Research article

Characterization of faba bean (Vicia faba L.) rhizosphere associating rhizobacteria against Botrytis fabae AAUBF-12 and their plant growth-promoting properties

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ABSTRACT

The rhizobacteria are known to protect plants from different pathogens acting as biocontrol agents and promote growth of plants. This study was conducted to isolate, screen and identify faba bean associating rhizobacteria for their antagonistic properties against Botrytis fabae AAUBF-12 and plant growth-promoting properties under in vitro conditions. In the dual culture assay, the isolates inhibited the mycelia growth of B. fabae AAUBF-12 (6–40 %) upon 3 days of incubation, and the inhibition increased to 9–43 %, 16–50 %, and 24–68 % after five, seven and 9 days of incubation, respectively. The inhibitory activity increased from 6 to 82 % using the culture filtrates of the isolates. Isolate AAUB95 displayed the highest mycelial inhibition (27 %) at 5 % concentration of culture filtrate, followed by AAUB146 that exhibited 21 % inhibition at the same concentration. AAUB146 and AAUB100 effectively inhibited B. fabae AAUBF-12 by 79 % and 80 % at 20 % concentrations of the culture filtrate. The qualitative study demonstrated 75 % of the isolates positive for protease and 60 % for lipase synthesis. Furthermore, the isolates that showed antagonistic activity against B. fabae AAUBF-12, produced IAA and ammonia with 65 % and 60 %, respectively. Moreover, 310–760 μg mL⁻¹ and 200–620 μg mL⁻¹ of tricalcium phosphate (TCP) was released on the 3rd and 6th days of incubation, respectively, due to rhizobacterial solubilization. Nevertheless, the Pearson’s correlation analysis between pH and TCP solubilization revealed an inverse relationship (r = -.422**). Based on 16S rRNA sequences analysis, isolate AAUB95, AAUB146b, AAUB100 and AAUB92 were identified as B. subtilis AAUB95, S. nematodiphila AAUB146b, B. tequilensis AAUB100 and B. subtilis AAUB92, respectively. Of the isolates, B. subtilis AAUB95 showed best antagonism of B. fabae AAUBF-12 with multiple plant growth-promoting properties.

1. Introduction

Faba bean (Vicia faba L.) is one of the pulse crops grown in the highlands of Ethiopia. However, abiotic and biotic stresses are implicated with low yield of faba bean (Mulugeta et al., 2019). Chocolate spot that is caused by Botrytis fabae is one of the most important fungal disease attacking faba bean. The yield loss by the disease can reach up to 50–100 % in highly susceptible faba bean varieties (Saber et al., 2011). In Ethiopia, the yield losses of about 67.5 % was reported in susceptible faba bean varieties (Sahile et al., 2010). The disease can be managed through the application of fungicides such as Mancozeb and Natura®250 EW. However, the extensive uses of fungicides are undesirable due to health concerns, the hazard effects they inflict on the environment and their high cost. For example, fungicides have been reported to cancer, respiratory and hormonal imbalance diseases in humans (Piel et al., 2019). So as to overcome these problems, biological control (BC) via the application of different microorganisms has drawn attention for the best growth promotion and yield of plants in addition to controlling the pathogens (Glare et al., 2012).

Plant growth-promoting rhizobacteria (PGPR) produce lytic enzymes such as protease, lipase, cellulase, chitinases and induction of systemic resistance against phytopathogens (Thomloudi et al., 2019). They also synthesize phytohormones such as, indole-3-acetic acid (IAA) and solubilize inorganic phosphate to promote plant growth (Gupta et al., 2015). The genera of Bacillus, Pseudomonas, Serratia and Enterobacter spp. are the well-studied biocontrol agents (BCAs) of several plant pathogens.

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Pseudomonas fluorescens isolates showed 88% mycelial growth inhibition of B. fabae (Alemu, 2016). Bacillus spp. also displayed 23–64% mycelial growth inhibition of B. fabae under in vitro conditions (Sahile et al., 2009), Ahmed (2015) and El-Banoby et al. (2013) have reported the inhibitory activity of B. subtilis on the mycelial growth of B. fabae by 62.6% and 67.03% respectively. Similarly, de Senna and Lathrop (2017) showed that Serratia plymuthica inhibited the growth of Botrytis cinerea by 51% under in vitro study.

The hitherto studies focused on screening of selected isolates such as Bacillus and Pseudomonas spp. antagonistic to B. fabae under laboratory conditions in Ethiopia (Sahile et al., 2009; Alemu, 2016). However, there is a dearth of information on the genetic identification and plant growth-promoting properties of rhizobacteria isolated from the rhizosphere of faba bean. Therefore, the present study was initiated to evaluate the in vitro inhibitory potential of rhizosphere bacterial isolates from the rhizosphere of faba bean. The representative colonies were picked, purified, preserved in 20% glycerol and incubated at -20°C for further studies.

2. Materials and methods

2.1. Sampling area, sample collection and isolation of rhizobacteria

Rhizospheric soil samples were collected from Arsi zone (DigelunaTijo and Tiyo districts) and Bale zone of Goba district (Figure 1). Ten gram (10 g) of soil sample was suspended in 90 mL of 0.85% of normal saline solution and further prepared to appropriate dilutions (10⁻¹-10⁻⁷), 100 μL was transferred to nutrient agar media containing 100 μg Amphotericin in triplicates, and incubated at 28±2°C for 24–72 hr. The representative colonies were picked, purified, preserved in 20% glycerol and incubated at -20°C for further studies.

