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Biochemical characterization and ecofriendly field application of the plant growth-promoting rhizobacterium *Bacillus amyloliquefaciens* MH046937 isolated from *Salsola imbricata* in Tur Sinai, Egypt

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ABSTRACT

The root-colonizing biocontrol bacterium *Bacillus amyloliquefaciens* was used to fight some plant root pathogens in agriculture. *Bacillus amyloliquefaciens* MH046937 isolated from *Salsola imbricata* rhizospheres was evaluated for promoting the growth of an important food legume broad bean (*Vicia faba* L.) in Egypt. *In vitro* antagonistic effect tests showed that *B. amyloliquefaciens* was most effective against *Sclerotium rolfsii* followed by *Alternaria alternata.* The tested strain produced a substantial quantity of indole acetic acid equivalent to 32.9 ± 0.01 µg/ml and ammonia, also synthesized hydrogen cyanide at a moderate quantity, and solubilized phosphate. The bacterial fermentation proved the capability of *B. amyloliquefaciens* to produce the antifungal metabolite chitinase enzyme after 48 hours which contributes to its antagonistic activities. The major compound **1** was purified and identified as 5-hydroxymethylfurfural on the basis of the Nuclear Magnetic Resonance (NMR) measurements and the Electron Ionizatio (EI) mass spectroscopy. Greenhouse application indicated that *B. amyloliquefaciens* (T_2) introduced a significant increase in germination percentage from 40% to 60% and reduced the disease incidence from 1.6 to 0.4 when compared with the control pots. The obtained results showed that *B. amyloliquefaciens* has both direct and indirect antagonism mechanisms against soil-borne fungi, depending on its multiple physiological traits and production of chitinase enzyme.

INTRODUCTION

Broad bean (*Vicia faba* L.) is an essential vegetable plant and is considered as a skim milk and meat substitute in diets due to its high protein and nutritional quality (Devi *et al*., 2011). Crop production is mainly affected by soil-borne pathogenic agents all over the world (Compant *et al*., 2005; Wu *et al*., 2015). Plant diseases as well as crops cause the loss of approximately 20% and even more (Kröber *et al*., 2014; Oerke, 2006) by plant pathogens worldwide depending on the type of the crop, the severity of the pathogen, the strategies of management, and environmental conditions.

Macrophomina phaseolina and *Rhizoctonia solani* are severe soil-borne plant pathogens associated with root rot and damping-off diseases of broad bean (Devi *et al*., 2011). The most essential tool to control plant phytopathogens is using chemical pesticides, but their tremendous overuse leads to serious environmental problems such as the development of pathogen resistance, decline in the number of nontarget beneficial microorganisms in the rhizosphere region, and negative human health effects (Dukare *et al*., 2020; Hesran *et al*., 2019).

Madhurankhi and Suresh (2020) stated that biofertilizers are the safest, most ecofriendly effective tool in comparison to chemical fungicides. They also enhance soil fertility as well as crop yield by 10%–40% (Bhardwaj *et al*., 2014). Furthermore, they have been found to aid in the long-term sustainability of the agricultural ecosystem. Biofertilizers include all the beneficial bacteria that can be either symbiotic or free-living bacteria in

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nature. They are together referred to as plant growth-promoting rhizobacteria (PGPR).

PGPR has been considered as one of the most promising tools to achieve the elimination and even prevent soil-borne disease and hence maximize crop yields (Aruna *et al*., 2020; Devi *et al*., 2011; Hass and Defago, 2005). It has been proved that PGPR has either indirect or direct effects on plants in order to promote their growth and to increase crop yields through several mechanisms (Devi *et al*., 2011), nitrogen fixing and phytohormones production (Cattelen *et al*., 1999), nitrogen availability stimulation, increase of phosphate solubilization and minerals in the soil (Richardson, 2003), and above all control or even prevention of plant pathogens' activities (Babu *et al*., 2015; Glick, 1995; Glick *et al*., 1999; Hayat *et al*., 2010). Several research works studied the mechanisms which microorganisms may use to overcome the disease stress in plants including the production of hydrolytic enzymes (Maurhofer *et al*., 1994; Mostafa and Abd El Aty, 2013) and siderophores production and hydrogen cyanide (HCN) synthesis (Buysens *et al*., 1996; Naureen *et al*., 2017). Some PGPR was responsible for promoting the growth of several plants indirectly by a mechanism known as induced systemic resistance (ISR) (Abdelrahman *et al*., 2016; Jogaiah *et al*., 2010; Kloepper, *et al*., 2004; Mendis *et al*., 2018). Kröber *et al*. (2014) added efficient bacterial strains to colonize the plant root system, accelerate plant growth, and activate their defense system. The phenomenon of initiating the systemic resistance activities of the plant has been termed rhizobacteria-ISR (Arguelles-Arias *et al*., 2009; Kröber *et al*., 2014).

