the study areas are presented (Figure 1, Table 1). The Goba district receives an annual range of rainfall that varies from 900 mm in lowlands and 1400 mm in highlands. It has bimodal rainfall occurring from March to April (a short rainy season) and from July through October (long rainy season). Mean monthly temperatures of the district range from 4°C to 25°C. The Dinsho district receives an annual rainfall that ranges from 800 mm- 4500 mm. It has a long rainy season (from March through October). The annual mean minimum and maximum temperature of the district is 2˚C and 20˚C, respectively. The Sinana district experiences an annual average temperature of 9˚C to 25˚C and an annual rainfall between 452.7 mm and 1129.5 mm. In all the three districts, the dominant soil type is clay soil and slightly acidic at pH of 6.5. Cereals (wheat and barley), legumes (faba bean and field pea) and oil crops (linseed and rapeseed) are the dominant crops cultivated in these regions. The three districts were selected for this study based on their production across south eastern Ethiopia and difference in altitude, weather, and soil characteristics (Eshetu et al. 2018; Mekuria and Ashenafi 2018).

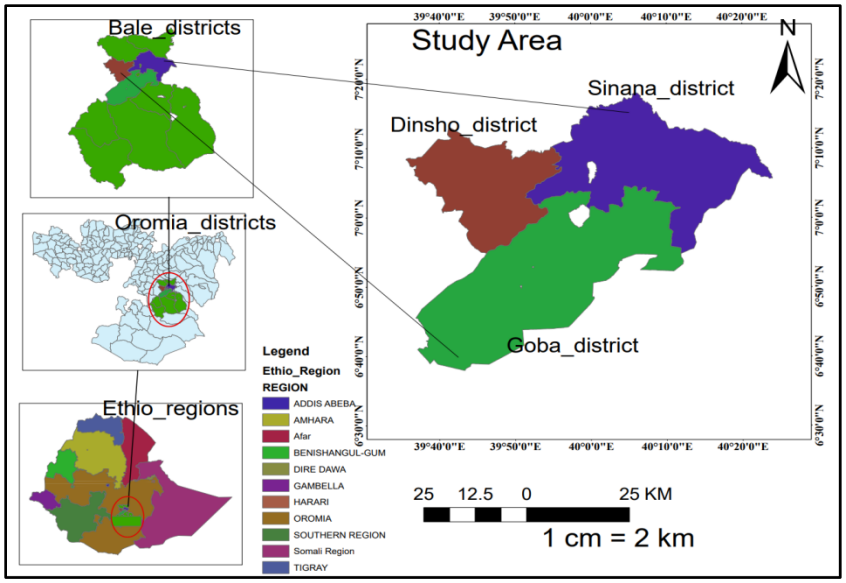

Figure 1: Map of the three district of the study areas in Bale Zone, Southeastern Ethiopia

c. Experimental design

The experiment was conducted using soil samples from the rhizosphere of the three faba bean varieties (Mosisa, Shallo and Wolki). The experiment was arranged in factorial completely randomized design (CRD) involving district, faba bean varieties and *Bacillus* isolates from such varieties as factors x PGPR traits as response variables with three replications. PGPR traits included percentage inhibition of radial growth (PIRG) of test fungal pathogen (*B. fabae*), enzymatic activity (amylase, chitinase, protease, lipase and cellulose production), IAA and production, Tri-calcium phosphate (TCP) and zinc oxide solubilization.

d. Soil sampling and sample collection

Soil sampling was done from a total of 16 study plots (faba bean farms) in the study areas. The sampling plot in each district was identified based on variation in altitude and suitability for optimum cultivation of faba bean crop. Each sampling plot consisted of a plot of 50 m x 50 m from which rhizospheric soil was sampled from 5 faba bean plants found diagonally and centrally in the core of small squares following the method described by HA (2014). The soil was sampled from rhizosphere of three faba bean varieties namely, Shallo (EH011-22-1) (moderately susceptible); Mosisa (EH-99047-1) and Wolki (EH9609-2) (moderately resistant) (Kora et al., 2017), which had no symptoms of chocolate spot disease. A 100 g of soil sample was collected from the root areas of individual faba bean plant by digging at the depth of 10 cm as described by Assefa et al. (2021). Soil sample collection was conducted from five healthy faba bean plants within the sampling plot and a total of 16 composite soil samples were prepared, then sieved, dried on sterile filter paper for 2–3 days and were kept at 4 ˚C until isolation of bacteria was done.

e. Rhizospheric *Bacillus* spp. isolation

Isolation of *Bacillus* spp. was conducted based on methods of driving endospore forming microorganisms from substratum (Mandic-Mulec et al., 2011). The soil sample was gently crushed into fine particles using sterilized mortar and pestle. A soil sample (10 g) was placed in a sterile 250 ml of Erlenmeyer flask consisting of 90 ml of sterilized distilled water. The mixture of the soil samples was shaken vigorously on a rotary shaker (IKA™ Orbital Shaker, UK) at 150 rpm for about

30 minutes. Then, the water bath was adjusted at temperature 80 ˚C and the soil sample was kept in a water bath for 10 minutes to induce the sporulation of putative *Bacillus* isolates. A 10 fold serial dilution was prepared by transferring 1 ml of soil suspensions in a 9 ml of sterilized distilled water. After vigorous vortexing of the suspension, aliquots of the soil suspension (0.1 ml) were spread-plated on Petri plates containing Nutrient agar (Himedia) amended with cycloheximide (100 mg/ml) to prevent fungal growth and finally incubated at a temperature of 35˚ C for 48 hours.

Isolation was done from Petri plates that produced a significant number of colony forming units per mL of sample (i.e., 30–300 cfu mL^{-1}) (Assefa et al., 2021). Morphologically different colonies of putative *Bacillus* isolates were randomly selected and streaked again on nutrient agar for the purpose of obtaining pure culture of bacteria. A single colony for pure culture of the putative *Bacillu*s isolate was transferred to the slant containing nutrient agar, incubated at 35˚C overnight and was then kept on in the refrigerator at 4˚ C for further analysis. The stock cultures of the putative *Bacillus* isolate were maintained in 20% (v/v) glycerol at -20°C in the deep-freezer.

f. Morphological and biochemical characterization of bacterial isolates

Morphological characters of the putative *Bacillus* isolates were done based on Gram-reaction, bacterial shape and staining features examined by microscopic observation and studying colony morphology. The presence of endospore was confirmed by staining of heat-fixed smear with a 10% aqueous malachite green for 45 min., followed by washing and counter-staining with 0.5% aqueous safranin for 30 s; spores are green with pink/red cells at 1000 × magnification (De Vos et al., 2009). Characterization of colony morphology (colony color, colony form, colony elevation, colony margin, and colony shape) was done on nutrient agar following the description in Bergey's Manual of Systematic Bacteriology (De Vos et al., 2009). The putative *Bacillus* isolates were characterized for biochemical reactions such as catalase test, oxidase test, motility test, citrate utilization test, oxidation fermentation test, methyl red test and Voges-Proskauer test following standard methods (Cappuccino and Sherman, 1992; De Vos et al., 2009).