2.2. Isolation of the study pathogen (Botrytis fabae) and pathogeneity test

The test pathogen (Botrytis fabae) was isolated from faba bean leaves (showing symptoms of chocolate spot) and stored seeds using faba bean dextrose agar (FDA) medium according to Shinde (2016). The identification was made based on the cultural characteristics (i.e. colony color and formation of sclerotia on the potato dextrose agar (PDA)). The purified cultures were preserved at 4°C using PDA slants and used for pathogenicity test according to Abdel-Aleem et al. (2011) and the isolate that showed the common symptom of chocolate spot was labeled as Botrytis fabae AAUBF-12.

2.3. Dual culture assay

The dual culture assay was done according to Zivkovic et al. (2010). A loopful of bacteria from 48 hr. old culture was streaked on one side of the Petri plate containing PDA modified with 10% sucrose and incubated at 28±2°C. After 48 hr. of incubation, 5 mm mycelial disk from 9 days old B. fabae AAUBF-12 culture was inoculated on the opposite side to the bacteria on the same plate and incubated until the control plates were fully covered by the growth of B. fabae AAUBF-12. The measurement of radial/mycelial growth was taken on the 3rd, 5th, 7th and 9th days of incubation. The assay was performed three times in triplicates and the percentage inhibition of radial growth (PIRG) was calculated using the following formula;

\[ \text{PIRG} = \frac{R_1 - R_2}{R_1} \times 100\% \]

where, \( R_1 \) - the radius of B. fabae AAUBF-12 in the control and \( R_2 \) - radial growth of B. fabae AAUBF-12 toward the rhizobacterial isolate in the treated Petri plates.

Figure 1. A map showing the study region (Oromia) and zones in Ethiopia (a), zones (b) and selected districts in each zone (c).
2.4. Culture filtrate-none volatile assay

The isolates that inhibited the growth of *B. fabae* AAUF-12 under dual culture assay were further screened for their antagonistic activity using cell filtrates according to Haggag and El Soud (2012). The filtrates were obtained from 48 hr. old nutrient broth culture grown at 28 ± 2 °C on a rotary shaker (ZJZD-III, Shanghai, China). The broth culture was centrifuged (Centrifuge, Wagtech international, United Kingdom) at 4000 rpm for 30 min, the supernatants were sterilized by passing through sterile filter papers (0.2 mm), the culture filtrates were prepared at the concentrations of 5, 10, 15 and 20 % (v/v) and supplemented to PDA amended with 10 % sucrose. The plates were then centrally inoculated with 5 mm mycelial cut from 7 days old culture of *B. fabae* AAUF-12 and incubated at 25 ± 2 °C. The experiment was performed three times and three replicates were made for each treatment. The percent inhibition of mycelial growth (PIMG) was calculated using the following formula;

\[ \text{PIMG} = \frac{D_1-D_2}{D_1} \times 100 \]

where, \( D_1 \) the diameter of *B. fabae* AAUF-12 in the control and \( D_2 \) the diameter of *B. fabae* AAUF-12 in the treated Petri plates.

2.5. In vivo-detached leaf assay method

A detached leaf assay was conducted according to Li et al. (2018). A healthy faba bean leaves were sterilized with 5% sodium hypochlorite for 1 min, followed by repeated rinsing in double distilled sterile water and air dried under fume hood. Then, 20 μL of culture suspension (CSS) of antagonistic bacteria (Approx. 1 × 10⁸ CFU/mL) was poured at the center of the leaves and allowed for two hours. Following that, the spores concentrations of *B. fabae* AAUF-12 was adjusted to 4.5 × 10² spore mL⁻¹ using haemocytometer. The inoculated leaves were kept on sterile moistened filter papers in Petri dishes with 85 cm of diameter and kept at 28 °C for 7 days. The size of the lesions was used to calculate the percentage of inhibition relative to the control. The experiment was performed three times in three replicates including the positive control (PC) and the negative control (NC).

2.6. Cultural characterisation of rhizobacterial isolates

The isolates were gram stained by taking a loopful of 24 hr. culture on glass slides, heat fixed, flooded with crystal violet for 1 min and rinsed with water. Slides were mordant dyed with Gram’s iodine for 1 min, decolorized with 95 % alcohol for 1 min, and counter stained with Safranin for 30 seconds after having been washed with water, air dried and observed under microscope (OLYMPUS-BX51, Germany) of oil immersion to distinguish the gram staining of the cells.

2.7. Extraction of rhizobacterial Deoxyribonucleic acid (DNA)

The extraction of rhizobacterial DNA was conducted according to Souza et al. (2017). The isolates were grown on nutrient agar medium at 25 °C for 48 hr. Genomic DNA was extracted from 2-3 colonies picked from the medium by transferring into 1.5 mL micro centrifuge tubes containing 200 μL of cell lysis buffer (0.05 M NaOH and 0.25 % SDS mixture), heated at 100 °C for 20 min, centrifuged at 10,000 rpm for 3 min and the DNA pellet was diluted 20x in sterile MilliQ water and stored at -20 °C for PCR reactions.

2.8. Primers, polymerase chain reaction (PCR) conditions for DNA amplification (16 rRNA) and identification of rhizobacteria

For PCR reactions, 3 μL of the extracted DNA was used as a template. The PCR reaction was conducted using the primers pair of 8NF (5’-AGAGTTTGATCCTGCTGTCAG-3’) and 1429R (5’-ACGGCTACCTTGTGTTACGACTT-3’) according to Estikova et al. (2002). The PCR cycle included an initial 2 min denaturation at 95 °C followed by 10 cycles of denaturation at 95 °C for 1 min, 1 min primer annealing at 65 °C with the annealing temperature decreased by 1 °C with each succeeding cycle, and 1 min elongation at 72 °C.

