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Screening of indigenous rhizobacteria as potential biological control against faba bean (*Vicia faba* L.) gall disease caused by *Olpidium viciae*

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Abstract

Background: Emerged faba bean gall disease attacks the stem and leaves of the plant and results in complete crop losses. This study was initiated to screen multi-trait rhizobacteria for their antagonistic efficacy under in-vitro and in-vivo conditions against *Olpidium viciae* to control the effect of gall disease on faba bean. Sixty antagonistic isolates were first examined for their morphological, biochemical, and phenotypic traits.

Results: *Pseudomonas fluorescens* AAUPF62, *P. aeruginosa* AAUS31, *Bacillus* AAUMF42, and *Bacillus* AAUAm28 showed greater than 68, 62, 57, and 54% suppression of *O. viciae* in dual culture, volatile metabolites, culture filtrate assay, and detached leaves experiments, respectively. The in-vivo study revealed that early treatment of the crop with *P. aeruginosa* AAUS31 reduced severity by 63% (in FB-Obse) and 54% (in FB-26869) faba bean varieties. The co-inoculation of *P. fluorescens* AAUPF62 and *P. aeruginosa* AAUS31 significantly enhanced the shoot ($P = 0.003$; mean = 122 cm) and root ($P = 0.018$; mean = 94 cm) length, increased shoot dry weight by 8 factors (83 g pot⁻¹), and reduced final disease severity by 92% in FB-Obse variety.

Conclusions: The results revealed that *P. fluorescens* AAUPF62 and *P. aeruginosa* AAUS31 strains could be the potential antagonistic agents of gall disease. The use and early treatment of moderately resistant faba bean varieties by co-inoculation of synergistic potential bioagents were recommended.

Keywords: Biocontrol, Co-inoculation, Gall disease, In-vivo, Rhizobacteria, *Vicia faba*

Background

Faba bean (*Vicia faba* L.) is a grain legume crop widely cultivated in the highlands of Ethiopia as the main source of protein for human nutrition (Asfaw et al. 2018). In recent years the newly emerged faba bean gall disease caused by plant debris and soil-borne fungal pathogen (*Olpidium viciae* Kusano) becomes one of the major faba bean production constraints in faba bean growing areas

at an altitude greater than 2400 m above sea level (Bogale et al. 2017). To curb this problem, various management strategies have been carried out which depend mainly on an application of chemical fungicides since its occurrence (Teferi et al. 2018). However, the use of a chemical fungicide is not ecofriendly and could result in the emergence of fungicide resistance plant pathogens (Singh et al. 2013). Despite various management efforts made by researchers, the distribution pattern of faba bean gall disease was continued at escalating speed.

Hence, searching for supplementary indigenous biological control methods is a promising option. Biological control is a potential non-chemical means for plant

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disease management that reduces the harmful effects of the pathogen through the use of other living entities (Ferreira et al. 2020). The application of biological controls using antagonistic micro-organism is ecofriendly (Zhou et al. 2016) and has proved to be successful in controlling various plant diseases in many countries (Khaledi and Taheri 2016). More recently a good result has been obtained with beneficial bacteria that are indigenous to the rhizosphere of plants. The rhizosphere supports a metabolically active group of bacteria known as plant growth-promoting rhizobacteria (Alsohim et al. 2014). Rhizobacteria are plant-associated bacteria, derived from many genera that can colonize roots and suppresses the growth of soil-borne phytopathogens through various mechanisms (Dinesh et al. 2015). *Pseudomonas* and *Bacillus* species are among the rhizobacteria that can act against phytopathogens in plant root vicinity (Mota et al. 2017).

Pseudomonas spp. are common Gram-negative, non-spore-forming, rod-shaped bacteria (Gómez-Lama et al. 2018) whereas *Bacillus* spp. are Gram-positive spore-forming, rod-shaped bacteria (Suthar et al. 2017). Due to their various genetic and phenotypic characteristics, members of these genera have good potential to be used as biocontrol agents (Zhou et al. 2016). Rhizobacteria employed either direct or indirect disease control mechanisms which include plant growth promotion, production of hydrolytic enzymes, siderophore, hydrogen cyanide (HCN), and competition with disease-causing microbes for niches and nutrients (Audrain et al. 2015). Finally, they developed induction of systematic resistance in the plants (Haddoud et al. 2017).

Rhizobacteria are extensively used in the control of several diseases of crop plants (Abjala et al. 2015). However, there is limited knowledge regarding the biological suppression of *Olpidium viciae* Kusano in faba bean by application of rhizobacteria in Ethiopia. This study aimed to isolate bacteria from faba bean rhizosphere and screen the organism for in-vitro and in-vivo antagonistic activity against faba bean gall disease causal agent. Hence, this may contribute to the application of rhizobacteria as a biological control of the test pathogen in faba bean and other leguminous plants in Ethiopia.

Methods

Collection of the test pathogen

Previously isolated *Olpidium* isolate 4 (AAUO4) was obtained from the stock cultures originally isolated from naturally infected faba bean leaves samples with gall disease symptoms, collected from different localities of Angolelana Tera and Sululta districts, North Shoa, Ethiopia. The Potato Dextrose Agar (PDA) medium supplemented with chloramphenicol was used for reactivation,

multiplication, and preservation of AAUO4 strain for further use.

Rhizobacterial antagonists

Isolation of rhizobacteria antagonists

A total of 150 health faba bean plant rhizosphere soil samples were randomly collected from Angolelana Tera (50 samples), Sululta (50 samples), and Medakegn (50 samples) districts at 20 cm depth. Using standard serial dilution techniques (Islam et al. 2018), 0.1 ml of each soil sample was pipetted into autoclaved nutrient agar medium. A total of 120 different bacterial colonies were sub-cultured and purified on the appropriate medium. The pure isolates were kept at 4 °C on nutrient agar slant for the next use.

Characterization and evaluation of the bacterial isolates for biological control traits

In the preliminary stage, in-vitro dual culture techniques were used to identify the potential antagonistic rhizobacteria strains against the test pathogen on potato dextrose agar medium. Then, 60 stains were selected, characterized, and identified based upon their morphological, phenotypic, and biochemical characteristics (Suthar et al. 2017).

The biochemical characteristics of isolates were assayed aimed to evaluate lipopolysaccharide (Mota et al. 2017), catalase (Dinesh et al. 2015), oxidase (Suthar et al. 2017) activities, and citrate utilization of the stains (Abjala et al. 2015). Phenotypes associated with biological control activities like hydrolytic enzymes amylase (Mota et al. 2017), protease (Audrain et al. 2015), cellulase (Gómez-Lama et al. 2018), and chitinase (Abjala et al. 2015) production capacity of the strains were examined to identify the potential antagonistic rhizobacterial isolates. Likewise, the plant growth promotion traits of the strains were evaluated for siderophore (Gómez-Lama et al. 2018), Hydrogen Cyanide (HCN) (Nandini et al. 2017), Indole-3-Acetic Acid (IAA) production (Dinesh et al. 2015), and phosphate solubilization (Suthar et al. 2017). The phosphate solubilization efficiency (SE) of the isolates was calculated and determined using the following formula (Suthar et al. 2017).

$$SE = \frac{(\text{diameter of solubilization halo zone}) \times 100}{\text{Diameter of colony} + \text{halo zone}}$$

Physico-chemical stress tolerance of rhizobacterial strains

Stress tolerance of selected strains was assessed on nutrient agar medium by determining their ability to grow at different pH values (Mota et al. 2017), temperature and salt (NaCl) concentrations (Kaur et al. 2018). Similarly,

fungicides (Mancozeb 80% WP and Copper Oxy Chloride 50% WP), and bactericides (Copper Hydroxide 53.8% WP) intrinsic resistance of the strains were evaluated at 50, 100, 200, 300, and 400 ppm of commercial formulations on nutrient agar medium followed by the food poison technique (Mishra et al. 2013). The medium without the test chemicals served as control. Spot inoculated plates with each bacterial isolate (loop full, 10^6 cells mL^{-1}) were incubated at 28 ± 2 °C for five days. Three replications were maintained for each experiment and the growth of the bacterial isolates were qualitatively evaluated as (-) for no growth and as (+) for the presence of growth.