g. Physico-chemical stress tolerance of *Bacillus* isolates

Temperature tolerance of *Bacillus* isolates was conducted following the procedure of Dugassa et al. (2021). A loopful of 24 hours old culture of each isolate was streaked on nutrient agar through a defined line and were incubated at temperature of 50 ˚C or 55 ˚C for 3-5 days to detect their temperature tolerance. Salt tolerance of the *Bacillus* isolates was determined by inoculating the bacterial culture in nutrient broth amended with a salt concentration of 7% NaCl or 10% NaCl solution (Mahdi et al., 2022). The bacterial isolates were examined for their growth under an anaerobic conditions in an anaerobic agar media (trypticase, 20 g; glucose, 10 g; NaCl, 5 g; sodium thioglycolate, 2 g; sodium formaldehydesulfoxylate, 1 g; agar 15 g; distilled water, 1 l; and pH adjusted at 7.2) following the procedure in De Vos et al. (2009).

h. Fungal pathogen and culture conditi-ons

Standard culture of *Botrytis fabae*, the etiologic agent for chocolate spot was kindly obtained from Holeta Agricultural Institute, Ethiopia. *B. fabae* was cultured on potato dextrose agar (PDA, Himedia) supplemented with 0.1 mg/l (chloramphenicol) and incubated for 7-10 days at 25 °C. The PDA culture was covered with Parafilm and was stored at 4°C till further use in the Applied Microbiology laboratory of Madda Walabu University.

i. In-vitro antifungal activity of *Bacillus* isolates against *B. fabae*

The *Bacillus* isolates were evaluated for their antifungal activity against *B. fabae* under in-vitro

conditions using a *dual* culture assay. From a fresh culture (≤ 7 days old) of test fungal pathogen (*B. fabae*), a mycelia plug of 2 cm^2 (1 cm x 2cm) was placed at the center of the PDA and Nutrient Agar plates mixed in 1:1 ratio. Exponentially grown (24-hrs-old) putative *Bacillus* sp. (one isolates/ plate) were streaked vertically as a straight short bar approximately at distance of 3 cm from the mycelial plug of the fungal pathogen at two opposite ends of the Petri dish (90 mm diameter) (Muleta et al., 2007). The inoculated Petri dishes were incubated at 25 °C for 7 days until the fungus in the control plate exceeded the growth size of fungal on plate streaked with bacterial isolate. The control plate was inoculated with the test fungal pathogen by omitting the bacterial culture. The dual culture assay was performed in three replicates. The radial growth of fungal mycelium and the zone of inhibition was measured in mm. The PIRG of the mycelium of the test fungal pathogen by the putative bacterial isolate in a relation to the control was calculated following similar formula to Firdu et al. (2022) as:

PIRG = (C − T)/C x100 where PIRG = percentage inhibition of radial growth (PIRG) of test pathogen; C = radial growth of the test pathogen in the control medium (mm); T = radial growth of the test pathogen in the test culture (mm).

j. Assay of hydrolytic enzymes and PGPR traits

The *Bacillus* isolates were evaluated qualitatively for expression of five enzymes (amylase, chitinase, protease, lipase and cellulase). Amylase production of the *Bacillus* isolate was conducted on the starch agar medium (peptone 5 g, beef extract 3 g, soluble starch 10 g, agar 15 g, distilled water 1,000 ml) and the final pH of the medium was adjusted to 7.5 ± 0.2 at 25°C (Yadav et al., 2021). The surface of the starch agar was flooded with 3% Gram's iodine solution after four days of incubation at 28°C. Amylase positive and negative result were shown by observation of a clear halo zone around the bacterial growth and a dark blue or black color surrounding the bacterial growth, respectively. The chitinase activity was tested on agar medium amended with colloidal chitin following standard methods (Renwick et al., 1991). The compositions of Agar-amended media are colloidal chitin 1% (w/v), Na_2HPO_4 (6 g L-1), NaCl (0.5 g L-1), KH_2PO_4 (3 g L-1), NH4Cl (1 g L-1), yeast extract (0.05 g L-1) and agar (15 g L-1). The *Bacillus* isolate was spot-inoculated on colloidal chitin amended agar media and incubated at 30± 1 °C for 5 days. Observation of the clear zone around the colonies is indicative of chitinase activity (Renwick et al. 1991). The protease activity was evaluated through inoculation of the isolates on 1% Skim milk agar (skim milk powder 10 g L-1, peptone 5 g L-1, yeast extract 2 g L-1, glucose 1.0 g L-1, and agar 15 g L-1 with pH 8.5 (Masi et al., 2021) and incubated at 30 °C for 48 hrs. The formation of a clear zone around the bacteria indicated the production of proteases.

The lipase activity was evaluated using agar-amended lipase media (g/L): calcium chloride (0.1 g), peptone (10 g), sodium chloride (5), Agar (15 g), distilled water (1 L) and 10 ml sterile Tween 80 following established methods (Muleta et al., 2007;Yadv et al., 2021). The *Bacillus* isolate was streaked on this medium and incubated at 27 °C for 48 hrs. Tween 80 hydrolysis is associated with the production of lipase enzyme and the appearance of a white precipitate (calcium soap) underneath and around the bacterial growth. The cellulase activity was evaluated through inoculation of the bacterial cultures in basal media containing the following ingredients: 0.01% $MgSO_4$, 0.1% yeast extract, 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.05% sodium citrate and amended with 1% carboxymethyl cellulose (CMC) following the method of Mohammed (2020). After incubation at 30 C for 72 hrs, the inoculated plates were flooded with 0.1% Congo red solution for 20 min and then washed with 0.1 M NaCl solution. The presence

of a clear zone around the colony indicated cellulase activity (Liang et al., 2014).

k. Tri-calcium phosphate (TCP) and Zn compounds solubilization

The qualitative study of TCP solubilization by the putative *Bacillus* isolates was done using Pikovskaya agar media (g/L): yeast extract (0.5 g), glucose (10 g), $Ca_3(PO_4)_2$ (5 g), $(NH_4)_2SO_4$ (0.5 g), $MgSO_4.7H_2O$ (0.1 g), $MnSO_4.2H_2O$ (0.002 g), KCl (0.2 g), $FeSO_4.7H_2O$ (0.002 g), and Agar (15 g) (Pikovskaya, 1948). A loopful of fresh culture of each bacterial isolate was spot-inoculated onto Petri plates containing Pikovskaya Agar media. The plates were incubated at 35 ± 2°C for 3-5 days and colonies with a clear halo were marked positive for TCP solubilization. The results were recorded as the diameter of the clear zone formed around each bacterial growth. Phosphate solubilization Index (PSI) was estimated following the method described in Pathak et al. (2018).