The sequences obtained were blasted against the GenBank database using the Basic Local Alignment Search Tool (BLAST at http://www.ncbi.nlm.nih.gov). The sequence similarity was identified from the best score of the compared species and sequences were aligned using multiple alignment fast Fourier transform (Katoh et al., 2002). The phylogenetic tree was constructed by molecular evolutionary genetic analysis (MEGA) version X. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to construct the phylogenetic tree using K2+G+I model.

2.9. Hydrogen cyanide (HCN) and ammonia production

Hydrogen cyanide production was detected according to Bakker and Schippers (1987). The isolates were grown on HCN induction medium for 2-5 days at 28 ± 2 °C. A hundred micro liter (100 μL) of the culture containing 1 × 10⁸ CFU mL⁻¹ was spread at the center of the Petri plates whose lids were over layered with filter paper (Whatman No. 1) strips impregnated with picric acid solution. Petri plates were sealed with parafilm and incubated at 28 ± 2 °C for 48–72 hr. Plates having the picric acid impregnated filter paper only were considered as controls. A change in color of the paper strip from yellow to orange-brown was considered positive for HCN production.

The qualitative assay of ammonia production was done according to Cappuccino and Sherman (1992). The isolates were grown in 5 mL peptone water at 28 ± 2 °C for 4 days, 1 mL of Nessler’s reagent was added and the development of yellow color was considered positive for ammonia production.

2.10. Qualitative and quantitative solubilization of tricalcium phosphate (TCP)

The qualitative study of phosphate solubilization was performed by using Pikovskaya’s (PVK) agar and quantified according to Nautiyal (1999) using National Botanical Research Institute Phosphate (NBRIP) medium containing tricalcium phosphate as the sole source of phosphate. Phosphate solubilization index (PSI) was calculated according to the following formula;

\[ \text{PSI} = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}} \]

The concentration of released phosphate was quantified against a standard curve constructed from known quantity of potassium dihydrogen orthophosphate, KH₂PO₄ (μg mL⁻¹). The pH of the supernatant was measured using a digital pH meter (NIG 333, New Delhi, India).

2.11. Qualitative and quantitative assay of indole-3-acetic acid (IAA)

The ability of isolates to produce IAA was checked qualitatively as described by Hartmann et al. (1983). Two hundred micro liter (200 μL) of bacterial culture containing 1 × 10⁸ CFU mL⁻¹ were grown in 50 mL nutrient broth amended with tryptophan (1 g L⁻¹), respectively. The cultures were, incubated at 25 °C on an orbital shaker (ZJZD III, Shanghai, China) for 72 hr. whereby uninoculated tubes served as controls. The cultures were centrifuged (Centrifuge, Wagtech international, United Kingdom) at 30000 rpm for 30 min, from which 2 mL of the supernatants were mixed with two drops of orthophosphoric acid and 4 mL of Salkowski’s reagent (50 mL of 70 % per chloric acid and 1 mL of 0.5 M FeCl3 and 49 mL sterilized distilled water), and incubated in dark for 30 min. The development of pink colour was visually checked as an indicator of IAA production and 1 mL of the supernatant was quantified spectrophotometrically (6405 UV/Vis., Jenway, England) at 530 nm. The
Table 1. Identification and gram reaction of rhizobacterial strains obtained from the rhizosphere soils samples of faba bean in Arsi and Bale zones.

| Isolate code | Identified strains/species | Accession number of the identified strain | Accession number of the nearest species obtained from GenBank (16S rRNA) |
|--------------|---------------------------|------------------------------------------|---------------------------------------------------------------------|
| AAUB100      | E. hormaechei              | EU036987.1                               | MW879446                                                            |
| AAUB113b     | S. nematodiphila           | EU036987.1                               | MW879450                                                            |
| AAUB113c     | S. nematodiphila           | EU036987.1                               | MW879454                                                            |
| AAUB115      | S. nematodiphila           | EU036987.1                               | MW879450                                                            |
| AAUB122a     | B. subtilis                | AJ276351.1                               | MW879450                                                            |
| AAUB122b     | B. subtilis                | AJ276351.1                               | MW879460                                                            |
| AAUB122c     | B. subtilis                | AJ276351.1                               | MW879460                                                            |
| AAUB130a     | A. johnsonii               | Z93440.1                                 | MW879457                                                            |
| AAUB130b     | A. johnsonii               | Z93440.1                                 | MW879457                                                            |
| AAUB146a     | A. nicotinovorans          | -                                        | AAUB146a                                                            |
| AAUB146b     | B. tequilensis             | AJ276351.1                               | MW879450                                                            |
| AAUB47       | E. faecalis                | -                                        | AAUB47                                                              |
| AAUB53       | B. macroides               | AJ628749.1                               | MW879445                                                            |
| AAUB55       | C. coccus                  | AJ508302.1                               | MW879451                                                            |
| AAUB77       | S. nematodiphila           | EU036987.1                               | MW879458                                                            |
| AAUB92       | B. tequilensis             | AJ276351.1                               | MW879450                                                            |
| AAUB94       | E. hormaechei              | -                                        | AAUB94                                                              |
| AAUB95       | B. subtilis                | -                                        | AAUB95                                                              |
| AAUB100      | E. hormaechei              | EU036987.1                               | MW879450                                                            |
| AAUB113b3    | B. subtilis                | -                                        | AAUB113b3                                                           |

The statistical analysis was performed by Two-Way ANOVA of SPSS version 24. The comparisons among means were done by Tukey honestly significant difference (HSD) at α = 0.05. All the values were considered statistically significant at p < 0.05.