One of the most antagonistic PGPR is *Bacillus* sp. with many advantages such as easy isolation and culturing, wide distribution, antiadversity ability, and their ability to produce a wide range of antibiotics and enzymes with broad-spectrum antimicrobial activity (Arguelles-Arias *et al*., 2009; Chowdhury *et al*., 2015; Kröber *et al*., 2014; Tiago *et al*., 2004; Yuan *et al*., 2013). *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus subtilis*, and so forth have been recorded as rhizosphere residents of a wide range of crops with plant growth-promoting properties (Abd El Aty and Zohair, 2020; Kloepper *et al*., 2004; Kumar *et al*., 2011). Previous studies of Kröber *et al*. (2014) documented *B. amyloliquefaciens* FZB42 as plant growth-promoting bacteria for their ability to promote plant growth as well as the ability to suppress different plant pathogens on cotton, cucumbers, lettuce, tobacco, and tomatoes (Chowdhury *et al*., 2015; Grosch *et al*., 1999; Guel *et al*., 2008; Idris *et al*., 2007; Koumoutsi *et al*., 2004; Wang *et al*., 2009; Yao *et al*., 2006).

The main goal of this work was to apply a novel rhizosphere bacterium with multiple plant growth-promoting traits and antifungal activity against phytopathogens for promoting the growth of *V. faba*, an important food legume in Egypt that can be developed as a commercial product.

MATERIALS AND METHODS

Bacterial strains

Five morphologically different rhizosphere bacterial strains isolated from outside the rhizosphere plant root tips of the species *Salsola imbricata*, collected from Tur Sinai in Egypt (Abd El Aty *et al*., 2018), were tested to select the most active strain with maximum antifungal activities against all tested phytopathogens. All rhizosphere strains were maintained on nutrient agar slants and subcultured periodically and preserved at 4°C.

In vitro antagonistic effects of isolated bacteria towards soil-borne plant pathogens

Different bacterial strains isolated by Abd El Aty *et al*. (2018) were screened against a panel of plant pathogenic fungal strains. The soil-borne plant pathogens *Alternaria alternata* NRC43, *Fusarium solani* NRC11, *Fusarium oxysporum* NRC23, and *Sclerotium rolfsii* NRC32 were supplied from the Culture Collection Unit at the Chemistry of Natural and Microbial Products Department, National Research Centre, Egypt (Abdel Wahab *et al*., 2018; Batrana *et al*., 2019). Petri dishes containing a Potato Dextrose Agar (PDA) (Difco) medium were inoculated with a 1 ml spore suspension containing about 1×10^6 spores of 7 days old fungal pathogens. After solidification of the agar medium, the tested bacterial colonies were inoculated individually in the center of each plate. Plates without bacteria were used as a control. The Petri dishes were incubated at a temperature of 28°C–30°C for 5 days. The mycelium growth inhibition and the diameter of bacterial colonies were measured and compared to the control without bacterial inoculation to select the most active bacterial strain (Zohair *et al*., 2018).

Bacillus amyloliquefaciens MH046937

The bacterial isolate identified as *B. amyloliquefaciens* MH046937 was used in the present study according to the antagonistic results obtained, and the plant growth-promoting tests were carried out only for that isolate.

Evaluation of B. amyloliquefaciens MH046937 for multiple plant growth-promoting traits

Indole acetic acid (IAA) production

Twenty-five milliliters of nutrient broth (NB) slants supplemented with 0.05% DL-tryptophan was inoculated with a 1 day old bacterial culture grown in a shaking incubator at 30°C at 200 rpm in the dark for 2 days (Ashour *et al*., 2016 a; Kumar *et al*., 2012a). The tested bacterial isolate was centrifuged in a refrigerated centrifuge at a range of 10,000–15,000 rpm for 10 minutes at 4°C. Indole-3-acetic acid [Indole-3-acetic acid (IAA)] was estimated in the supernatant by using the colorimetric assay test (Loper and Schroth, 1986). Half millilitre of bacterial supernatant was added and well mixed with 2 ml of Salkowski's reagent and the produced pink color was measured after half an hour at 535 nm in a UV-visible spectrophotometer. The production of the pink color in the test tube was considered as an indication of IAA production which was previously indicated by Gordon and Weber (1951).