$$PSI = \frac{\text{Colony diameter (mm)} + \text{Diameter of the halo zone (mm)}}{\text{Colony diameter (mm)}}$$

Solubilization of inorganic forms of Zn compound (ZnO) was performed on tris-minimal salt media composed of the ingredients such as D-glucose (10 g), Tris HCl (6.06 g), NaCl (4.68 g), KCl (1.49 g), NH_4Cl (1.07 g), Na_2SO_4 (0.43 g), $MgCl_2.2H_2O$ (0.2 g), $CaCl_2.2H_2O$ (30 mg), and Agar (15 g) dissolved in deionized water to make 1000 mL volume following the method of Mumtaz et al. (2017). The ZnO (0.1%) was added in the media as the sole source of inorganic zinc Mumtaz et al. (2017). Briefly, each B*acillus* isolate was spot-inoculated in this medium and incubated in the dark at 28°C for seven days to observe a clear halo zone around colonies. Zinc solubilization index (ZSI) was estimated as following the method described by Yadav et al. (2021).

l. IAA and HCN Production

The capability of the bacterial isolates to produce Indole acetic acid (IAA) was estimated using the method in Chandra et al. (2018). A 500 µl of 1-day-old bacterial culture was placed into 50 ml of nutrient broth having 0.1% dl-tryptophan and was then incubated at 28 ± 2 C for 72 hours. The culture suspension was finally centrifuged at 10,000 rpm and 4 C for 10 min. Into the supernatant (2 ml), 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) and two drops of ortho-phosphoric acid was added. The formation of pink color indicated the isolates were positive for Indole acetic acid. Production of HCN was evaluated following the method of Bakker and Schippers (1987). Nutrient agar was amended with glycine (4.4 g/l) and bacteria were streaked on modified agar plates. Whatman's filter paper no.1 soaked in 2% Sodium carbonate in 0.5% picric acid was placed at the inner surface of the lid of the petri plates. Plates were sealed with Parafilm and were incubated at 30 °C for 48 hrs. Development of orange to red color indicated HCN production.

m. Data Quality Control

All standard apparatus and equipment were used for in-vitro microbiological work. About 3% of the prepared medium was kept in an incubator (at 25 °C for fungus for 2-3 days and at 28 °C for bacteria) overnight to check the sterility of the media. In-vitro test was done after confirming the sterility of the medium. A medium without the biocontrol agents (*Bacillus* isolates), but with test fungal pathogen (*Botrytis fabae*) was used as untreated control.

n. Ethical clearance

As the study did not involve human and animal subjects, ethical clearance was not needed (not applicable) in this study.

o. Statistical Analysis

All the experiments were carried out in triplicates. The data was recorded and entered into Statistica software version (Stat Soft. INC., Tulsa, OK, 74104, USA). Analysis of variance (ANOVA) with a general linear model (GLM)

was employed to test the statistical difference in the means of the radial growth, inhibition zone and PIRG of *B. fabae* among *Bacillus* isolates and the difference was significant at $P < 0.05$. In ANOVA test, Fisher Least Significant Difference (LSD) test was used for multiple comparisons of means.

3. Results

3.1. Morphological and biochemical characterstics of the bacterial isolates

A total of 80 *Bacillus* isolates were isolated and identified as *Bacillus* spp. Among them, 16 bacterial isolates were selected for their potential antagonistic activity against the fungus *B. fabae* and expression of multi-PGPR traits under in-vitro conditions. The bacterial isolates were rod-shaped, Gram-positive in their Gram-reaction, and positive in endospore staining. In addition, all isolates were motile which were shown by diffusion of the isolate into the medium from the original area of stabbing the pure culture (Table 1).

Table 1: The geographical locations and the morphological characteristics of *Bacillus* isolates from rhizosphere of faba bean in some districts of Bale Zone, Southeastern Ethiopia

Study area	Isolate	Faba bean variety	Latitude/ Longitude	Altitude (m.a.s.l)	Colony characteristics				Gram-reaction	Endo-spore	Cell shape
					Colony Color	Colony form	Colony elevation	Colony margin			
Dinsho	D3	Shallo	7ºN,39.52ºE	2732	White	Regular	Flat	Entire	+	+	Rod
	D5	Shallo	7ºN,39ºE	2730	White	Regular	Convex	Entire	+	+	Rod
	D10	Shallo	7ºN,39.52ºE	2735	Cream	Regular	Flat	Entire	+	+	Rod
	D12	Mosisa	7ºN,38.9ºE	2729	White	irregular	Flat	Entire	+	+	Rod
	D14	Wolki	7ºN,39.3ºE	2728	Cream	irregular	Flat	Entire	+	+	Rod
Goba	G2	Shallo	7ºN,39.58ºE	2550	Cream	irregular	Flat	Undulate	+	+	Rod
	G3	Mosisa	7ºN,39.58ºE	2575	White	Regular	Flat	Ciliated	+	+	Rod
	G4	Mosisa	7ºN,39.53ºE	2563	White	Regular	Flat	Curled	+	+	Rod
	G9	Wolki	7ºN,39.58ºE	2565	White	Regular	Flat	Branching	+	+	Rod
	G10	Shallo	7ºN,39.58ºE	2562	Brown	Regular	Flat	Entire	+	+	Rod
Sinana	S2	Shallo	7ºN,39.98ºE	2513	White	irregular	Radial	Undulate	+	+	Rod
	S3	Shallo	7ºN,39.98ºE	2511	Cream	Regular	Flat	Branching	+	+	Rod
	S5	Wolki	7ºN,39.95ºE	2510	Cream	Regular	Flat	Ciliated	+	+	Rod
	S6	Shallo	7ºN,39.96ºE	2508	White	irregular	Flat	Entire	+	+	Rod
	S11	Mosisa	7ºN,39.96ºE	2509	White	irregular	Flat	Branching	+	+	Rod
	S16	Shallo	7º N,39.96ºE	2506	White	irregular	Flat	Entire	+	+	Rod

m.a.s.l = meter above sea level; D = Isolates from Dinsho District; G = Isolates from Goba District; S = Isolates from Sinana District. The numbers represent isolate numbers from each district

The biochemical tests of bacterial isolates of this study are summarized in Table 2. All of the 16 bacterial isolates were catalase positive and oxidase positive. Fifteen (93.75%) isolates utilized citrate as a carbon source through changing the color of the media from deep green to blue within 24 hours; 13 (81.25%) bacterial isolates showed positive result for methyl red test through a color change of MR-VP medium to red or pink color. Nine isolates were VP positive indicating formation of acetoin in 2,3-butanediol fermentation. All of *Bacillus* spp. metabolized glucose oxidatively, but there was a variation among the isolates.