3. Results and discussion

3.1. Cultural characterisation and molecular identification of the rhizobacterial isolates

Rhizospheric soil samples from faba bean (Vicia faba L.) plants were collected from Arsi and Bale Zone in Oromia region to screen bacterial isolates for biological control agents of Botrytis fabae AAUBF-12, the causal agents of chocolate spot disease in faba bean and evaluate their antagonistic and plant growth promoting properties. A total of twenty rhizobacterial strains were isolated from the rhizosphere of faba beans from sixteen locations that are found in Arsi and Bale zones (Table 1). Based on the gram reaction, the rhizobacterial strains were grouped into Gram positive (45 %) and Gram negative (55 %). The gram negative strains were grouped into the genera of Acinetobacter, Enterobacter, Serratia, and Enterococcus, and the gram positive isolates were the genera of Bacillus and Arthrobacter. The genera Bacillus were the dominant rhizobacteria with eight (8) species representing 40 % of the isolates, followed by the Serratia isolates that represented with five (5) species containing 20 % of the isolates. It is interesting to note that the commonest rhizobacteria genus Pseudomonas contained only one (1) representative isolate.

The 16S rRNA sequence analysis assigned the isolates into the genera of Bacillus, Serratia, Enterobacter, Arthrobacter, Acinetobacter and Enterococcus. The sequences of the isolates were deposited in NCBI with GenBank accession number listed in Table 1 and the phylogenetic tree of all the isolate is presented below having some related representative of reference strains retrieved from NCBI (Figure 2). However, three isolates (AAUB152, AAUB150 and AAUB113b3) were not identified by the sequence analysis, whose DNA amplification failed which might be associated with the primers and/or the PCR conditions employed during DNA amplification. However, these isolates were identified to the genus level through treating at a temperature of 70 °C for 14 min to check their temperature tolerance and an ability to grow when re-streaked on nutrient agar to be considered as Bacillus species and inability to grow on King B medium for Pseudomonas species.

3.2. The antagonistic properties of rhizobacterial isolates against Botrytis fabae AAUBF-12 through dual culture assay

In the present study, the rhizobacterial isolates were evaluated against B. fabae AAUBF-12 isolated from chocolate spot infected faba...
beans. The isolates showed significant difference to inhibit the test pathogen with the percentage of mycelial growth inhibition that ranged from 6-68 % (Table 2) upon 3–9 days. They differed in their effectiveness to inhibit the mycelial growth of the pathogen by 6–40 %, 9–43 %, 16–50% and 24–68 % on the 3rd, 5th, 7th and 9th days of incubation, respectively. All the strains showed in the percentage of the pathogen mycelial growth inhibition as the days of incubation increased. Among the identified strains, Bacillus subtilis AAUB95, Bacillus subtilis AAUB122b and Bacillus macroides, AAUB122a exhibited the highest inhibition in all the incubation days (3–9 days). Bacillus macroides AAUB122a displayed the highest inhibition (40 %) over the control within the shortest incubation time (3rd days), whereas Enterobacter hormaechei AAUB53 showed the least percentage of B. fabae AAUBF-12. However, Bacillus tequilensis AAUB100, Serratia nematophila AAUB115, Bacillus sp. and AAUB150 showed mild inhibitory activities throughout the incubation days. However, the other isolates did not show significant antagonistic activity as a function of incubation days. In addition, in this study, of all the strains, the Bacillus strains were found to show the highest percentage of B. fabae AAUBF-12 mycelial growth inhibition compared to the other strains. This might be caused by the ability of Bacilli spp. to produce several volatile and non-volatile antifungal compounds to inhibit plant pathogens, indicating their potential use as biocontrol agents to suppress phytopathogenic. For example, the genera of Bacillus are known to produce lipopeptides, classes of surfactin, iturin, and fengycin. Microbes that produce such lipopeptides are good biocontrol agents (Malviya et al., 2020).

Moreover, the Serratia strains were relatively effective rhizobacteria to inhibit the mycelial growth of B. fabae AAUBF-12 compared to others, of which the Bacillus strains displayed 10–68 % of inhibition upon 3–9 days of incubation. A study conducted in Ethiopia, Sahile et al. (2009) have reported that Bacillus spp. obtained from faba bean leaves indicated 23–64 % of B. fabae mycelial growth inhibition upon 5–10 days of incubation. Furthermore, Ahmed (2015) and El-Banoby et al. (2013) have reported that Bacillus subtilis inhibited the mycelial growth of B. fabae by 62.6 % and 67.03 % within five (5) days of incubation respectively, which is different from the present study. The difference might be associated with the inherent characteristics of strains used in this study, the inoculum size and/or the experimental conditions used in the studies. Similarly, the different strains of Serratia nematophila exhibited the percent inhibition of the B. fabae AAUBF-12 mycelia that ranged from 8-57 % upon 3–9 days. These strains displayed similar pattern of inhibitory activity with Serratia plymuthica that showed 51 % inhibition of Botrytis cinerea (de Senna and Lathrop, 2017) and Serratia marcescens with 54.81 % inhibition against Rhizoctonia solani after seven (7) days (Ahmed, 2016). In this study, only one strain of Pseudomonas AAUB152 was found with the inhibitory activity of 13–53 % upon 3–9 days of incubation. Pseudomonas species are one of the commonest rhizobacteria that have effective plant growth-promoting and antagonistic properties (Altinok et al., 2016). In contrast, Alemu (2016) has isolated several Pseudomonas spp. whereby P. fluorescens-10 from the rhizosphere of faba bean showed 88 % mycelial growth inhibition of B. fabae on the 7th days of incubation using King B medium which is different from the present study. The difference might have associated with the type of medium used to test the antagonistic activity that favors the better performance of P. fluorescens than the studied pathogen. However, in our study, potato dextrose agar (PDA) modified with 10 % sucrose was used to study the antagonistic activity of the rhizobacterial strains against B. fabae AAUBF-12.