Production of ammonia

Bacillus amyloliquefaciens was investigated for the production of ammonia (NH_3) as reported by Ashour *et al*. (2016a) and Cappuccino and Sherman (1992). A 1-day-old bacterial culture was inoculated in 5 ml peptone broth media then incubated at 30°C for 2 days in a shaking incubator. Then 0.25 ml of Nessler's

reagent was added to flasks and mixed well. The appearance of a faint yellow color to a dark brown color was considered as an indicator of ammonia production.

Production of HCN

Production of HCN was estimated by streaking the *B. amyloliquefaciens* bacterial isolate on King's B agar medium amended with glycine. The internal side of the Petri plate's lid was covered with filter paper soaked in a picric acid solution (0.05% solution in 2% sodium carbonate). Then the plates were sealed air-tight with Parafilm stretch and incubated at 30°C ± 1°C for 2 days. The change of the filter paper color from deep yellow to reddish-brown indicated HCN production (Bakker and Schipperes, 1987; Geetha *et al*., 2014).

Production of siderophore

Siderophore productivity was estimated according to Sujatha and Ammani (2013). The tested bacterial isolates were streaked on the (King's B) medium with and without the addition of (50 mg/l) $FeCl_3$ then incubated at temperature 28°C ± 1°C for 2 days. The formation of fluorescent pigment was considered as evidence of siderophore production.

Phosphate solubilization

The phosphate solubilization qualitative test was carried out using Pikovskaya's (PVK) medium containing (in g/l) glucose 10.0, $Ca_3(PO_4)_2$ 5.0, $(NH_4)_2SO_4$ 0.5, KCl 0.2, $MgSO_4.7H_2O$ 0.1, $MnSO_4$ 0.002, $FeSO_4$ 0.002, yeast extract 0.5, agar 16.0, and distilled water. The freshly prepared bacterial culture was streaked on PVK's agar medium and incubated at a temperature of 30°C for 12 days. The formation of a clear zone surrounding the tested culture proved the phosphate solubilization ability (Kumar *et al*., 2012a).

Determination of the fungal cell-wall hydrolytic enzymes

Bacillus amyloliquefaciens was screened for chitinase and 1,3-β-glucanase enzymes production using an NB medium at different incubation periods (Hao *et al*., 2012). Fermentation was carried out in 250 ml conical flasks containing 50 ml of a sterile medium. For the cultivation process, a 24-hour-old culture was adjusted to ~1.0 at 600 nm, and each flask finally was inoculated with a 5 ml bacterial suspension. Flasks were incubated at 32°C, 180 rpm for 24, 48, and 72 hours. After incubation for different time intervals, the fermentation broth was centrifuged for chitinase and 1,3-β-glucanase activities determination.

Assay of chitinase

The chitinase assay was performed using the colorimetric method for the estimation of N-acetyl amino sugar using the modified dinitrosalicylic acid [Dinitrosalicylic acid (DNS)] method. The unit (U) of chitinase productivity was defined as the quantity of enzyme required to liberate 1.0 µmol/minute of N-acetyl glucosamine (Ashour *et al*., 2016b; Shehata *et al*., 2018).

Assay of 1,3-β-glucanase

Glucanase productivity was determined according to Burner (1964). The reaction mixture contained 250 µl of the freshly prepared crude enzyme solution and 250 µl of 1% laminarin (Sigma Chemical Co., St. Louis, MO) in a 0.2 M sodium acetate buffer at pH 5. The previous mixture was incubated with gentle shaking at 37°C for 30 minutes. The reaction was arrested by the addition of 1% dinitrosalicylate (DNS) and then boiled for another 10 minutes. One unit (U) of the glucanase productivity was estimated based on the quantity of enzyme that is needed to liberate 1 µmol/minute of glucose.

Extraction and detection of bioactive compounds with antifungal properties

The B. amyloliquefaciens fermentation medium was investigated for antifungal metabolites production.