3.2. Physio-chemical tolerance of Bacillus spp.

All the 16 bacterial isolates showed growth at temperatures of 50°C and 55°C. All of them had high salt tolerance (7% NaCl and 10% NaCl). However, none of the bacterial isolates showed growth under anaerobic condition. Based on the morphological and biochemical characteristics, all of the 16 bacterial isolates were identified as belonging to the genus *Bacillus* (Table 2). Molecular analyses are in progress for the identification at species level.

Table 2: The biochemical tests and growth requirements of *Bacillus* spp. isolated from the rhizosphere of faba bean in some districts of Bale Zone, Ethiopia.

Isolates	Catalase	Oxidase	Citrate	†MR Test	‡VP Test	*OF Test	Anaerobic growth	Stress tolerance			
								Temperature		NaCl	
								50 °C	55 °C	7%	10%
D3	+	+	+	+	–	++	–	+	+	+	+
D5	+	+	+	+	+	+++	–	+	+	+	+
D10	+	+	–	–	–	+++	–	+	+	+	+
D12	+	+++	+	+	+	+++	–	+	+	+	+
D14	+	++	+	+	+	++	–	+	+	+	+
G2	+	+	+	+	–	+	–	+	+	+	+
G3	+	+++	+	+	+	+++	–	+	+	+	+
G4	+	+	+	+	–	+	–	+	+	+	+
G9	+	+	+	+	+	+++	–	+	+	+	+
G10	+	+	+	+	–	+	–	+	+	+	+
S2	+	+	+	-	+	+++	–	+	+	+	+
S3	+	++	+	+	+	+	–	+	+	+	+
S5	+	++	+	+	+	+++	–	+	+	+	+
S6	+	+	+	+	–	++	–	+	+	+	+
S11	+	++	+	+	+	+++	–	+	+	+	+
S16	+	+	+	+	–	++	–	+	+	+	+

In the table above: + refers to a positive result: – refers to negative result; in *OF-test: +++ = very strong activity, ++ = strong activity, + = weak activity, – = absence of activity; In †MR test and ‡VP tests, + means the isolates are producing mixed acid-fermentation and acetoin, respectively, – in both cases imply they are not producing both of them

3.3. In-vitro antifungal activity of Bacillus isolates against B. fabae Sard.

Generally, all *Bacillus* isolates displayed an inhibitory activity against *B. fabae* Sard., a pathogen causing a chocolate spot of faba bean (Table 3; Figure 2). The test pathogen on the control plate was grown and filled the 90 cm Petri dish with a week of incubation at 25°C. The *Bacillus* isolates and the faba bean varieties significantly affected the means of radial growth of *B. faba*e and the inhibition zone against *B. faba*e ($P < 0.05$). However, the district had no significant effect on such factors of *B. fabae* ($P > 0.05$). The smallest and the largest radial growth of *B. fabae* was recorded when subjected to *Bacillus* isolates G3 (1.56 ± 0.6 mm) and S16 (16.66 ± 4.93 mm), respectively. Similarly, the smallest and the largest inhibition zone of *B. fabae* was recorded when subjected to *Bacillus* isolate S16 (28.33 ± 1.44 mm) and isolate G3 (43.44 ± 1.23 mm), respectively (Table 3; Figure 2).

The means of PIRG of *B. faba*e significantly varied among *Bacillus* isolates and faba bean varieties ($P < 0.05$), but not among districts ($P > 0.05$). The highest PIRG was revealed by isolate G3 from Goba district (96.53 ± 1.14), followed by isolate D12 from Dinsho district (91.11 ± 2.94) and by isolate S5 from Sinana district (81.48 ± 3.20). The lowest PIRG of the *B. fabae* was achieved by *Bacillus* isolates S16 from Sinana district (62.96 ± 3.20). *Bacillus* isolates such S6, D5, G9, S11, D14 and S3 showed moderate PIRG that ranged from 66.66 ± 1.11 to 71.48 ±1.28. However, the means of PIRG excreted by the *Bacillus* isolates pairs D3, G2, G4 and S2; D5, G9 and S6; and D10, G10 and S16 against *B. fabae* were not significantly different from each other ($P > 0.05$) (Tables 3). The pooled mean ± SD of PIRG against *B. fabae* was higher *i*n *Bacillus* isolates from rhizospheric soil of faba variety Mosisa (81.38 ± 15.42%) followed by Wolki variety (73.33 ± 7.7%). However, *Bacillus* isolates from soil of Shallo faba bean variety produced the lowest pooled mean ± SD of PIRG against faba bean *B. fabae* (65.51 ± 3.87) (Table 3).