In-vitro antagonism assay of rhizobacterial strains against *Olpidium viciae*

To determine the in-vitro antagonism activity of bacterial strains, dual cultures were performed in PDA media plates as described by Islam et al. (2018). Production of volatile metabolites having antagonistic activity against fungal pathogens was tested by the paired plate technique (Cernava et al. 2015). In an antagonistic assay of bacterial culture filtrates, the culture was centrifuged at 10,000 rpm for 30 min to get the cell-free filtrate (Mota et al. 2017). The culture filtrate was tested for its efficacy against fungal pathogens at 15, 25, and 40% concentrations. The tested pathogen was inoculated at the center of the plates both in the antagonistic assay of volatile antifungal metabolites and culture filtrates. Control was maintained and incubated for 14 days at 23 ± 2 °C. Three replications were maintained for each treatment. After the period of incubation, the percentage of inhibition of the test pathogen was calculated followed the following formula (Islam et al. 2018).

$$\% \text{ inhibition} = \frac{(R1 - R2) \times 100}{R1}$$

where $R1$ —mycelial growth in control, $R2$ —mycelial growth in dual culture.

Detached leaves experiments

In-vitro detached leaf dual culture antagonistic assay of rhizobacterial strains against *O. viciae* was conducted on susceptible health faba bean (FB-26869) leaf let's according to Ali et al. (2020) in two independent experiments. The cell suspensions (10^8 CFU mL^{-1}) of each bacterial strain were prepared by standardized techniques according to Mota et al. (2017). Each surface-sterilized detached leaflet was soaked in bacterial cell suspensions and placed face-up on the filter paper impregnated with sterile distilled water in the sterilized Petri plates to serve as a moist chamber. Three leaflets were placed per plate.

Experiment 1 A 4 mm agar plugs of 14 days old pure test pathogen was placed at the center leaflets by using a sterile scalpel.

Experiment 2 Faba bean stems with gall disease symptoms were collected and 4 mm discs of the infected portion were surface sterilized and placed at the center of leaflets.

In both experiments, the leaflets only inoculated with the pathogen served as a positive control. Whereas, leaflets without bacterial cell suspension and the fungal pathogens were served as the negative control. Three replicates were maintained for each experiment. They were kept in an incubator for 6 days at room temperature (20 ± 3 °C). The length and width diameters of the leaf lesions formed around each agar plug and infected faba bean stem discs were measured. Percentages of lesion inhibition were calculated according to Ali et al. (2020).

In-vitro compatibility test of rhizobacteria

The in-vitro compatibility among different rhizobacterial strains was tested according to Ferreira et al. (2020). A 0.1 ml of one bacterial suspension (10^8 CFU mL^{-1}) was spread inoculated into Petri plates containing nutrient agar medium. Then four mm size sterilized filter paper (Whatman paper No. 1) discs impregnated with another bacterial suspension were placed on spread inoculated plate as indicated by Mota et al. (2017). Three discs impregnated with a different bacterial suspension were placed at different places at equal distances from each other on a single plate. Spread inoculated Petri plate treated with the disc impregnated with the same bacterial isolate was used as the control. Three replicates were maintained for all treatments. They were incubated at 28 ± 2 °C for 96 h. Then, the zone of inhibition was analyzed and recorded as incompatible (-) for the presence of inhibition zone, whereas compatible (+) for the absence of the inhibition zone.

In-vivo biocontrol assay in pot experiments

Two independent experiments (1 and 2) were carried out under greenhouse conditions to test the biocontrol performance of rhizobacterial strains individually and in combination. The selected rhizobacterial strain was inoculated one (experiment 1) and three (experiment 2) weeks after faba bean seedling emergence. In this experiment, both susceptible and moderately resistant faba bean varieties were used to evaluate the synergistic effect of rhizobacterial strains and moderately resistant varieties for the management of gall disease. Faba bean seeds susceptible to gall disease (FB-26869) were collected from the Ethiopian Biodiversity Institute (EBI). Whereas the moderately resistant variety (FB-Obse.) was collected from Holota Agricultural Research Institute (HARI).

Table 1 Treatment details

S/N	Treatment codes	Treatments
1	P1	Positive control: tested pathogen (AAUO4), no bioagents
2	P1 + B1	AAUO4 + <i>P. aeruginosa</i> AAUS31
3	P1 + B2	AAUO4 + <i>P. fluorescens</i> AAUPF62
4	P1 + B3	AAUO4 + <i>Bacillus</i> spp. AAUAm28
5	P1 + B4	AAUO4 + <i>Bacillus</i> spp. AAUMF42
6	P1 + B1 + B2	AAUO4 + <i>P. aeruginosa</i> AAUS31 + <i>P. fluorescens</i> AAUPF62
7	P1 + B1 + B3	AAUO4 + <i>P. aeruginosa</i> AAUS31 + <i>Bacillus</i> spp. AAUAm28
8	P1 + B1 + B4	AAUO4 + <i>P. aeruginosa</i> AAUS31 + <i>Bacillus</i> spp. AAUMF42
9	P1 + B2 + B3	AAUO4 + <i>P. fluorescens</i> AAUPF62 + <i>Bacillus</i> spp. AAUAm28
10	P1 + B2 + B4	AAUO4 + <i>P. fluorescens</i> AAUPF62 + <i>Bacillus</i> spp. AAUMF42
11	P1 + B3 + B4	AAUO4 + <i>Bacillus</i> spp. AAUAm28 + <i>Bacillus</i> spp. AAUMF42

Preparation of pathogen inoculum

Three 4 mm agar plugs of one of our *Olpidium* isolate 4 (AAUO4) was obtained from the purified culture slant and inoculated in a 250 ml flask containing 100 ml Coon's broth and incubated at 23 ± 2 °C for 14 days. The level of conidia suspensions was adjusted to (10^5 spores/ml) by using a hemocytometer. For mass production of the pathogen, 10 ml of the fungal suspension were added to 100 g sterilized powdered (0.5 mm) faba bean stems and incubated at 23 ± 2 °C for 21 days.