Fermentation and extraction of metabolite compounds

For bacterial fermentation, 500 ml conical flasks containing 100 ml of sterile NB of total volume 500 ml were inoculated with a *B. amyloliquefaciens* suspension. The flasks were incubated for 7 days at 33°C and 180 rpm. Then the fermentation medium was centrifuged at 7,000 rpm for 15 minutes and the filtrate obtained was partitioned with ethyl acetate three successive times. The ethyl acetate layer was concentrated under a vacuum to obtain the ethyl acetate extract.

Fractionation, purification, and characterization of compounds

The crude ethyl acetate extract was separated on silica gel 60 with particle size 0.063–0.2 mm (70–230 mesh) column chromatography. *N*-Hexane and ethyl acetate with ratio (1,1) were applied to give 20 ml of three subfractions (A1–A3) and then ethyl acetate (100%) to give another three subfractions (A4–A6). Subfractions coded A3 and A4 were gathered and further purified by using YL9100 High-performance liquid chromatography (HPLC) equipped with a YL9112 isocratic pump and YL9120 UV/visible detector at λ = 254 and 280 nm. The used column was a normal phase (ZORBAX RX Silica 5 µm, 250 × 9.4 mm). The used solvent system was *n*-hexane, EtOAc with 2:1 ratio, and flow rate 4 ml/minutes. The detected eluted peaks at different retention times by the UV detector were recorded and the major compound **1** (4.0 mg) was purified. Structure elucidation of **1** was carried out using ^1H and ^{13}C NMR measured in $CDCl_3$ using a Bruker 500 MHz.

In vitro antifungal activity

Nine fractions separated from the HPLC column were assayed for their antifungal properties using the agar diffusion method (Mohamed *et al*., 2017; El-serwy *et al*., 2015). Each fraction was loaded on a paper disc of blotting paper (5 mm in diameter) with a concentration of 100 µg/5 µl ethyl acetate/disc. The phytopathogen *F. solani* NRC11 was inoculated into plates containing a sterile PDA medium. The discs were placed on the surface of the inoculated plates and left for compounds diffusion for about 30 minutes at 4°C. Plates were incubated for 3 days at 28°C for the growth of pathogenic fungi. The diameters of the inhibition zone formed around the discs at three different points were measured in millimeters and the average values were recorded as mean ± SD by using the MS Excel program.

***In vivo* biocontrol estimation of root rot disease**

The biocontrol activity of *B. amyloliquefaciens* against phytopathogens (*F. oxysporum*, *F. solani*, and *S. rolfsii*) was

Table 1. Greenhouse experiment design.

Treatments	Pathogen	Seeds soaked in			Pathogenic fungi	Control agents	
		Sterile water	Chemical fungicide[a]	Bacterial filtrate (*B*)		Bacterial filtrate (B)	Chemical fungicide[a]
NC[b]	-	+	−	−	−	−	−
	FO	−	+	−	+	−	+
PC[c]	FS	−	+	−	+	−	+
	Sr	−	+	−	+	−	+
	FO	+	−	−	+	−	−
T_0	FS	+	−	−	+	−	−
	Sr	+	−	−	+	−	−
T_1	-	−	−	+	−	+	−
	FO	−	−	+	+	+	−
T_2	FS	−	−	+	+	+	−
	Sr	−	−	+	+	+	−

[a]Fungicide, RIZOLEX-T 50% WP, Sumitomo Chemical Ltd., Japan, in concentration 1 g/l.

[b]Negative control.

[c]Positive control.

FO, *F. oxysporum*; FS, *F. solani*; Sr, *S. rolfsii.*

confirmed in the greenhouse experiment. The healthy *V. faba* seeds were cultivated in pots (each containing 400 g sterilized soil previously autoclaved at 121°C for 15 minutes). The infection technique was applied using 1-week-old soil-borne pathogenic fungi cultures on PDA. A 5-ml sterilized distilled water suspension was inoculated with a 5 mm diameter disc of soil-borne pathogenic fungi to prepare the inocula. To create an artificial infection, the prepared inocula were added to pots. The treatments were designed as mentioned in Table 1: T_0: treatment of pots with pathogenic fungi without the addition of any control agents, T_1: treatment of pots with the biocontrol agent *B. amyloliquefaciens* without infecting pots with pathogens, and T_2: treatment of pots with both the biocontrol agent *B. amyloliquefaciens* and three different pathogenic fungi separately. The pots were kept under greenhouse conditions with a temperature of 25°C.

Each treatment was carried out in triplicate. The tested vegetative growth parameters were estimated and recorded at the end of the experiment. The disease index (DI) was estimated on a scale from 0 to 4. The statistical design work was completely randomized according to the work of Harveson *et al*. (2014).