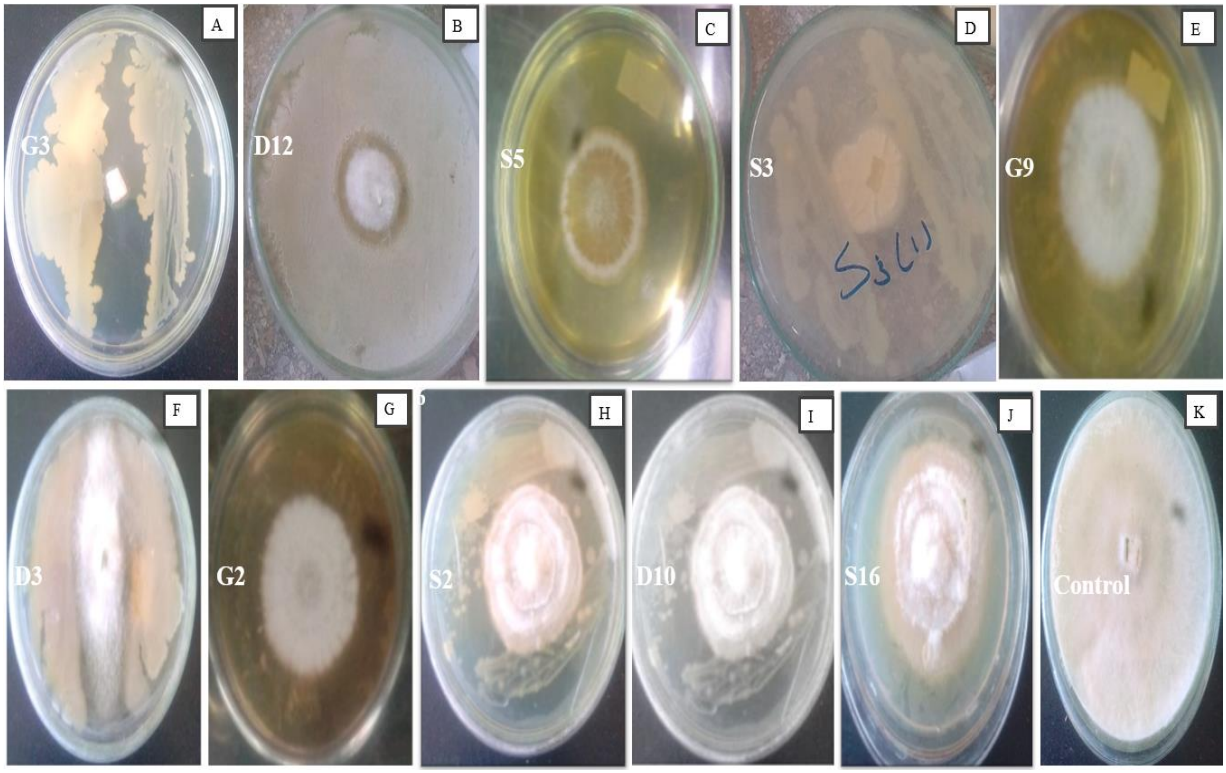

Figure 2: Antagonistic activity of *Bacillus* isolates against *Botrytis fabae* against causing chocolate spot of faba bean, Bale Zone, Southeastern Ethiopia after seven days of incubation (dual culture); Isolate G3; (B) Isolate D12; (C) Isolate S5; (D) Isolate S3; (E) Isolate G9; (F) Isolate D3; (G) Isolate G2; (H) Isolate S2; (I) Isolate D10; (J) Isolate S16; (K) Untreated control (culture of *B. fabae* on PDA and Nutrient Agar plate in 1:1)

Table 3: Mean ± SD of radial growth, inhibition zone and percent inhibition of radial growth (PIRG) of *Botrytis fabae* by *Bacillus* isolates, from rhizosphere of faba bean, Bale Zone, Southeastern Ethiopia

Category	Source	Radial growth of *B. fabae* (mm) Mean± SD	Inhibition zone(mm) (Mean ± SD)	PIRG (Mean± SD)
Isolates	D3	15.84 ±1.44 e-g	29.17 ± 1.44 a-c	64.81 ± 3.20 a-c
	D5	14.33 ± 2.75 d-g	30.67 ± 2.75 a-d	68.14 ± 6.11 a-d
	D10	16.5 ± 1.32 g	28.50 ± 1.32 a	63.33 ± 2.94 a
	D12	4.0 ± 1.32 b	41.00 ± 1.32 f	91.11 ± 2.94 f
	D14	13.50 ± 3.77 de	31.50 ± 3.77 c-d	70.00 ± 8.34 c-d
	G2	16.16 ± 1.25 e-g	28.83 ± 1.25 a-c	64.07 ± 2.80 a-c
	G3	1.56 ± 0.6 a	43.40 ± 1.23.0 g	96.53 ± 1.14 g
	G4	15.84 ± 1.44 e-g	29.17 ± 1.44 a-c	64.81 ± 3.20 a-c
	G9	14.16 ± 1.44 d-g	30.83 ±1.44 a-d	68.51 ± 3.20 a-d
	G10	16.35± 1.44 f-g	28.67 ± 1.44 a	63.7 ± 3.20 a
	S2	16.0 ± 1.73 e-g	29.00 ±1.73 a-c	64.44 ± 2.22 a-c
	S3	12.84 ± 0.57 d	32.17 ± 0.57 d	71.48 ± 1.28 d
	S5	8.34 ± 1.44 c	36.67 ±1.44 e	81.48 ± 3.20 e
	S6	15.0 ± 0.5 d-g	30.00 ± 0.50 a-d	66.66 ± 1.11 a-d
	S11	13.66 ± 1.28 d-f	31.33 ± 1.26 b-d	69.63 ± 2.79 b-d
	S16	16.66 ± 4.93 g	28.33 ± 1.44 a	62.96 ± 3.20 a
	Mean	13.07 ± 4.93	31.93 ± 4.93	70.95 ± 10.96
	Control[a]	45.00 ± 0.0	-	-
District	Dinsho (n = 15)	12.83 ± 5.10 a	32.17 ± 5.10 a	71.5 ±11.33 a
	Goba (n = 15)	12.50 ± 6.60 a	32.50 ± 6.60 a	72.2 ± 14.67 a
	Sinana (n = 18)	13.75 ± 3.02 a	31.25 ± 3.01 a	69.44 ± 6.70 a
	Mean	13.07 ± 4.95	31.93 ± 4.93	70.94 ± 10.96
Faba bean variety	Mosisa	8.37 ± 6.94 a	36.62 ± 6.94 a	81.38 ± 15.42 a
	Wolki	12.0 ± 3.5 b	33.0 ± 3.5 b	73.33 ± 7.77 b
	Shallo	15.52 ±1.75 c	29.48 ± 1.74 c	65.51 ± 3.87 c
	Mean	13.07 ±4.93	31.93 ±4.93	70.94 ±10.96

Means ± SD followed by the same letter (s) in the same column of the same source were not significantly different from each other at $P < 0.05$ in Factorial ANOVA using Fisher LSD test for multiple mean comparison; Control[a] is the radial growth (mm), inhibition zone (mm) and PIRG of test *B. fabae* in the control medium

3.4. Assay of hydrolytic enzymes and PGPR traits

All of the *Bacillus* isolates produced at least one hydrolytic enzymes (amylase, chitinase, protease, cellulose and/or lipase). Amylase production was observed in 8 (50.0%) of the isolates. *Bacillus* isolate S5 displayed the strongest amylase activity as compared to the rest of the isolates revealed by formation of the largest clearing zone around the colony (Table 4). The mean ± SD of starch hydrolysis indices varied significantly among the *Bacillus* isolates ($P< 0.001$) and ranged from 1.18 ± 0.02 to 3.52 ± 0.71. Eleven *Bacillus* isolates (68.8%) were chitinase positive revealed by a clearing zone in the presence of colloidal chitin in the medium, among which isolates S5, G2, G3, and D5 were the strongest chitinase producers. The chitinolytic indices also significantly varied among the isolates ($P< 0.001$) and was higher in isolate S5 (4.37 ± 0.0) followed by G2 (4.0 ± 0.0) (Table 4). Protease activity was detected in 11 (68.8%) of the isolates and the strongest activity was exhibited by the isolates D5, D12, D14, S5 and S11. The proteolytic indices varied among the isolates ($P < 0.0001$) and the highest indices were recorded in isolate D14 (6.70 ± 0.82) followed by S5 (5.73 ± 0.25). Lipase

activity was recorded in 14 isolates (87.5%). Five isolates (D5, D12, G3, S3 and S5) displayed strongest lipase activity among other isolates that was revealed by deposition of a large area of calcium precipitate around the colony (Table 4). Cellulase activity was displayed by 7 (43.75%) the *Bacillus* isolates and such isolates displayed generally low to moderate activity (Table 4). A moderately high cellulose hydrolysis index was exhibited by *Bacillus* isolate S2 (2.76 ± 0.04) followed by S11 (2.58 ± 0.02) (Table 4).