Preparation of rhizobacterial inoculum

Four in-vitro effective antagonistic rhizobacteria strains (AAUS31, AAUPF62, AAUAm28, and AAUMF42) against the test pathogen (*O. viciae*) which exhibited plant growth promotion traits were selected to study their in-vivo effects both in mono and dual inoculation treatments on gall disease suppression and growth parameters under greenhouse conditions (15–28 °C) in connection with faba bean varieties by pot culture method. For single isolate inoculum production, a loopful of each isolate was separately inoculated in 250 ml flasks containing nutrient broth. After inoculation with bacteria, the flasks were incubated on a rotator shaker at 120 rpm and 28 °C for 72 h. Before use, bacterial concentration was adjusted to 10^8 cells ml⁻¹ (OD₆₂₀ 0.8–0.9) using spectrophotometer. Bacterial concentrations were confirmed by dilution plating. The combination of bacterial inoculums was prepared by mixing with an equal volume (1:1 v/v ratio).

Experimental design and treatments details

The pots were arranged in a complete randomized block design (CRBD) with 3 replications and watered daily. The pots were surface-sterilized for 3 min with 5% sodium hypochlorite and washed three times with sterile water and left to dry before use. A mixture of (1:1 m/m ratio)

clay soil and sand were autoclaved at 121 °C for 1 h. and 2 kg of them were filled per 20 cm diameter plastic pots. For treatments of the tested pathogen, four selected individual rhizobacteria strains, and six combined rhizobacteria strains were used with the control treatments. The control group consists of test pathogen without bioagents (Table 1).

Inoculum inoculation

At the initial stage, surface-sterilized healthy-looking faba bean seeds variety were impregnated with the test pathogen and incubated at 23 ± 2 °C for 48 h. before sowing. Seeds impregnated with sterile distilled water were used as control. Four seeds were sown per potted soil. Sowing was made during the first week of May 2019. Secondly, 20 g of test pathogen colonized faba bean stem powder was used to infest 2 kg of soil (Ommati and Zaker 2012) five days after the emergence of seedlings at 75% relative humidity to increase the inoculum source. A 5 ml of bacterial strain (10^8 CFU/ ml) was inoculated per seedling to the base of the stem at 2 cm depth of soil.

Disease assessment and growth parameters of faba bean

Disease incidence and severity were evaluated after 25 days of crop emergence. Percent disease incidence was evaluated by counting the number of diseased plants from the total number of inspected plants. The disease percent severity index (PSI) was recorded using a 0–9 scale to determine the area of the infected plant part according to Sheik et al. (2015). Six resistance level were used in this experiment to calculate PSI

$$PSI = \frac{a + 3b + 5c + 7d + 9e}{9(a + b + c + d + e)} \times 100$$

where 9 = highest rating value, *a* = number of plants in class 1, *b* = number of plants in class 3, *c* = number of

Table 2 Phenotypic traits related with biocontrol and plant growth promotion in some strains

Isolates code	Activities								
	Amylase	Protease	Cellulase	Chitinase	Siderophore	IAA	HCN	Phosphate solubilization	SE (%)
AAUPF62	+	+	+	+	+	+	+++	+	75.00 ^a
AAUS31	–	+	–	+	+	+	+++	+	74.07 ^a
AAUMF42	+	+	–	–	–	+	+++	+	73.60 ^a
AAUAm28	–	–	+	–	–	+	+++	+	69.39 ^b
AAUW1	–	+	+	–	–	–	++	+	68.42 ^b
AAUF2	+	+	–	–	–	+	–	+	63.64 ^c
AAUC2	–	+	–	–	–	+	+	+	63.16 ^c
AAUAm23	–	+	+	+	+	–	+++	+	63.16 ^c
AAUAm27	–	+	–	+	–	–	+++	+	61.76 ^c
AAUAm26	+	+	–	–	–	+	+++	+	60.00 ^c
AAUMF41	–	–	–	+	+	+	+	+	58.06 ^c
AAUF3	–	+	–	–	+	–	+++	+	57.14 ^c
AAUPS61	+	–	+	–	+	–	–	+	52.63 ^d
AAUMF51	–	+	+	–	–	–	++	+	52.50 ^d
AAUW24	–	+	–	–	+	–	++	–	0.00 ^e
AAUW2	–	+	–	+	+	+	++	–	0.00 ^e

+ presence, – absence, +++ strong, ++ moderate, SE solubilization efficiency, the same letter in the same column indicated the absence of significance difference at $P > 0.05$

plants in class 5, d = number of plants in class 7, e = number of plants in class 9.

0 = Healthy plant, 1 = only 1–15% of leaf surface infected, 3 = 15.1–40% leaf surface infected with some defoliation, 5 = 40.1–60% leaf surface infected with defoliation, 7 = 60.1–80% leaf surface infected with defoliation, 9 = more than 80% of leaf surface infected with high defoliation.

The seedlings were harvested 70 days after the last inoculation without damaging the root system. A total of four seedlings per treatment were harvested and used to measure the biomass. For each seedling, the biomass index included calculations of the shoot length, shoot fresh weight, pods per plant, shoot dry weight, root length, root fresh weight, and root dry weight at harvest. Once the fresh weight had been measured, the seedlings were oven-dried at 85 °C for 24 h. to measure the dry weight (Ommati and Zaker 2012).

Statistical analysis

Collected data were subjected to SPSS statistical software version 20 and one-way ANOVA. The effect of rhizobacterial strains on fungal pathogens was compared using the least significant difference (LSD) at a 5% probability level ($P \leq 0.05$).

Results

Among 60 potential antagonistic rhizobacteria obtained from rhizosphere of different faba bean were found belong to *Pseudomonas* (55%) and *Bacillus* (25%) genera. Twenty percent of strains couldn't be identified. All the isolates were studied for hydrolytic enzyme production and plant growth promotion traits. However, due to the vastness of the data, we provide here only multi-trait rhizobacteria. The most potential biological control rhizobacteria obtained were related to *P. fluorescens* (AAUPF62), *P. aeruginosa* (AAUS31), and the genera *Bacillus* (AAUMF42 and AAUAm28).

Morphological and phenotypic characterization

The potential antagonistic rhizobacterial isolates exhibited a wide variation in morphological and phenotypic characters. The majority (40%) of the bacterial isolates were identified as rod-shaped gram-negative and 30% were identified as rod-shaped gram-positive bacteria. The colony form, elevation, and margin of the majority of bacterial isolates were identified as irregular (28%), flat (30%), and entire (38%), respectively.

In the KOH test, most (55%) isolates revealed sticky lipopolysaccharides. About 63% of the isolates had utilized citrate and changed Simon's Citrate agar slant from

green to blue color. All strains were positive for catalase activities. In the Oxidase test, 48% of the isolates produced an intense deep-purple blue within 5–10 s and exhibited the presence of a cytochrome oxidase.

Assay for hydrolytic enzymes and plant growth promotion traits

About 25% of strains were positive for starch hydrolysis, protease (81%), cellulase (14%), and chitinase (28%) activities. The majority (63%) of strains exhibited green to yellow florescent and showed positive for siderophore production. Likewise, 56% were produced IAA. Most (60%) of the isolates were HCN producers and changed the color of filter paper from yellow to brown with different intensities (Table 2).

Sixty percent of the total isolates solubilize tricalcium phosphate although the solubilization efficiency was varied across the strains. The PF62, S31, and MF42 stains were able to solubilize tricalcium phosphate more than the remaining isolates with solubilization efficiency 75.00, 74.07, and 73.60%, respectively (Table 2).