Statistical analysis

The recorded data were subjected to an analysis variation test and were analyzed by using the a computer based Statistical software packages (MSTATC) program. The differences of recorded means of the three replicates were compared applying the least significant differences (LSD) test at a 5% level of significance.

RESULTS AND DISCUSSION

Evaluation of bacterial strains antagonistic effects

All bacterial strains isolated from the rhizosphere zone of *S. imbricata* in Tur Sinai, Egypt (Abd El Aty *et al*., 2018), were tested *in vitro* against four different pathogenic fungi: *A. alternata*, *F. solani*, *F. oxysporum*, and *S. rolfsii*. Out of all rhizosphere strains, the bacterial isolate identified as *B. amyloliquefaciens was found to have* broad-spectrum antifungal activities against all pathogens (Fig. 1). *Antifungal effects depend* on the production of cell-wall hydrolytic enzymes such as chitinases, a major enzyme group capable of degrading the polymeric chitin components of pathogenic fungal cell walls (Dubey *et al*., 2014; Kumar *et al*., 2012b). In this context, *B. suly* produced the chitinase enzyme that reduced the infection severity of *Fusarium* sp. under greenhouse conditions (Hariprasad *et al*., 2011).

Bacillus amyloliquefaciens showed high antagonistic activities against *S. rolfsii* (a) followed by *A. alternata* (b) and showed similar effects against the *Fusarium* sp. (c and d). Our results agree to some extent with Gowtham *et al*. (2018) who indicated that *Pseudomonas aeruginosa* has the maximum percentage of inhibition capability against *Colletotrichum truncatum* growth, followed by *B. amyloliquefaciens* and *Burkholderia cepacia*. On the other hand, Akinrinlola *et al*. (2018) exhibited limited antagonism of *B. pumilus* strains R174, R183, and R190 and *B. megaterium* R181 against *F. graminearum*, while the two *Pythium* spp. and *R. solani* were not affected.

Evaluation of *B. amyloliquefaciens* for plant growth-promoting properties

Physiological traits of *B. amyloliquefaciens* were estimated *in vitro* for the production of IAA, HCN, siderophores, and ammonia and capability for phosphate solubilization.

Production of IAA

The PGPR secreting IAA through the interaction with the plant enhance and accelerate plant growth and help in the plant defense responses mechanism (Mohite, 2013; Tekalign *et al*., 2016). Our studies proved that *B. amyloliquefaciens*

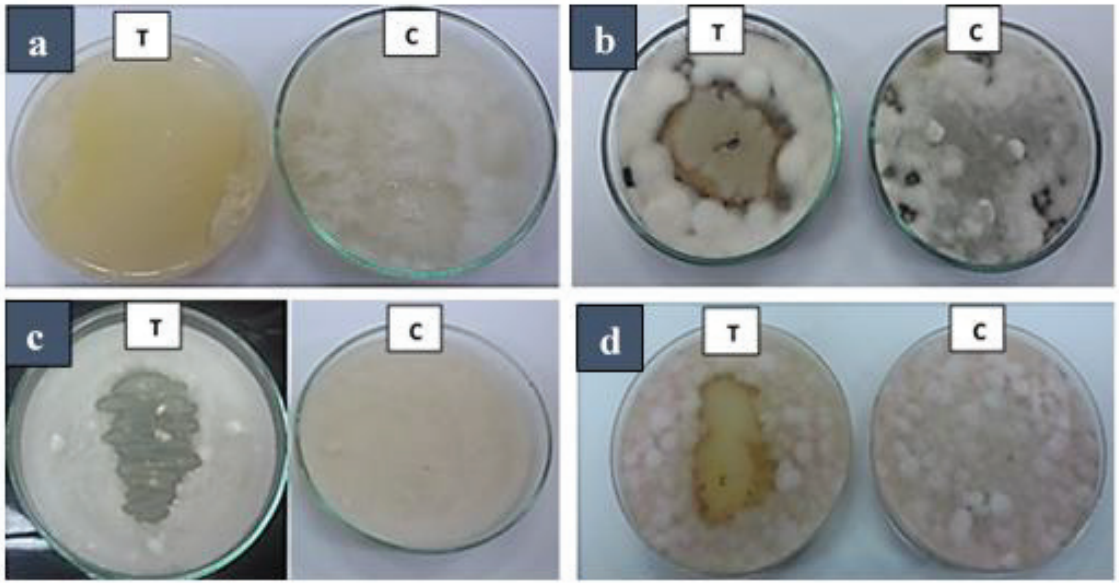

Figure 1. Antagonistic effect of the rhizospheric *B. amyloliquefaciens* against (a) *S. rolfsii,* (b) *A. alternata*, (c) *F. solani*, and (d) *F. oxysporum. T* tested plates inoculated with bacteria. C, control plates without bacterial inoculation. All plates incubated at 28°C–30°C for about 5 days.