Table 4: Enzymatic activity and Hydrolytic enzymes activity indices of some *Bacillus* isolates from rhizospheric soil of faba bean in some districts of Bale Zone, Southeastern Ethiopia

Bacillus Isolates	Amylase	Chitinase	Protease	Lipase	Cellulase	[a]Amylolytic Index	[b]*Chitinolytic* index	[c]Proteolytic Index	[d]Cellulolytic Index
D3	-	-	+	-	-	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	2.47 ± 0.15^{cd}	0.0 ± 0.0^{a}
D5	++	+++	+++	+++	+	1.69 ± 0.10^{c}	3.07 ± 0.11^{g}	3.50 ± 0.25^{fg}	2.12 ± 0.23^{c}
D10	+	-	+	-	-	1.40 ± 0.21^{ab}	0.0 ± 0.0^{a}	1.75 ± 0.15^{bc}	0.0 ± 0.0^{a}
D12	+	++	+++	+++	+	1.18 ± 0.02^{b}	2.63 ± 0.23^{f}	$3.34 \pm 0.24^{e-g}$	1.43 ± 0.96^{b}
D14	-	-	+++	++	++	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	6.70 ± 0.82^{i}	2.44 ± 0.15^{d}
G2	+	+++	+	+	+	1.46 ± 0.12^{ab}	4.0 ± 0.0^{i}	1.53 ± 0.03^{b}	2.11 ± 0.28^{c}
G3	-	+++	++	+++	-	0.0 ± 0.0^{a}	3.53 ± 0.23^{h}	2.38 ± 0.12^{cd}	0.0 ± 0.0^{a}
G4	-	-	-	+	-	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
G9	-	-	++	+	+	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	$3.0 \pm 0.0^{d-f}$	1.5 ± 0.0^{b}
G10	+	+	-	+	-	2.0 ± 0.0^{d}	2.47 ± 0.23^{ef}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
S2	+	++	-	+	+	2.0 ± 0.0^{d}	2.10 ± 0.17^{b}	0.0 ± 0.0^{a}	2.76 ± 0.04^{e}
S3	-	+	-	+++	-	0.0 ± 0.0^{a}	$2.25 \pm 0.0^{b-d}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
S5	+++	+++	+++	+++	-	3.52 ± 0.71^{f}	4.37 ± 0.0^{j}	5.73 ± 0.25^{h}	0.0 ± 0.0^{a}
S6	+	++	++	+	-	1.42 ± 0.02^{ab}	2.14 ± 0.0^{bc}	2.73 ± 0.09^{de}	0.0 ± 0.0^{a}
S11	-	++	+++	+	+++	0.0 ± 0.0^{a}	$2.30 \pm 0.0^{c-e}$	4.0 ± 0.0^{g}	2.58 ± 0.02^{de}
S16	-	+	-	+	-	0.0 ± 0.0^{a}	2.40 ± 0.0^{de}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

+++ = Very strong activity; ++ = Strong activity, + = weak**;** = **-** no activity; [a] Amylolytic Index ($F = 117.74$; df =15,32; *P*-value < 0.001); [b]*Chitinolytic* index ($F = 535.59$; df = 15,32; *P*-value <0.001); [c]Proteolytic Index ($F = 223.36$; df = 15,32, P-value = 0.0001); [d]Cellulolytic Index ($F = 193.03$; df = 15,32; *P*-value < 0.001). Mean ± SD with the same letter(s) in the same column are not significantly different from each other in One Way ANOVA at $P < 0.05$ using Fisher LSD test for multiple comparison of means

3.5. IAA and HCN production

Indole acetic acid (IAA) was produced by 13 (81.25%) of the *Bacillus* isolates. However, no IAA production was detected in the isolates G2, S11 and S16, while HCN was produced by 10 *Bacillus* isolates (62.5%) (Table 5).

3.6. TCP and ZnO solubilization

Ten (62.25%) isolates were found to solubilize TCP, among which two isolates (D14 and G9) showed moderate to strong positive results. The TCP solubilization Index (PSI) ranged from the lowest (1.14 ± 0.03) by isolate D12 to the highest value (3.00 ± 0.5) by isolate G9 (Table 5). Solubilization of the inorganic zinc (ZnO) was exhibited by 8 (half) of the isolates among which isolate G2 followed by D14 exhibited a high ZnO

solubilization activity. The mean ± SD of ZnO solubilization indices varied from 1.06 ± 0.0 to 3.17 ± 0.28 (Table 5).

3.7. Synergistic activity of faba bean rhizospheric *Bacillus* species

This study revealed that each of *Bacillus* isolate in addition to suppression of the fungal pathogen *B. fabae*, it displayed positive results in at least two PGPR traits (enzymatic activity, IAA and HCN production and solubilization of TCP and /or ZnO). Eleven (68.8%) of the isolates were positive for at least five of PGPR traits in addition to suppression of the phytopathogenic fungus, *B. fabae* (Tables 3-5).