Physico-chemical stress tolerance of the bacterial isolates

About 45% of the tested bacterial isolates were unable to grow within 24 h. at low pH (pH = 5). The strain AAUS31 and AAUPF62 grow fast (within 24 h.) over a wide pH range (5–6.5) whereas grow slowly (after 72 h.) well at pH 7 and 7.5. All the tested rhizobacterial strains were able to grow within 24 h. at 25 and 30 °C whereas grown after 72 h. at 20 and 35 °C. However, 27% of stains (AAUPF62 and AAUS31) grown fast at 20 °C whereas 73% of stains

(AAUAm23, AAUMF42, AAUAm26, AAuAm28, AAUF3, AAU W2, AAUC2, and AAuW24) grown slowly at 20 °C. The bacterial strains exhibited varied tolerance to NaCl. All strains have grown fast at 0.1 M. About 45% of the strains (AAUS31, AAUPF62, AAUMF42, AAUAm26, and AAUW2) grown fast at 0.2 M. About 90% and 45% of the strains grown slowly at 0.5 M, and 0.75 M, respectively. However, there were no bacterial strains grown at 1 M.

All the tested rhizobacterial strains were resistant to Mancozeb 80% WP and grown fast within 24 h. at 50 and 100 ppm concentrations. About 36% of the strains (AAUMF42, AAUPF62, AAUS31, and AAUAm28) were grown fast at 200 ppm whereas 64% of them grown after 72 h. of incubation at 28 ± 2 °C. No isolates were found to grow within 24 h. incubation at 300 ppm. There were no isolates found to resistant 400 ppm concentration of Mancozeb 80% WP. Similar patterns were followed by bacterial strains against Copper Oxy Chloride 50% WP. On the other hand, 100% of rhizobacterial strains were resistant to the bactericide (Copper Hydroxide 53.8% WP) at 50 and 100 ppm concentrations. About 18% of strains (AAUS31 and AAUPF62) were grown fast at 200 ppm while no bacterial strains were found resistant to 400 ppm concentrations of Copper Hydroxide 53.8% WP.

In-vitro antagonistic assay of rhizobacterial stains on culture media

The dual culture of bacterial strains against *O. viciae* revealed that a significant reduction in the radial growth over the control. The maximum radial growth inhibition of the pathogen was recorded in the presence of the

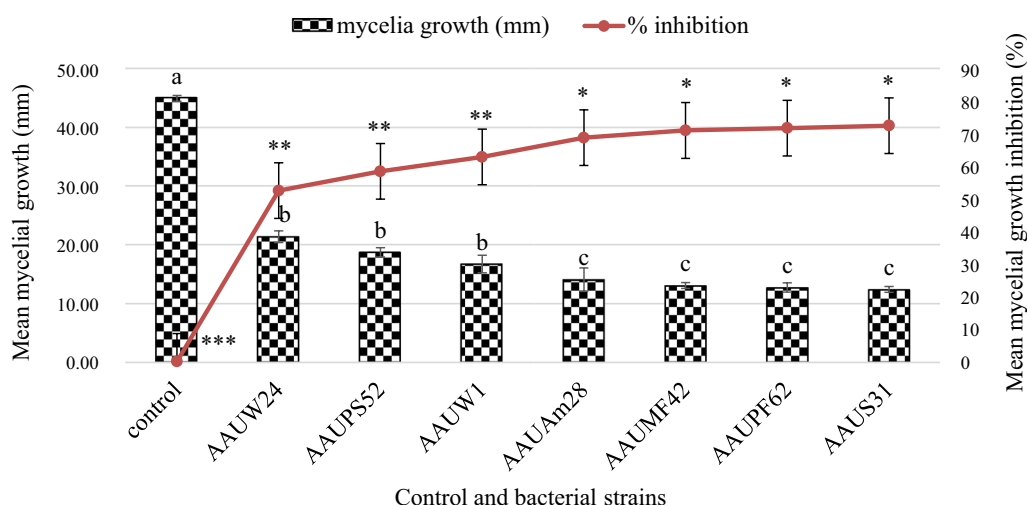
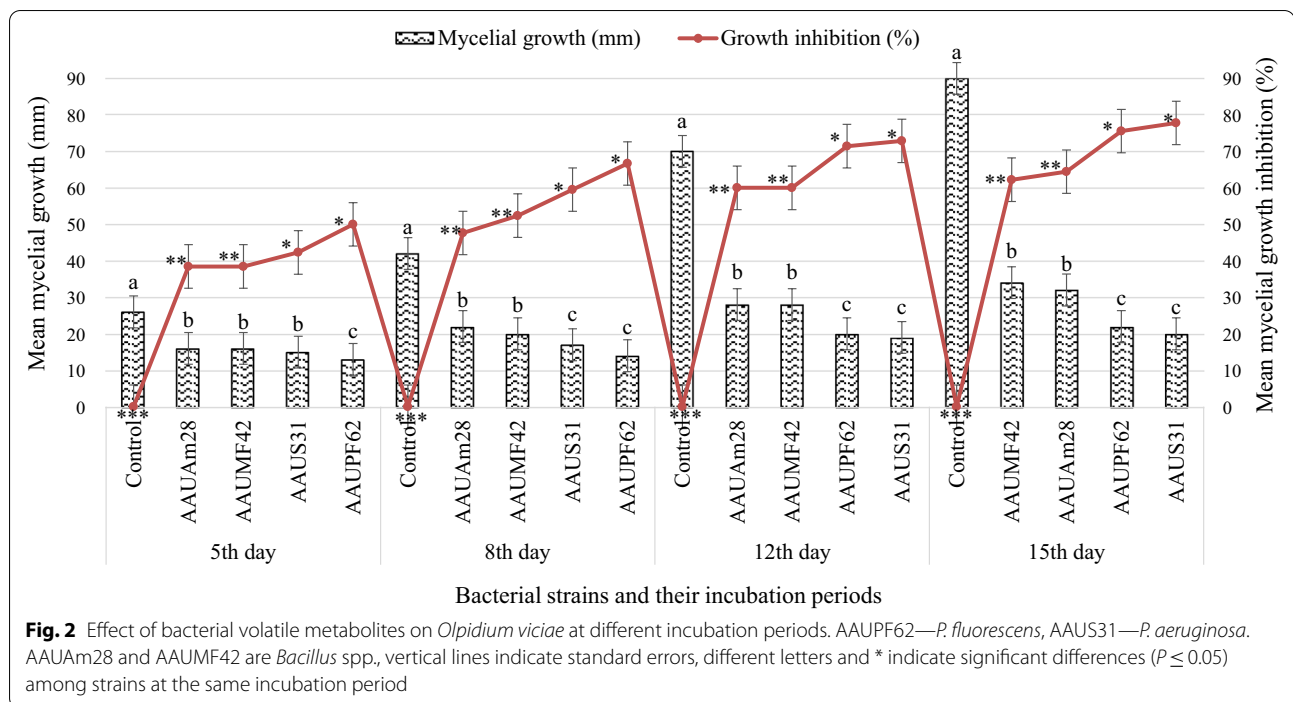
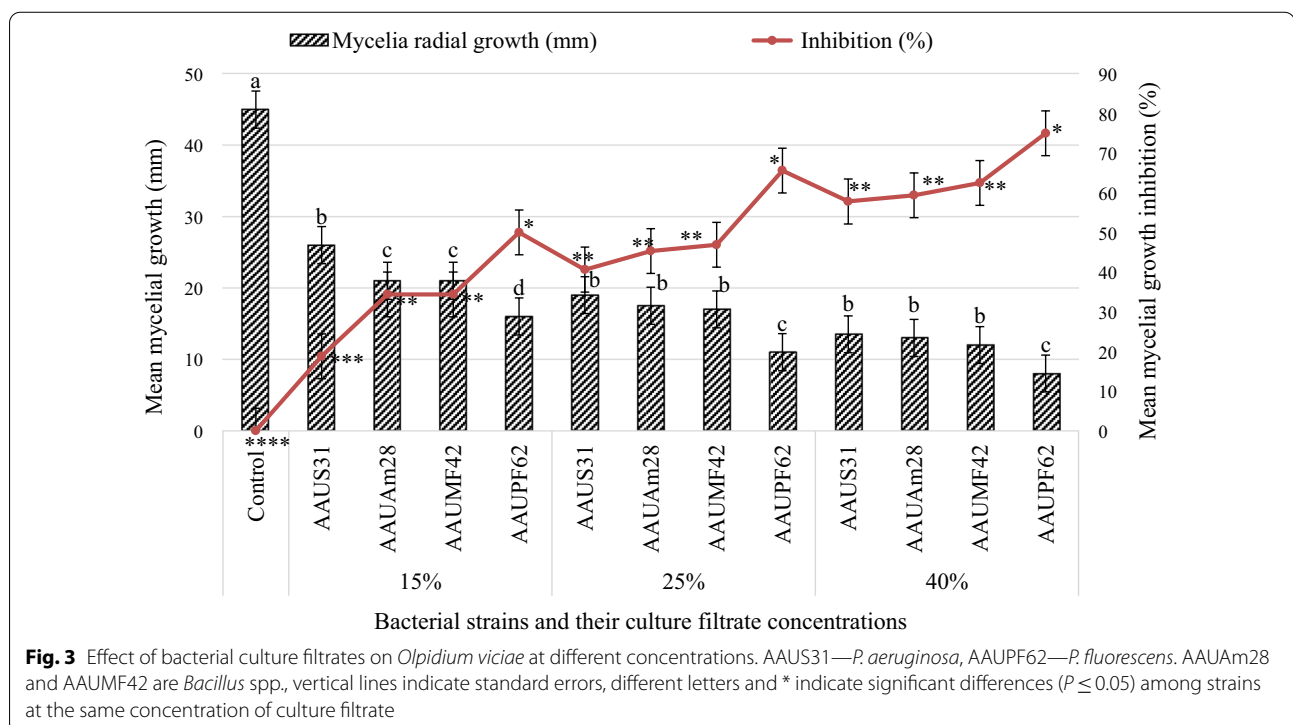