3.3. The antagonistic activity of the rhizobacterial strains against Botrytis fabae AAUBF-12 using culture filtrates

The antagonistic properties of the rhizobacterial isolates were further screened by using culture filtrates of different concentrations (5–20 %, v/v...
v). As noted from the data shown below (Table 3), the culture filtrates showed better mycelial growth inhibition of *B. fabae* AAUF-12 than observed in the dual culture assay (Table 2). The culture filtrate inhibited the mycelial growth of the test pathogen in the range of 6–82 %. The highest inhibition (27 %) was recorded from the culture filtrate of *Bacillus subtilis* AAUB95 at 5 % of the filtrate concentration. Similarly, in the dual culture assay, the strain exhibited the highest antagonistic activity (68 %) and an inhibition of 82 % at 20 % concentration of its filtrate was

Table 2. Percent inhibition of radial growth (PIRG) displayed by the antagonistic bacterial isolates against *B. fabae* AAUBF-12 upon 3–9 days of incubation using dual culture method.

| Sample  | Isolate code | Strains/species         | Percent of inhibition over control in days |
|---------|--------------|-------------------------|------------------------------------------|
|         |              |                         | 3 days | 5 days | 7 days | 9 days |
| FRSS-04 | AAUB100      | *Bacillus tequilensis*  | 19bc   | 25cd   | 41abc  | 59ab  |
| FRSS-01 | AAUB113b     | *Serratia nematodiphila*| 20bc   | 32bc   | 52a    | 57bc  |
| FRSS-08 | AAUB146b     | *Serratia nematodiphila*| 10sd   | 17bc   | 30bd   | 53bc  |
| FRSS-10 | AAUB92       | *Bacillus subtilis*     | 28ab   | 30bc   | 49bc   | 56bc  |
| FRSS-15 | AAUB113c     | *Serratia nematodiphila*| 12cd   | 17de   | 22ed   | 31bf  |
| FRSS-02 | AAUB53       | *Enterobacter hormaechei*| 06d   | 09a    | 16d    | 31bf  |
| FRSS-13 | AAUB122a     | *Bacillus macroides*    | 40\*   | 48\*   | 49ab   | 68\*  |
| FRSS-06 | AAUB122b     | *Bacillus subtilis*     | 37\*   | 50a    | 59ab   | 55bc  |
| FRSS-03 | AAUB115      | *Serratia nematodiphila*| 20bc   | 40ab   | 49bc   | 46bc  |
| FRSS-07 | AAUB94       | *Enterobacter hormaechei*| 13cd   | 23de   | 24cd   | 24f   |
| FRSS-16 | AAUB95       | *Bacillus subtilis*     | 30b    | 39ab   | 47ab   | 68b   |
| FRSS-11 | AAUB77       | *Serratia nematodiphila*| 08cd   | 15de   | 25cd   | 45cd  |
| FRSS-14 | AAUB55       | *Enterobacter hormaechei*| 12cd   | 14de   | 18d    | 23f   |
| FRSS-08 | AAUB152      | *Pseudomonas sp.*       | 13cd   | 20de   | 34abcd | 53bc  |
| FRSS-05 | AAUB130b     | *Arthrobacter johnsonii*| 14ed   | 16de   | 21ed   | 25f   |
| FRSS-07 | AAUB130a     | *Arthrobacter johnsonii*| 10cd   | 16de   | 29cd   | 41bc  |
| FRSS-09 | AAUB47       | *Enterococcus faecalis* | 16cd   | 19def  | 20ef   | 22f   |
| FRSS-12 | AAUB146a     | *Arthrobacter nicotinovorans* | 15cd   | 19cde  | 20cd   | 27f   |
| FRSS-03 | AAUB150      | *Bacillus subtilis*     | 13b    | 14de   | 24cd   | 31def |
| FRSS-13 | AAUB150b     | *Arthrobacter nicotinovorans* | 10cd   | 14de   | 24cd   | 31def |
| FRSS-09 | AAUB47       | *Enterococcus faecalis* | 16cd   | 19cde  | 20cd   | 27f   |
| FRSS-11 | AAUB113b3    | *Bacillus subtilis*     | 10cd   | 14de   | 24cd   | 31def |
| CV      |              |                         | 0.12   | 0.10   | 0.11   | 0.12  |

Table 3. Percent inhibition of radial/mycelial growth (PIRG) displayed by the antagonistic bacterial isolates against *B. fabae* AAUBF-12 upon different culture filtrate concentrations (5–20 %).