Table 5: Some PGPR traits of *Bacillus* isolates from soil of faba bean in some districts of Bale Zone, Southeastern Ethiopia

Bacillus Isolates	IAA	HCN	Phosphate solubilization	ZNO solubilization	[e]PSI (mm) Index	[f]ZnO solubilization Index
D3	+	-	-	-	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
D5	+	+	+	+	1.18 ± 0.04^{bc}	1.28 ± 0.10^{bc}
D10	+	-	+	-	1.40 ± 0.10^{bc}	0.0 ± 0.0^{a}
D12	+	+	+	-	1.14 ± 0.03^{a}	0.0 ± 0.0^{a}
D14	+	+	++	++	2.67 ± 0.6^{d}	1.93 ± 0.30^{d}
G2	-	-	+	+++	1.28 ± 0.03^{bc}	3.17 ± 0.28^{e}
G3	+	+	+	+	1.26 ± 0.04^{bc}	1.06 ± 0.0^{b}
G4	+	-	-	-	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
G9	+	+	+++	+	3.0 ± 0.5^{e}	1.32 ± 0.02^{c}
G10	+	-	-	+	0.0 ± 0.0^{a}	1.38 ± 0.27^{c}
S2	+	+	+	+	1.20 ± 0.16^{bc}	1.40 ± 0.0^{c}
S3	+	+	-	-	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
S5	+	+	+	-	1.15 ± 0.09^{bc}	0.0 ± 0.0^{a}
S6	+	+	+	-	1.26 ± 0.07^{bc}	0.0 ± 0.0^{a}
S11	-	+	-	+	0.0 ± 0.0^{a}	1.44 ± 0.31^{c}
S16	-	-	-	-	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

+++ = Very strong activity; ++ = Strong activity, + = weak**;** = **-** no activity; One way ANOVA*:* [e]PSI ($F = 66.19$; df = 15,32; P-value = 0.0001); [f]ZnO solubilization Index ($F = 15,32$; df = 15,32; $P < 0.001$). Means ± SD with the same letter(s) in the same column are not significantly different from each other in One Way ANOVA at $P < 0.05$ using Fisher LSD test for multiple comparison of means.

4. Discussion

In our study, all of our bacterial isolates were Gram-positive in Gram-reaction, rod-shaped and endospore former, indicating the attributes of *Bacillus* spp. Biochemically, the 16 bacterial isolates were catalase positive and oxidase positive; they metabolized glucose oxidatively, but there was a variation among the isolates. All of them were isolated at very high temperature (80 ^{o}C for 10 minutes), could be due to their ability to form endospores (Bressuire-Isoard et al., 2018), though, all isolates didn't grow under anaerobic conditions, revealing that they were strict aerobes in their metabolism. The morphological and biochemical characteristics of our bacterial isolates (Tables 1-2) were fairly enough to designate as belonging to the genus of *Bacillus* and in agreement with the description of *Bacillus* species (Cappuccino and Sherman, 1992; De Vos et al., 2009). Using 16S rRNA sequence analysis, Firdu et al. (2022) identified 7

Bacillus spp. representing 35% of PGPR isolates from faba bean in Arsi and Bale Zones, Ethiopia, showing the importance of this bacterial species in becoming the dominant members of faba bean rhizosphere, supporting the result of our study. *Bacillus* spp. were identified as the major bacteria in the rhizosphere region of roots of many crop plants (Yadav et al., 2021).

All 16 *Bacillus* isolates in this study grew at temperatures of 50ºC and 55ºC (Table 2). Study of PGPR isolates from the rhizosphere of heat-stressed wheat genotypes has shown us that they display heat tolerance owing to enhanced activity of antioxidant and proline (Ashraf et al. 2019). Our *Bacillus* isolates also tolerate high salinity (7% and 10% NaCl) (Table 2), in concordance with rhizobacterial isolates of faba bean from South Wollo (Adal et al., 2022). *Bacillus* spp. undertake induced regulation of several genes, proteins, antioxidant enzymes, pigments, hormones, nutrient transport and prevention of excess sodium transport in the plant system thereby supporting plants to survive soil salinity (Radhakrishnan et al., 2017). Therefore, the isolation of salt-tolerant *Bacillus* isolates in our study demonstrates their potential in mitigating salt-stress especially in saline soil.Among 80 *Bacillus* isolates evaluated for in-vitro antifungal activity, 16 *Bacillus* isolates showed significant biocontrol efficacy against *B. fabae* under in-vitro conditions (Table 3; Figure 2). *Bacillus* isolates G3, D12 and S5 exhibited the maximum radial growth inhibition of the fungal pathogen. The ranges of means of radial growth of *B. fabae* achieved by *Bacillus* isolates were largely lower than *Bacillus* strains of faba bean leaves from farmers' fields in 12 districts of Amhara Regional State, Ethiopia (16.2-34.6 mm) on the same pathogen (Sahile et al., 2009), showing higher efficacy of our rhizospheric *Bacillus* isolates.

The PIRG of our *Bacillus* isolates ranged from the lowest value (62.96 ± 3.20) by isolate S16 from Sinana district to the highest value by isolate G3 from Goba district (96.53 ± 1.14).. The range of PIRG in our study was higher than the rhizobacteria from faba bean in South wollo, Ethiopia (52.5-85.8%) (Adal et al. 2022), rhizospheric *Bacillus* isolates faba bean in Arsi and Bale zone against *B. fabae* AAUBF-12 (34-50%) within seven days of incubation (Firdu et al., 2022) and from Gondar-Zuria, Wogera, Dabat, and Debark districts in the 2021 main cropping season (25.57-72.38%) (Mengstie et al., 2024). The antifungal activity of *Bacillus* spp. against various phytopathogens were reportedly attributed to production of lipopeptides and different secondary metabolites including antimicrobial peptides (AMPs), polyketides, lytic enzymes, and volatile compounds (Miljaković et al., 2020; Zhang et al., 2023). Surfactin, iturin and fengycin are among the major lipopeptides produced by *Bacillus subtilis*, which have a remarkable role in regulating many phytopathogens (Mahapatra et al., 2022).

The pooled mean ± SD of PIRG against *B. fabae* was higher in *Bacillus* isolates from rhizospheric soil of faba variety Mosisa followed by Wolki variety. However, *Bacillus* isolates from soil of Shallo faba bean variety produced the lowest pooled mean ± SD of PIRG against faba bean *B. fabae* (Table 3). Two moderately resistant varieties of faba bean, i.e., an unsprayed Mosisa faba bean variety from Sinana Agricultural Institute (Kora et al., 2017) and unsprayed Wolki variety from Tigrayi, North Ethiopia (Wubshet and Chala 2021) showed a low level disease severity index and area under disease progress curve (AUDPC) of chocolate spot disease. Such faba bean varieties may have been associated with PGPR including *Bacillus* isolates that have contributed for higher disease resistance against *B. fabae*.