Fig. 1 Antagonistic effect of a bacterial strain's against *Olpidium viciae* in dual culture on PDA medium. AAUW1—*Pseudomonas* spp., AAUPF62—*P. fluorescens*, AAUS31—*P. aeruginosa*, AAUW24, AAUPS52, AAUAm28 and AAUMF42 are *Bacillus* spp., vertical lines indicate standard errors, different letters and * indicate significant differences ($P \leq 0.05$) among strains



strain AAUS31 (72.59%), followed by AAUPF62 (71.85%) and AAUAm28 (68.89%). However, the fungal mycelial growth inhibition by strains AAUW24 (52.59%) and AAUPS52 (58.52%) were lower than others. This showed

that the presence of significant variations ($P \leq 0.05$) among bacterial strains in reducing the growth of the test pathogen (Fig. 1).



The volatile metabolites produced by the rhizobacterial strains were showed an antagonistic effect against the tested fungal pathogen. As the incubation period increased, the volatile metabolites of bacterial strains significantly reduced the rate of mycelial growth of test pathogens. Percent inhibition of mycelial growth by bacterial strains linearly increased with the incubation period. In the first 8 days of incubation, maximum growth inhibition was recorded by the strain AAUPF62, followed by AAUS31, while after eight days of incubation period reverse results were recorded by the two strains (Fig. 2).

The cell-free culture filtrates (diffusible antifungal metabolites) of the evaluated rhizobacterial strains significantly reduced the mycelial growth of the test pathogen than the control groups. This effect was linearly increased with the concentration of culture filtrates. The diffusible antifungal metabolites of strain AAUPF62 showed a higher antagonistic effect against the test pathogen than other strains throughout the evaluated concentrations, followed by AAUMF42. The 15% of AAUPF62 culture filtrates exhibited a better antagonistic effect than 25% of other strains. Similarly, 25% of AAUPF62 culture filtrates inhibited the mycelial growth of the test pathogen more than the 40% culture filtrates of other strains (Fig. 3).

In-vitro antagonistic assay of rhizobacterial strains on detached leaves

The leaflets inoculated with the mycelial disc (Experiment 1) of fungal pathogen and infected faba bean stem

disc (Experiment 2) produced progressive lesion from the inoculation point through leaflet. They produced similar leaf lesions symptoms. Significant variation ($P \leq 0.05$) was recorded among bacterial isolates in leaf lesions inhibition. Lower degree of leaf lesions recorded on leaves impregnated with bacterial cell suspensions than the control leaflets. The level of length diameter (LD) and width diameter (WD) of leaf lesions was varied based on the bacterial strains used. In experiment 1, The maximum length and width diameter of leaf lesions inhibition was recorded in leaves treated with AAUPF62 (LD = 58%, WD = 46%), followed by AAUS31 (LD = 57%, WD = 46%), AAUAm28 (LD = 56%, WD = 39%), and AAUMF42 (LD = 54%, WD = 47%). Though, AAUPF62, AAUAm28, AAUMF42, and AAUS31 exhibited similar patterns in experiment 2, the level of LD and WD leaf lesions inhibition were different with the values AAUPF62 (LD = 78%, WD = 73%), AAUS31 (LD = 75%, WD = 73%), AAUAm28 (LD = 66%, WD = 60%), and AAUMF42 (LD = 58%, WD = 65%) (Fig. 4).

In-vitro compatibility assay among bacterial strains

The in-vitro antagonistic assay among potential bacterial strains indicated that there was no zone of inhibition recorded between AAUS31, AAUAm28, AAUMF42, and AAUPF62 strains. Whereas between AAUPF62 and AAU W1 about 18.3 mm zone of inhibition was recorded (Table 3). Hence, those exhibited no zone of inhibition against each other was compatible