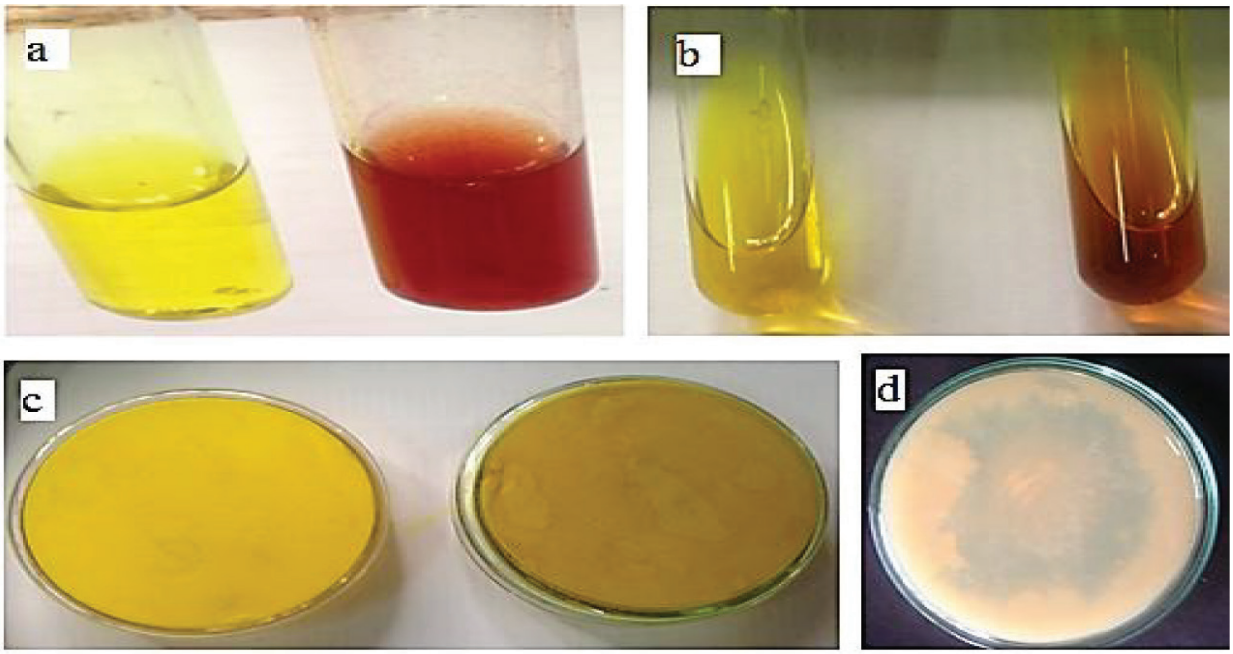

Figure 2. *In vitro* physiological traits of *B. amyloliquefaciens* MH046937 for the production of IAA (a), production of ammonia (b), production of HCN (c), and phosphate solubilization (d).

produced a substantial quantity of IAA equivalent to 32.9 ± 0.01 µg/ml (Fig. 2a). Idris *et al*. (2007) indicated that the Gram-positive bacterial strain *B. amyloliquefaciens* synthesized significant quantities of IAA, while mutants of *B. amyloliquefaciens* E101 (ΔtrpED) and E102 (ΔtrpAB) synthesized IAA in small quantities. The amount of IAA produced by *B. amyloliquefaciens* MH046937 was more than that reported by Asma *et al*. (2013) (7–14 µg/ml) due to the positive effect of tryptophan and higher than that recorded by Shao *et al*. (2015), 9.46 µg/ml, when L-tryptophan was added, while Chandra *et al*. (2018) proved that only three bacterial isolates out of the different isolates were IAA producers. Isolates CA2004 and CA1001 exhibit significant production capability of IAA (91.7 µg/ml) followed by 81.7 µg/ml and the isolate coded CA1001 produced 32 µg/ml of IAA which was similar to some extent to our results. Akinrinlola *et al*. (2018) showed the ability