| Strains/species                        | Inhibition over control at 5-20 % v/v of the culture filtrate |
|----------------------------------------|---------------------------------------------------------------|
|                                        | 5 %   | 10 %  | 15 %  | 20 %  |
| *Bacillus tequilensis* AAUB100         | 15\*edef | 30\*cd | 52\*abc | 80\* |
| *Serratia nematodiphila* AAUB113b      | 15\*cd | 27\*cd | 54\*bcd | 75\*bc |
| *Serratia nematodiphila* AAUB146b      | 21\*bc | 41\*bc | 60\*abc | 79\* |
| *Bacillus subtilis* AAUB92             | 13\*defg | 32\*cd | 53\*bcdef | 73\*bdef |
| *Serratia nematodiphila* AAUB113c      | 13\*defg | 24\*def | 39\*efg | 63\*efg |
| *Enterobacter hormaechei* AAUB53       | 18bdef | 38\*abcd | 49\*cdef | 53g |
| *Bacillus macroides* AAUB122a          | 26a    | 31bdef | 46\*cdef | 66\*bde |
| *Bacillus subtilis* AAUB122b           | 17cdef | 41\*abc | 50\*bdef | 77\*b |
| *Serratia nematodiphila* AAUB115       | 09f    | 35bde | 64\*bde | 65\*def |
| *Enterobacter hormaechei* AAUB94       | 11e  | 20\*f | 47\*bdef | 77\*ab |
| *Bacillus subtilis* AAUB95             | 27a    | 47\*ab | 65\*ab | 82\* |
| *Serratia nematodiphila* AAUB77        | 17\*de | 50\*f | 66\*f | 75\*abc |
| *Enterobacter hormaechei* AAUB55       | 16\*def | 31\*de | 62\*bcdef | 66\*bde |
| *Pseudomonas sp.* AAUB152              | 16\*def | 29\*cdef | 50\*bcdef | 72\*bdef |
| *Arthrobacter johnsonii* AAUB130b      | 25\*b | 32bde | 38\*f | 42\* |
| *Arthrobacter johnsonii* AAUB130a      | 06\*f | 32\*bde | 38\*f | 59\*efg |
| *Enterococcus faecalis* AAUB47         | 09f   | 15\*f | 27\*f | 29f |
| *Arthrobacter nicotinovorans* AAUB146a | 26\*f | 40\*abc | 48\*bcdef | 61\*efg |
| *Bacillus sp.* AAUB150                 | 19bdef | 33bdef | 51\*bdef | 63\*def |
| *Bacillus sp.* AAUB113b3               | 16\*cdef | 27\*cdef | 46\*cdef | 55\* |
| CV                                     | 0.16   | 0.12  | 0.15  | 0.18  |

Mean values in the same column labeled with the same letter(s) as superscript are not significantly different (p > 0.05) by Tukey honestly significant difference (HSD) analysis of One-Way ANOVA. CV-indicates the coefficient of variation among means in the same column.
found after incubation for 9 days. This may indicate the ability of the strains to produce none volatile secondary metabolites that can inhibit the mycelial growth of the phytopathogenic fungi. Thus, further study need to be conducted to identify the compounds that are responsible for the antifungal activity of B. fabae AAUBF-12 in the present study.

Serratia nematophila AAUB146b was also effectively inhibited the test pathogen under this in vitro study with the inhibition of 21 % at (5 %) and 79 % at (20 %) culture filtrate concentrations. Although, Bacillus tequilensis AAUB100 was relatively weak in mycelial growth inhibition of AAUBF-12 at 5 % of its culture filtrate concentration, it displayed 80 % inhibition with 20 % of the filtrate. In addition, Serratia nematophila AAUB146b (79 %) was the best antagonistic rhizobacterium against B. fabae AAUBF-12 next to Bacillus subtilis AAUB95 (82 %) and Bacillus tequilensis AAUB100 (80 %) at 20 % concentration of the culture filtrate (Table 3). El Khaldi et al. (2015) have indicated that the culture filtrate of Serratia marcescens suppressed the mycelial growth of Rhizoctonia solani by 65.6 %, through the application of 100 μL of the culture filtrate after 7 days of incubation. On the other side, Serratia sp. C4 displayed antifungal activity with 19.52 % decrease in the mycelial growth of Fusarium oxysporum f. sp. lycopersici (FOL) through dual culture assays. However, the culture filtrate supplemented to PDA medium at 20 % (v/v), slightly improved the inhibition of the pathogen by 23 % at 20 % of concentration on the 5th days of incubation (Aydi-Ben Abdallah et al., 2017). This support our finding that indicated the best inhibition performance by the studied different concentrations of the culture filtrate instead of using the strains directly. This might be due to the early coming together of the metabolites within the filtrate with the study pathogen unlike in the dual culture method of antagonistic activity evaluation/determination.

3.4. Inhibition of B. fabae AAUBF-12 on faba bean leaves by antagonistic bacteria and their extracellular culture filtrate-detached leaf assay