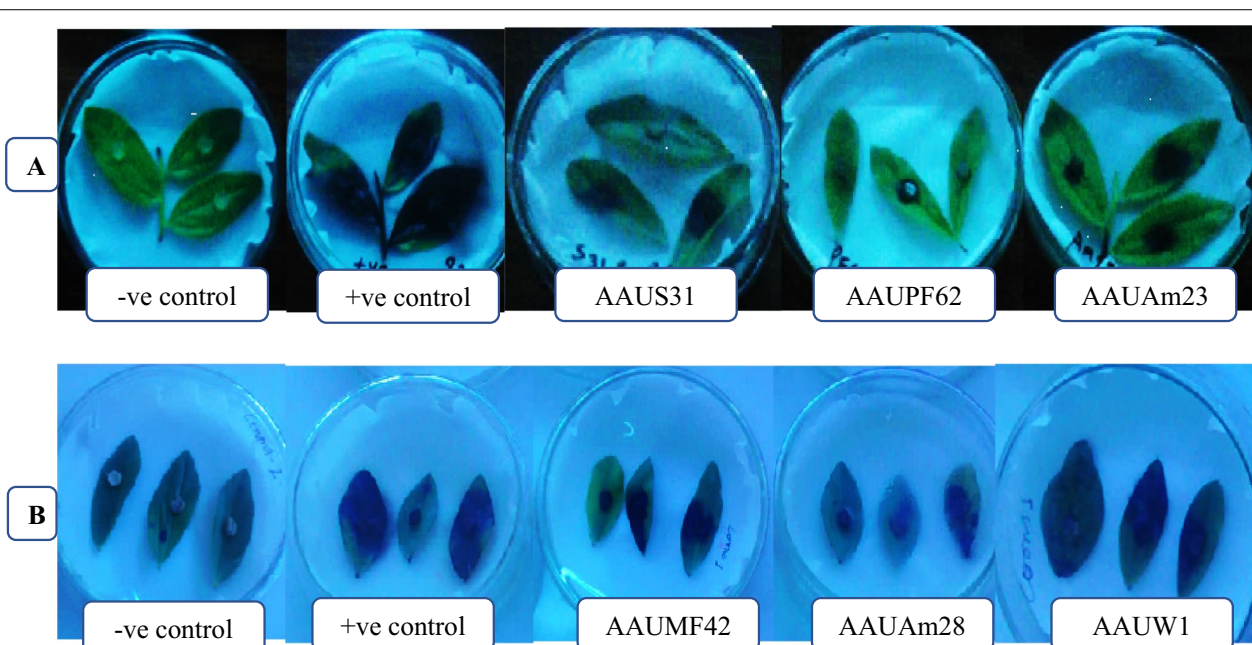


Fig. 4 Dual culture of some bacterial strains against gall-forming disease on detached leaves. **a** experiment 1, **b** experiment 2, AAUW1—*Pseudomonas* spp., AAUS31—*P. aeruginosa*, AAUPF62—*P. fluorescens*. AAUAm23, AAUAm28, AAUMF42 are *Bacillus* spp

Table 3 In-vitro compatibility assay among bacterial strains

Bacterial isolates code	Bacterial isolates with a zone of inhibition in mm							
	B1	B2	B3	B4	B5	B6	B7	B8
AAUS31 (B1)	+							
AAUAm28 (B2)	+	+						
AAUW1 (B3)	+	+	+					
AAUMF42 (B4)	+	+	+	+				
AAUPF62 (B5)	+	+	– (18.3)	+	+			
AAUF3 (B6)	+	– (11.0)	– (6.7)	– (14.3)	+	+		
AAUW24 (B7)	+	+	– (8.3)	– (7.3)	+	+	+	
AAUF2 (B8)	+	+	+	+	+	+	+	+

+ compatible, – incompatible, values in the bracket are zone of inhibition recorded in mm between the bacterial isolates

and selected for dual inoculation assay under greenhouse condition.

In-vivo efficacy evaluation of rhizobacteria against gall disease and growth parameters

In the present investigation, four rhizobacterial strains, from the rhizosphere soil of faba bean plants were

assessed for their efficacy in faba bean gall disease reduction and growth parameters enhancement under greenhouse conditions. Non-inoculated faba bean seedlings with rhizobacterial strains showed gall disease symptoms four weeks after crop emergence in the two independent experiments performed (1 and 2). The first characteristic disease symptoms (galls) were observed

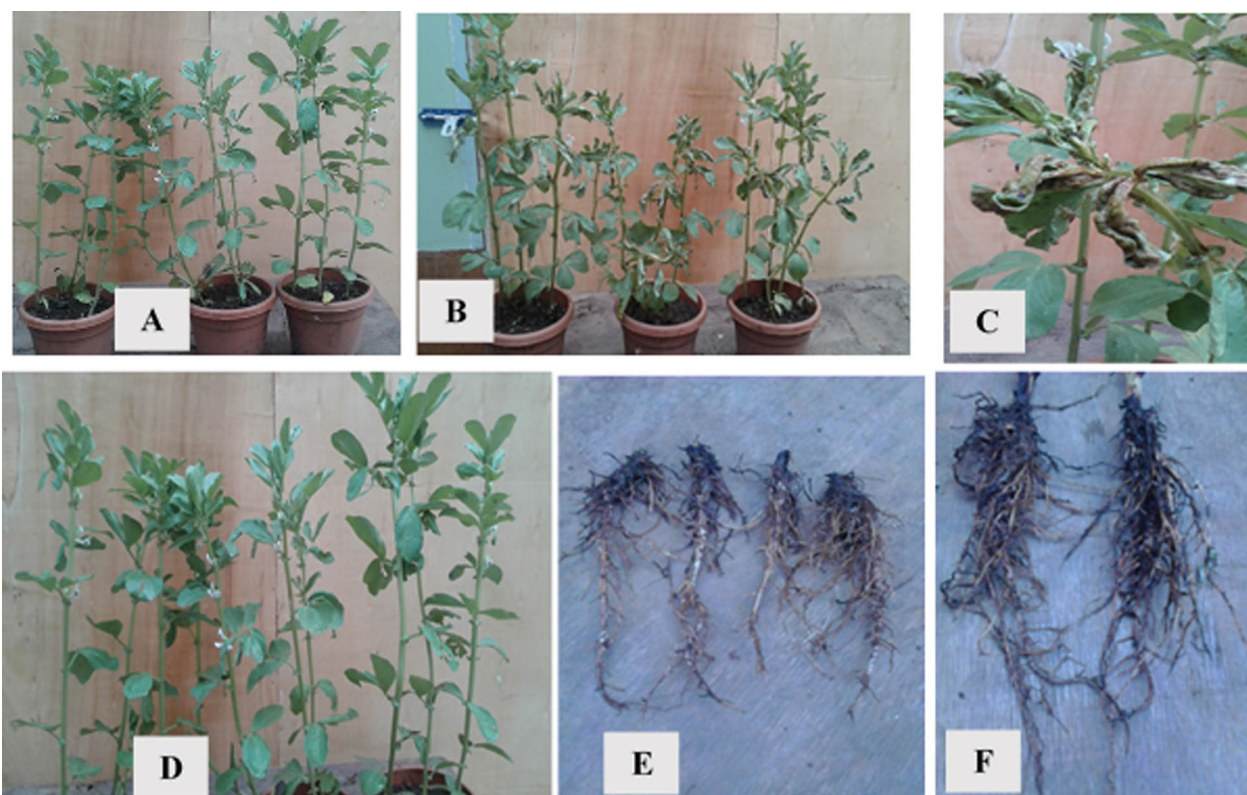


Fig. 5 Rhizobacterial efficacy against gall disease severity, and shoot and root growth promotion in faba bean. **a** Pathogen non-inoculated control; **b** and **e**, pathogen inoculated control; **c** severely infected leaves; **d** and **f** were treated with co-inoculation of *P. aeruginosa* AAUS31 and *P. fluorescens* AAUF62 strains