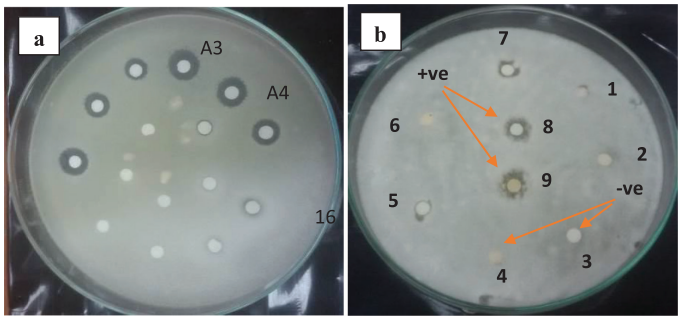

Figure 3. Agar diffusion assay of subfractions obtained from silica gel column chromatography (a) and nine fractions separated from HPLC column (b) against the phytopathogen *F. solani*. Each fraction was loaded with a concentration of 100 µg/5 µl ethyl acetate/disc. +ve, positive for inhibiting *F. solani* growth. −ve, negative for inhibiting *F. solani* growth.

of *Bacillus safensis* and *B. megaterium* to synthesize IAA and none of the *B. pumilus* tested strains exhibited this activity.

Production of ammonia

Our results proved that *B. amyloliquefaciens* MH046937 produced a dark brown color with Nessler's reagent indicating a considerable amount of ammonia production as shown in Figure 2b, and these results were found to agree with the Sushil *et al.* (2013) study which proved that *B. amyloliquefaciens* produced ammonia.

HCN and siderophore production

Bacillus amyloliquefaciens MH046937 was able to synthesize HCN at a moderate amount as shown in Figure 2c and this result goes along with Defago *et al.* (1990) who stated that *B. amyloliquefaciens* produced HCN at a moderate amount. Nadège *et al.* (2015) reported that over 75% of HCN producers were found to be *Bacillus* sp. strains isolated from the rhizosphere of rice.

Siderophore production was not recorded either in the presence or in the absence of $FeCl_3$. These results were similar to some extent to Nadège *et al.*'s (2015) results, who showed siderophore synthesis without the addition of 1 µM $FeSO_4.7H_2O$ to the culture flask. However, other *Bacillus* strains coded R181, R180, R232, and R190 were proved to be capable of siderophore synthesizing when cultivated on a Chrome Azurol S medium (CAS) agar medium (Akinrinlola *et al.*, 2018).

Phosphate solubilization activity

In the work of Schwartz *et al.* (2013) and Khalid *et al.* (2004) the phosphate solubilization traits were connected to high differentiation, growth, and development efficacy. As shown in Figure 2d, the tested bacterial isolate had efficiency in phosphate solubilization and produced a large inhibition zone after 10 days. Akinrinlola *et al.*'s (2018) and Nadège *et al.*'s (2015) data declare that *B. amyloliquefaciens* and *B. megaterium* strains, respectively, exhibited phosphate solubilization activity on an (PVK's) agar medium. These results were in excellent accordance with those recorded by other scientists (Fernandez *et al.*, 2012; Riberio and Cardoso, 2012).

Results showed that *B. amyloliquefaciens* has many physiological traits like IAA, ammonia, and HCN production in addition to the ability to hydrolyze phosphate, which helps the bacterium prevent the plant pathogens activities and increase plant growth-promoting properties.

Evaluation of *B. amyloliquefaciens* hydrolytic enzymes activities

The bacterial isolate was screened for chitinase and 1,3-β-glucanase enzymes activities at different incubation periods using the NB medium without any induction by substrate addition. The result showed that *B. amyloliquefaciens* was able to produce the chitinase enzyme (68 IUl^{-1}) after 48 hours. In contrast, the tested bacterial isolate did not exhibit 1,3-β-glucanase enzyme activity. Other studies of Ashour *et al.* (2016a) and Wang *et al.* (2006) reported chitinase productivity in many *Bacillus* species such as *B. diminuta* KT277492, *B. cereus* KU058893, and *B. megaterium* using the NB medium, where other species were reported as β-glucanase producers, such as *B. licheniformis* (Chaari *et al.*, 2012) and *B. subtilis* (Manjula and Podile, 2005; Narasimhan *et al.*, 2013).