The detached leaf assay is a simple in vitro method that is used to evaluate the pathogenicity and virulence of pathogenic microbes that cause foliar diseases in plants and that used to screen potential biological control agents for the management of plant pathogens (Cowley et al., 2012). In the present study, the detached leaf assay showed that culture suspension of B. subtilis AAUB95 inhibited the occurrence of faba bean leaf lesions by 85 %. In the same way, the culture filtrates (CF) of the strains inhibited leaf lesions by 94. 25 % (Figure 3). This is in agreement with Li et al. (2018) who have reported that the culture filtrate and culture suspension of B. tequilensis GYLH001 significantly inhibited the lesions length caused by M. oryzae on the detached leaves of rice plants. This result suggested that, the antagonistic rhizobacterial strain (B. subtilis AAUB95) can be used as biological control agents of B. fabae AAUBF-12. The strain was further studied under field and greenhouse conditions in combination with other plant beneficial fungi (Trichoderma harzianum AATU14) against chocolate spot disease, caused by B. fabae so as to evaluate the disease severity/incidence and its effect on the growth promotion of faba bean. Our study indicated that, the incidence and severity of chocolate spot was reduced to 23–46 % and 37–48 % in faba beans, respectively (Zewdineh et al., 2020). Further, the study showed 62
and 34 % increment of hundred seed dry weight and grain yield respectively over the uninoculated controls under the field study. Under greenhouse study, the same study revealed 50 % reduction of chocolate spot disease incidence in Ashebeka variety of faba beans. Thus this study could suggest that, B. subtilis AAUB95, can be re-tested against chocolate disease, formulated and used as bioinoculant for the control of chocolate spot disease in faba beans. In this regard, Bacillus spp. based products, such as Kodiak (Bacillus subtilis GB03), Quantum-400 (B. subtilis GB03), Rhizovital (Bacillus amyloliquefaciens FZB42), Serenade (B. subtilis QST713), and YIB (Bacillus spp.), have also been commercialized for improving crop production (Cawoy et al., 2011).

3.5. The antagonistic and plant growth-promoting properties displayed by the rhizobacterial strains

The antagonistic rhizobacterial strains were characterized for their possible exerted mechanisms associated with the control of B. fabae AAUBF-12 and plant growth-promoting traits. The qualitative assay demonstrated that 75 % and 60 % of the strains were positive for protease and lipase synthesis, respectively (Table 4). These are the possible antagonistic features displayed by the strains against B. fabae AAUBF-12 to control its mycelial growth as observed in our present study. Enzymes such as cellulase, protease, and chitinase are reported to limit the growth and an abnormal hyphal morphology of several plant pathogens (Shrestha et al., 2015). Therefore, in our study, protease and lipase secreted by the strains can cause the hyphal deformation and growth suppression of B. fabae AAUBF-12 which need to be confirmed by extra studies. Of the studied strains, 100 % and 75 % of the Bacillus were positive for protease and lipase secretion, respectively. It has been revealed that, the Bacillus species has great potential in biotechnology as they can produce a large number of commercially hydrolytic enzymes ab bioactive substances which are beneficial for plant growth health (Ali et al., 2020). In addition, Pseudomonas sp. AAUB152, Bacillus macroides AAUB122a and Bacillus subtilis AAUB95 produced HCN as well as bioactive substances which are beneficial for plant growth health (Ali et al., 2020).

Kaur et al. (2018) have implicated the production of HCN by Bacillus spp. with the mycelial growth inhibition of Fusarium oxysporum growth under in vitro study. In the present study, Serratia nematodiphila AAUB113b and Serratia nematodiphila AAUB115 produced HCN against the test pathogen (B. fabae AAUBF-12). The production of HCN by the antagonistic Serratia nematodiphila NII-0928 obtained from the rhizosphere of black pepper (Piper nigrum L) was also reported by Dastager et al. (2010). In addition, the author reported the biocontrol related metabolite (siderophore) production by the studied Serratia nematodiphila strains. Hence, the antagonistic property of the Serratia nematodiphila strains against B. fabae AAUBF-12 might be assisted by the HCN production and the other none tested metabolites such as the production of siderophore in the present study too. The variability in the inhibition potential of the study strains against B. fabae AAUBF-12 was attributed to the synthesis of lytic enzymes (protease and lipase) together with HCN production. Although the antagonistic potential of Arthrobacter nicotinovorans AAUB146a was 26–61 % using the culture filtrate assay at different concentrations. The strain did not produce either HCN and ammonia and/or enzymes (protease and lipase). This may indicate that the antagonistic mechanisms acquired by the strain might be different from the antagonistic property investigated in this study. Plant growth promoting rhizobacterial isolates produces enzymes that degrade fungal cellular components including chitinase, β-1,3 glucanase, and are cellulolytic (Heydari and Pessarakli, 2010) which can cause the loss of structural integrity, lysis, fragmentation and perforations of hyphae, and sclerotial degradation in phytopathogenic fungi (Sharma and Dubey, 2017).

In addition, 65 % and 60 % of the study strains produced IAA and ammonia, respectively (Table 4). The production of IAA was in the range of 0.18–15.51 μg mL⁻¹. Kaur et al. (2018) have reported that different plant growth-promoting bacterial isolates to produce IAA that varied from 5 to 24 μg mL⁻¹. In our study, Bacillus subtilis AAUB92 produced the highest IAA (15.51 μg mL⁻¹), followed by Bacillus macroides AAUB122a (13.93 μg mL⁻¹), Serratia nematodiphila AAUB77 (11.62 μg mL⁻¹) and Bacillus subtilis AAUB95 (10.52 μg mL⁻¹). The study also designated as
Bacillus strains produced the highest quantity of IAA compared to the other strains. Similarly, Naibi et al. (2013) have reported B. licheniformis and Bacillus sp. as the best producers of IAA, 78 and 101 mg g⁻¹, respectively, in addition to their ability to induce lytic enzyme and solubilize inorganic phosphates under in vitro conditions. Similarly, different strains of Serratia nematodiphila produced IAA that varied from 0.27–11.62 μg mL⁻¹. This was much lower than the IAA concentration of 64.75 μg mL⁻¹ and 56.60 μg mL⁻¹ produced by S. marcescens subs. marcescens strain KB01 and S. marcescens subs. marcescens strain KB05, respectively (Hasuty et al., 2017). The production of IAA by the studied strains can promote faba bean growth through reducing the adverse effects of ethylene and plant growth-promoting rhizobacteria can improve plant disease resistance (Li et al., 2018). Bashan and de-Bashan (2005) stated that mechanisms including nitrogen fixation, solubilization of phosphate, production of phytohormones such as auxins, cytokinins and gibberellins, production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase promote direct plant growth.