Table 4 *P* values of contrast analysis between treatments

Contrast	DF	Disease data			DF	Shoot parameter			Root parameter		
		AUDPC	Final DI	Final S		Length	Fresh W	Pods/P	Dry W	Length	Fresh W
[P1] versus [B1; B2]	43	< 0.05	< 0.05	< 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
P1 versus B3	43	0.046*	0.059	0.101	0.348	0.381	0.528	0.604	0.654	0.599	0.712
P1 versus B4	43	0.017*	0.029*	0.090	0.298	0.354	0.681	0.586	0.766	0.904	0.806
P1 versus B1B2	43	< 0.001*	< 0.001*	< 0.001*	0.003*	0.019*	0.007*	0.009*	0.018*	0.045*	0.003*
P1 versus B1B3	43	< 0.001*	0.002*	0.003*	0.006*	0.102	0.042*	0.064	0.092	0.100	0.026*
[P1] versus [B1B4; B2B3]	43	< 0.05	< 0.05	< 0.05	< 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
[P1] versus [B2B4; B3B4]	43	< 0.05	< 0.05	< 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
B1 versus B1B2	43	0.016*	0.001*	0.094	0.022*	0.097	0.028*	0.027*	0.092	0.185	0.009*
B2 versus B1B2	43	0.053	0.032*	0.071	0.024*	0.112	0.021*	0.020*	0.048*	0.125	0.007*
B3 versus B1B2	43	0.014*	0.000*	0.017*	0.016*	0.401	0.023*	0.022*	0.194	0.235	0.051
B4 versus B1B2	43	0.038*	0.000*	0.019*	0.019*	0.103	0.015*	0.162	0.032*	0.122	0.004*
B1B2 versus B2B4	43	0.107	0.015*	0.314	0.060	0.161	0.034*	0.024*	0.033*	0.097	0.006*
B1B2 versus B3B4	43	0.069	0.003*	0.230	0.050*	0.133	0.026*	0.020*	0.030*	0.092	0.005*
FB-Obse versus FB-26869	43	0.022*	0.014*	< 0.001*	0.010*	< 0.001*	0.017*	0.006*	0.005*	0.001*	0.028*
Early versus Late application	43	< 0.001*	0.002*	0.010*	ND	ND	ND	ND	ND	ND	ND

DF—degrees of freedom, ND—no data, AUDPC—area under disease progress curve, DI—disease incidence, S—severity, W—weight, P—plant, P1—control (inoculated with *Olpidium viciae* isolate 4), B1—*P. aeruginosa* AAUS31, B2—*P. fluorescens* AAUPF62, B3—*Bacillus* spp. AAUAm28, B4—*Bacillus* spp. AAUMF42, FB—faba bean variety

*A significant difference at 5% probability level

in six (experiment 1) and two (experiment 2) weeks after rhizobacterial inoculation (Fig. 5c). The combined inoculation of *P. aeruginosa* AAUS31 (B1) and *P. fluorescens* AAUPF62 (B2) delayed the incidence of disease symptoms by one week in both experiments than others. In both experiments, significant ($P < 0.001$) lower disease pressure was recorded in FB-Obse than FB-2689 variety (Table 4). The early application of bioagents more significantly reduced AUDPC ($P < 0.001$), final disease incidence ($P = 0.002$), and final disease severity ($P = 0.010$) than late applications.

A single inoculation of rhizobacterial strains had non-significant effect on gall disease severity as compared to the control. Co-inoculation with B1 and B2, and B1 and *Bacillus* spp. AAUAm28 (B3) had shown a significant reduction in final disease severity as compared with the control treatment (Fig. 5d; Table 4). The combined inoculation of B1 and B2 into FB-Obse had exhibited the maximum final disease incidence (84.08%) and severity (92.36%) reduction in experiment 1. Whereas in experiment 2 the disease incidence and severity were reduced by 70.02 and 77.09%, respectively.

Rhizobacterial treated FB-Obse variety had shown significantly higher shoot length, shoot fresh weight, pods/plant, shoot dry weight, root length, root fresh weight, and root dry weight over FB-26869 faba bean variety (Table 4). There was no difference in shoot and root growth parameters between the single inoculations (B1, B2, B3, and *Bacillus* spp. AAUMF42 (B4)) and

the control treatment, but combined inoculation of B1 and B2 resulted in significantly ($P < 0.05$) higher shoot and root growth parameters (Table 4). The co-inoculation of B1 and B2 was significantly increased shoot length ($P = 0.006$; mean = 108.8 cm) (Fig. 5d), pods/plant ($P = 0.042$; mean = 6.2 pods) and root dry weight ($P = 0.026$; mean = 10.1 g) (Fig. 5f) over the control treatment. Whereas the dual inoculation of B1B4, B2B3, B2B4, and B3B4 had shown no significant effect on shoot and root growth parameters (Table 4).

The single inoculation with B1 and B2 had showed significant effect both on shoot and root growth parameters of the two faba bean varieties (Table 4). The co-inoculation of B1B2 had significantly increased shoot length ($P = 0.022$; mean = 122 cm) pods/plant ($P = 0.028$; mean = 6.5 pods), shoot dry weight ($P = 0.027$; mean = 83 g), and root dry weight ($P = 0.009$; mean = 14.5 g) over single treatments by B1. Similarly, B1B1 had enhanced shoot length ($P = 0.024$; mean = 122 cm), pods/plant ($P = 0.021$; mean = 6.5 pods), shoot dry weight ($P = 0.020$; mean = 83 g), and root dry weight ($P = 0.007$; mean = 14.5) over B2 treatment (Table 4).

Discussion

In this study, indigenous rhizobacterial strains which exhibited antifungal and plant growth promotion activities were isolated from faba bean rhizosphere. The study covers the results of effective potential antagonistic

strains under both laboratory and greenhouse conditions. Isolates for varieties of biological control and plant growth promotion traits were tested. This offers awareness into the useful variances among isolates and is essential for the careful selection of valuable indigenous strains. The rhizobacterial strains AAUPF62, AAUS31, AAUMF42, and AAUAm28 exhibited more than one biological control and plant growth-promoting trait. This is advantageous to apply them both for disease control and plant growth promotion under adverse conditions. The selected strains were positive for the production of hydrolytic enzyme and able to produce siderophore, IAA, and HCN, and solubilize inorganic phosphate. They were also tolerant to environmental and chemical stresses. Hence, the above-mentioned strains can play the role of antifungal activities and plant growth promotion both directly or indirectly. The dual traits of the strains made them preferable to be selected for further evaluation as a potential biological control agent in this study.

The phenotypic traits and hydrolytic enzymes associated with biological control agents were evaluated as well as their intrinsic tolerance to environmental stress and chemical sensitivities. Faba bean rhizobacteria exhibited tolerance variation in different pH values, temperature, and NaCl concentrations. The pattern of these variations among the bacterial isolates could be associated with their genetic makeup. However, Kaur et al. (2018) reported that rhizosphere bacterial strains were relatively sensitive to lower pH (4). Previous studies also showed the tolerance of some bacterial isolates to 40–45 °C irrespective of their growth rate (Singh et al. 2013). Also, Mota et al. (2017) reported the NaCl tolerance variation of various bacterial isolates. We found resistant rhizobacterial isolates to 50–200 ppm concentrations of the tested agrochemicals implying their better survival chance upon exposure to the chemicals and would make it feasible to incorporate these strains in integrated disease management. This is in agreement with Mishra et al. (2013) who reported the compatibility of plant growth-promoting rhizobacteria with fungicides.