The *Bacillus* isolates were tested for the qualitative enzymatic activity under in-vitro conditions (Tables 4). Half four isolates (50%) hydrolyzed starch in starch agar plates shown by clearing of the medium and this was higher than the proportion of rhizobacteria (*Pseudomonas*

spp. and *Bacillus* spp.) hydrolyzing starch from faba bean in North Shoa, Ethiopia (25%) (Dugassa et al., 2021), but nearly similar to the frequency of starch hydrolyzing rhizospheric, endophytic and epiphytic bacteria from chilli cultivars of telangana, India (48.8%) (Akshitha et al., 2024). Eleven *Bacillus* isolates (68.8%) were chitinase positive revealed by a clearing zone in the presence of colloidal chitin in the medium. This result was higher than the result of rhizospheric *Bacillus* isolates from South Wollo, Ethiopia (57.69%) (Adal et al., 2022) and North Shewa, Ethiopia 28% (Dugassa et al., 2021). The chitinase enzyme breaks down the protective coats and weakens the defense system of several pathogenic microorganisms and insects (Hamid et al., 2013). Eleven (68.8%) and 14 (87.5%) of our *Bacillus* isolates exhibited protease activity and lipase activity, respectively. Firdu et al. (2022) reported protease activity in 7 strains (100%), a higher activity than our study, but lipase activity was detected in 71.4% of the rhizospheric *Bacillus* isolates, a result lower than our isolates. Cellulase activity was displayed by 7 (43.75%) of our *Bacillus* isolates, a result higher than rhizospheric, endophytic and epiphytic bacteria isolates from chilli cultivars of telangana, India from India where 15 (34.8%) were cellulase positive (Akshitha et al., 2024). *Bacillus* spp. produce several hydrolytic enzymes (amylase, lipase, cellulase, xylanase, glucanase, pectinase, and protease enzyme) that exhibited a strong inhibitory activity against many fungal plant pathogens, owing to their attack on the glycosidic bonds of the cell wall components (e.g., chitin, glucan, and protein) (Jamali et al., 2020; Tsotetsi et al., 2022), which was also exhibited by most of our *Bacillus* isolates.

Most of our *Bacillus* isolates (81.25%) could produce IAA (Table 5), in agreement rhizobacteria from faba bean in South Wollo, Ethiopia (80.8%) (Adal et al., 2022), but slightly lower than the IAA producing by *Bacillus* isolates (85.7%) from Bale highlands and Arsi Zone (Firdu et al., 2022). It was suggested that rhizospheric bacterial isolates are more efficient auxin or indole-3-acetic acid (IAA) producers due to rich supplies of substrate released from the roots compared to non-rhizospheric soils (Chandra et al., 2018), and this could be the explanation for IAA production by most of our *Bacillus* isolates. Among our *Bacillus* isolates, 10 (62.5%) of them were positive for HCN production, a result higher than that reported by Firdu et al. (2022) where 4 out of 7 seven *Bacillus* isolates (57.1%) and by Adal et al. (2022) where 6 out of 26 (23.1%) rhizobacteria were HCN producers. Many rhizobacteria including *Bacillus* spp. produce HCN to bio-control phytopathogens (Santigo et al., 2015; Abd El-Rahman et al., 2019).

In our study, ten (62.25%) isolates were found to solubilize TCP (Table 5). Our result of TCP solubilization activity was slightly higher than the result from North Shewa (60%) (Dugassa et al. 2021), but smaller than report by Firdu et al. (2022) (in 71.4% of *Bacillus* isolates. The TCP solubilization Index (PSI) ranged from the lowest (1.14 ± 0.03) by isolate D12 to the highest value (3.00 ± 0.5) by isolate G9 (Table 5), and were in a higher range than reported by *Bacillus* isolates in earlier studies (Muleta et al., 2013; Firdu et a l., 2022). *Bacillus* spp. are among the most powerful phosphate solubilizing microbes; hence the use of biofertilizers containing N_2-fixing and/or P-solubilizing *Bacillus* spp. helps in mitigating the adverse effects of synthetic fertilizers without compromising food safety (Miljaković et al., 2020). *Bacillus* spp. employ the secretion of phosphatases and low molecular weight organic acids, which acidify the environment to support the change of inorganic phosphates into free phosphate, thereby enhancing the uptake of phosphates by the roots (Tsotetsi et al., 2022).

ZnO solubilization was revealed in 50% of our *Bacillus* isolates (Table 5), higher than the result of Yadav et al. (2021) who reported solubilization

of inorganic zinc compounds (ZnCO3, ZnO and $Zn_3(PO4)_2$) in 42 (24%) *Bacillus* spp. from rhizosphere of wheat in Indo-Gangetic plains of Northern India. The mean ± SD of ZnO solubilization indices varied from 1.06 ± 0.0 to 3.17 ± 0.28 (Table 5) and were significantly varied among *Bacillus* isolates. Overall, the ZnO solubilization indices were lower than results of *Bacillus* isolates from the rhizosphere of maize in Bahawalpur, Punjab, Pakistan which were in the range of 2.33-4.03 (Mumtaz et al.. 2017). Rhizobacterial *Bacillus* spp. are the predominant organisms facilitating the transformation of insoluble/unavailable Zn compounds in the soil into the soluble ones (Yadav et al., 2021; Yadav et al., 2023) mainly through production of 2-ketogluconic acid and gluconic acid, the main acids accountable for Zn solubilization (Yadav et al., 2023).

Our study revealed that each of *Bacillus* isolate was positive for at least two PGPR traits and >68% of the isolates were positive for at least five of PGPR traits in addition to suppression of the phytopathogenic fungus, *B. fabae* (Figure 2, Tables 3-5). The secretion of antifungal substances, hydrolytic enzymes and growth substances, and nutrient utilization by most of *Bacillus* isolates showed the possibility of synergistic activity among these characters. This is in agreement with the result of Yadav et al. (2021) who reported similar mechanisms of biocontrol activity and plant growth promotion traits of *Bacillus* isolates against fungal plant pathogens. Dugassa et al. (2021) reported *Pseudomonas* spp. and *Bacillus* spp. from the rhizosphere of faba bean that exhibited multi-PGPR traits and antifungal activity and tolerance to environmental and chemical stresses. It was also revealed that PGPR can indirectly promote the growth of plants by suppressing the harmful effects of biotic stress through production of PGPR traits including HCN, and other lytic enzymes, viz., chitinase, protease, pectinase, and cellulase (Ribeiro and Cardoso, 2012; Sing et al., 2019), which was also noted from our in-vitro study.

5. Conclusion and Recommendations

This study revealed the isolation and characterization of rhizospheric *Bacillus* isolates with a potent inhibitory activity against the phytopathogenic fungus *B. fabae* and that express multi-PGPR traits under in-vitro conditions. *Bacillus* isolates G3, D12 and S5 exhibited a high inhibition zone and PIRG against *B. fabae* whilst isolates S3, D14, S11, G9, D5 and S6 showed a moderate inhibition zone and PIRG. Many of our *Bacillus* isolates were positive in at least five PGPR traits, revealing a synergistic activity among PGPR traits. We recommend that the three *Bacillus* isolates (G3, D12 and S5) could be used as bio-inoculant strains in the growth of faba bean after further investigations for inhibition of the test pathogen and their growth promotion under greenhouse and field conditions.

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Declaration of competing interest

The authors declare no competing interests.

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