3.6. The potential of rhizobacterial strains to solubilize tricalcium phosphate (TCP)

Phosphorus (P) is the most important nutrient that plants need at an adequate rate from the early stages of their development. This nutrient plays key roles in root development, root traits anatomy modification and root hair density with a significant contribution in increasing yield of crops and plants resistance against multiple diseases (Ma et al., 2001). Phosphate solubilizing bacteria (PSB), as a group of PGPR, facilitate the hydrolysis of a wide range of phosphorus compounds leading to higher availability of phosphorus from inorganic phosphates under conditions. Similarly, different strains of Serratia nematodiphila produced IAA that varied from 0.27–11.62 μg mL⁻¹. This was much lower than the IAA concentration of 64.75 μg mL⁻¹ and 56.60 μg mL⁻¹ produced by S. marcescens subs. marcescens strain KB01 and S. marcescens subs. marcescens strain KB05, respectively (Hasuty et al., 2017). The production of IAA by the studied strains can promote faba bean growth through reducing the adverse effects of ethylene and plant growth-promoting rhizobacteria can improve plant disease resistance (Li et al., 2018). Bashan and de-Bashan (2005) stated that mechanisms including nitrogen fixation, solubilization of phosphate, production of phytohormones such as auxins, cytokinins and gibberellins, production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase promote direct plant growth.

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### Table 5. Phosphate solubilization efficiency of different Rhizobacterial strains obtained from faba bean (Vicia faba L.) rhizosphere soil; the amount of phosphorus released (μg mL⁻¹) (NBRIP liquid) from inorganic tricalcium phosphate based upon days (3–6) of incubation (for strains with PSI > 2) and change in the initial pH of the medium (pH0 = 7).

| Strains/species | PVKA (solid) PSI | NBRIP (liquid) (μg mL⁻¹) in days | Change in pH (3–6 days) |
|----------------|-----------------|---------------------------------|------------------------|
|                | Day 3           | Day 6                           |                         |
| Control        | 1.00a           | 55b                             | 6.40a                   |
| Bacillus tequilensis 100 AAUB | 2.67a         | 75a                             | 5.00de                  |
| Serratia nematodiphila AAUB113b | 2.21cd         | 410a                            | 4.50f                   |
| Serratia nematodiphila AAUB146b | 2.24abcd        | 650a                            | 5.01f                   |
| Bacillus subtilis AAUB92 | 2.51abc         | 75a                             | 3.53c                   |
| Serratia nematodiphila AAUB113c | 2.16d         | 460a                            | 5.91b                   |
| Bacillus macroides AAUB122a | 2.64a          | 700a                            | 4.98de                  |
| Bacillus subtilis AAUB122b | 2.14d          | 650a                            | 4.90de                  |
| Serratia nematodiphila AAUB115 | 2.18d          | 600a                            | 5.19d                   |
| Bacillus subtilis AAUB95 | 2.09d          | 410a                            | 5.18de                  |
| Serratia nematodiphila AAUB77 | 2.43d          | 440b                            | 4.94de                  |
| Pseudomonas sp. AAUB152 | 2.06d          | 310b                            | 5.00de                  |
| CV             | 0.12            | 0.15                            | 0.18                    |

PVKA- Pikovskaya’s agar, NBRIP- National Botanical Research Institute Phosphate, PSI- Phosphate solubilization index and μg mL⁻¹: Microgram per millilitre. Mean values in the same column labeled with the same letter(s) as superscript are not significantly different (p > 0.05) by Tukey honestly significant difference (HSD) analysis of One-way ANOVA. CV-indicates the coefficient of variation among means in the same column.

The Pearson’s correlation analysis between phosphate solubilization and the change in pH were summarized in Table 6.

### Table 6. The Pearson’s correlation analysis between phosphate solubilization and the change of pH.

| Correlations | solubilized phosphate | pH change |
|--------------|-----------------------|-----------|
| TCP          | Sig. (2-tailed)       | -.422a    |
| N            | 24                    | 24        |
| pH change    | Pearson Correlation   | -.422a    |
| Sig. (2-tailed) | .040                | 1         |
| N            | 24                    | 24        |

*Correlation is significant at the 0.05 level (2-tailed).
4. Conclusion

The present study, pointed out Bacillus and Serratia species as the potential isolates in showing the best inhibitory activity against Botrytis fabae AAUBF-12 along with the antagonistic and plant growth-promoting traits. Of the Bacillus strains, B. subtilis AAUB95 showed the best antagonistic feature against B. fabae AAUBF-12 with plant growth-promoting traits production and thus can be utilized as biofungicides either under greenhouse and/or field conditions after testing the strains several times. Even though, the strains of Serratia showed better performance against B. fabae AAUBF-12 and plant growth-promoting traits, further study should be conducted on the safety aspects of the strains prior to greenhouse and/or field application of the strains.

Declarations

Author contribution statement

Larissa Maia: conceived and designed the experiments; Zewdineh Firdu: performed the experiments; Wrote the paper.

Jorge Teodoro: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Fassil Assefa: Analyzed and interpreted the data.

Tesfaye Alemu: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at NCBI with GenBank under the accession number.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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