Rhizosphere-associated bacteria were positive for protease, siderophore, HCN production, starch hydrolysis, and phosphate solubilization (Singh et al. 2013). Similarly, Gómez-Lama et al. (2018) reported that siderophore and HCN producing bacterial isolates from the plant rhizosphere showed remarkable inhibitory effects against the plant fungal pathogens.

Siderophore-producing rhizobacterial strains could stimulate plant growth and inhibit the growth of plant pathogens (Abjala et al. 2015). Siderophore prevents plant pathogens from acquiring enough amount of iron by chelating the ferric ions with high specific activity (Dinesh et al. 2015). Siderophores directly stimulate the

biosynthesis of antimicrobial compounds by increasing the availability minerals to the rhizobacteria, which, in turn, suppresses the growth of pathogenic organisms (Abo-Elyousr et al. 2019). The production and release of HCN by rhizobacterial strains were studied as a biocontrol mechanism (Nandini et al. 2017). HCN is the common secondary metabolite produced by gram-negative *Pseudomonas* (Abo-Elyousr et al. 2019), which acts as an inducer of plant resistance and is postulated to play a role in the biological control of several plant pathogens (Gómez-Lama et al. 2018). In this study, the strains AAUPF62 and AAUS31 were able to produce protease, chitinase, siderophore, and HCN. These could capable to antagonize and reduced the mycelia growth of the test fungal pathogen.

The strains AAUPF62, AAUS31, AAUMF42, and AAUAm28 exhibited positive IAA producing abilities and phosphate solubilization at various intensities. IAA promotes plant growth and mainly affects the root system by increasing its size and weight (Dinesh et al. 2015). The phosphate solubilizing bacteria would be resulting in increased availability of phosphate ions in the soil and improves plant nutrition (Mota et al. 2017). This could capable to antagonize the test fungal pathogen indirectly and promote healthy plant growth.

The present work found volatile antifungal metabolites produced by strains AAUPF62, AAUS31, and AAUMF42 significantly inhibited the mycelia growth of test fungal pathogen with greater than 67% inhibition. This antagonistic efficacy could be related to their capacity for HCN production, which is toxic to the test pathogen (Islam et al. 2018). This is in line with the findings of Gómez-Lama et al. (2018) who studied the production and release of HCN by beneficial rhizosphere bacteria as a biocontrol mechanism. Volatile compounds produced by soil microbes promoted plant growth, showed high antimicrobial activities, and induced systemic resistance in several crops (Abo-Elyousr et al. 2019). In this regard, rhizobacteria are well known for producing large arrays of antifungal and plant growth-promoting metabolites (Raza et al. 2016).

On another hand, the cell-free culture filtrates (diffusible metabolites) of AAUPF62, AAUS31, AAUMF42, and AAUAm28 strains significantly inhibited the mycelial growth of the test pathogen. This inhibitory effect might be associated with the production of hydrolytic enzymes, siderophore, and other diffusible secondary metabolites (Islam et al. 2018). Production of diffusible and volatile antifungal molecules along with hydrolytic enzymes and compounds like siderophore and HCN seems to be the primary source of growth inhibition of the test pathogen. This is in agreement with the findings of Audrain et al. (2015) and Cernava et al. (2015) who reported the

antagonistic effect of diffusible and volatile antifungal secondary metabolites of soil microbes against fungal plant pathogens.

In experiments (1 and 2) of in-vitro detached leaf experiments the average leaf length and width diameter lesion were reduced to more than 50% by AAUPF62, AAUS31, AAUMF42, and AAUAm28 strains as compared to the positive control. The in-vitro detached leaf experiment was effective to evaluate biocontrol activity of indigenous rhizobacterial strains against fungal pathogen and permitted to find out and select effective strains. The result reported by Ali et al. (2020) the antifungal effect of Rhizospheric bacterial species against phytopathogens on detached leaves and found the result complementing the current work.

The strains AAUPF62, AAUS31, AAUMF42, and AAUAm28 were compatible with each other under in-vitro conditions. This could be attributed to the existence of synergism between the metabolites produced by the strains. Combining compatible biocontrol agents are a strategic approach to control plant disease. The dual inoculation of compatible biocontrol agents reduced plant disease severity more than mono inoculation of the potential antagonistic rhizobacterial strains (Mota et al. 2017).

In in-vivo biocontrol assay, single inoculation of rhizobacterial strains delayed the gall disease incidence by 3 weeks in experiment 1 and by one week in experiment 2 than in the control treatments. Early incubation of antagonistic rhizobacteria strains (AAUPF62, AAUS31, AAUMF42, and AAUAm28) reduced the disease incidence and severity rates. This could be associated with the induction of systematic resistance in the plants by various secondary metabolites of biocontrol agents (Mohamed et al. 2020). The highest disease severity reduction by AAUPF62 and AAUS31 strains could be related to the siderophore and HCN production capacity of the strains. On the other hand, the dual inoculation of rhizobacterial strains delayed faba bean gall disease incidence by one week than a single treatment. This exhibited that the existence of synergism among rhizobacterial strains in disease control. In both experiments' lower disease severity was recorded in FB-Obse than in FB-26869 variety. This could be associated with the genetic makeup of the faba bean varieties in addition to the effect of rhizobacterial strains.

Regarding the plant growth-promotion, AAUS31 and AAUPF62 strains increased faba bean shoot and root growth parameters. This implied that the two strains could colonize the root system and play a role in promoting plant growth through IAA production and phosphate solubilization to the useable forms (Abo-Elyousr et al. 2019). There was a large difference between dry weight

and fresh weight of faba bean seedlings. The increase in fresh weight could be related to water absorption, while the increase in dry weight could be related to organic matter accumulation. As indicated in the results section, both FB-Obse and FB-26869 faba bean seedlings treated with AAUS31, and AAUPF62 had a long root, which enabled them to obtain sufficient water and nutrients from the soil, the production and regulation of phytohormones lead to increase of biomass (Elkelany et al. 2020). However, the accumulation of organic matter is a slow process and is less in the early stage of plant growth (Aydi-Ben-Abdallah et al. 2020). The dual inoculation of AAUS31 and AAUPF62 significantly increased shoot and root parameters. This could be due to the existence of synergistic effects among bacterial strains.

Conclusions

Based on in-vitro and greenhouse experiments, 2 rhizobacteria, *P. fluorescens* AAUPF62 and *P. aeruginosa* AAUS31 markedly enhanced faba bean gall disease suppression and increased growth parameter of faba bean than the control group. The 2 strains showed stress tolerance and exhibited multiple biological control and plant growth promotion traits. Hence, they were identified as a potential candidate to be used as a microbial inoculant for the control of faba bean gall disease. The early treatment of the crop by microbial inoculum was advisable. To suppress the effect of gall disease severity by greater than 92%, use of moderately resistant faba bean variety and early co-inoculation of *P. fluorescens* AAUPF62 and *P. aeruginosa* AAUS31 strains is recommended.

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Authors' contributions

AD, TA and YW: planning of experiments, AD: manuscript preparation and data interpretation, TA and YW: correction of the manuscript with scientific advice. All authors read and approved the final manuscript.

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Availability of data and materials

The data set used/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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