The ability of *B. amyloliquefaciens* to produce the hydrolytic enzyme chitinase, in addition to the previously mentioned physiological traits, increased its ability to overcome the disease stress in plants.

PGPR-based mechanisms employed in the biological control of phytopathogens include the production of cell-wall degrading chitinase enzyme (Husson *et al.*, 2017), synthesis of antimicrobial metabolites (Couillerot *et al.*, 2009), and HCN (Nandi *et al.*, 2017), and siderophores (Shen *et al.*, 2013) production is important for induction of ISR that causes a faster and stronger plant response to attacks by different pathogens (Olanrewaju *et al.*, 2017).

Purification and characterization of *B. amyloliquefaciens* bioactive metabolites

The crude ethyl acetate extract of the tested bacterial isolate was first purified using silica gel column chromatography with different solvent systems. The antifungal activity of all obtained subfractions against *F. solani* was detected. Results indicated that subfractions A3 and A4 have the highest inhibition zone diameters (IZD) of 16 and 15 mm (Fig. 3a), respectively, that indicated the availability of bioactive antifungal metabolites. The A3 and A4 subfractions were combined and further purified using YL9100 HPLC, and the eluted peaks at various retention times were collected.

Nine fractions separated from the HPLC column at different retention times were assayed using the agar diffusion method. The fungal pathogen *F. solani* was used as an indicator to explore and determine the bioactive fractions for the identification of the major antifungal compound. Results in Figure 3b showed that fractions eight and nine were positive for inhibiting the fungal growth (IZD of 12 ± 0.71 mm). Fraction seven showed week antifungal activity (IZD of 8 ± 0.21 mm), where the other fractions were negative for inhibiting *F. solani* growth.

Eluted peaks 8 and 9 with good antifungal activities were subjected to structural elucidation analysis, and the major compound (**1**) was obtained. The structural analysis of the major compound (**1**) on the basis of the NMR measurements and EI mass spectroscopy was identified as 5-hydroxymethylfurfural (Fig. 4).

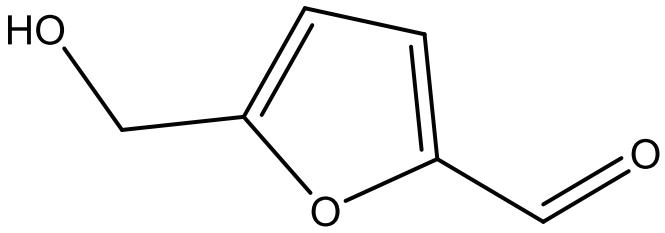

Figure 4. Chemical structure of 5-hydroxymethylfurfural.

^{1}H NMR (500 MHz, $CDCl_3$) δ, 2.15 (br s, 1H), 4.74 (s, 2H), 6.52 (d, J = 3.6 Hz, 1H), 7.22 (d, J = 3.6 Hz, 1H), 9.61 (s, 1H), ^{13}C NMR (125 MHz, $CDCl_3$) δ, 57.7, 110.0, 122.6, 152.4, 160.4, 177.7, Low Rate- Electron Ionization (LR-EI) mass, 126 *m/z*.

Other studies of Rane Zab *et al*. (2012) stated that the ethyl acetate fraction of *Punica granatum* rind containing abundant 5-hydroxymethylfurfural showed antimicrobial activity by the agar diffusion method. Rigal and Gaset (1983) showed that the compound 2-furancarboxaldehyde,5-(hydroxymethyl) has antifungal and antibacterial activity and is applicable in many fields, including pesticides, cosmetics, and pharmaceuticals. Also, previous studies indicated that furfural could be used as a flavor and in many other products, such as herbicides, pesticides, fungicides, germicides, and insecticides, as mentioned by Morales (2008).

Plant growth-promoting and biocontrol properties of *B. amyloliquefaciens*

In greenhouse experiments, *V. faba* seeds treated with PGPR *B. amyloliquefaciens* were estimated for their positive effect on differentiation and vegetative growth parameters such as shoot dry and fresh weights and plant height, along with the number of leaves per plant. Our results showed that the bacterial treatment had significant stimuli on the height of infected plants with pathogens *S. rolfsii*, *F. oxysporum*, and *F. solani* at 34, 27, and 24 cm, respectively. The vegetative growth had been affected positively by the application of bacterial treatment.

The highest shoot dry and fresh weights were 0.4 and 4.2 g, respectively, in treated plants with the tested bacterial strain and infected with *S. rolfsii* compared with the positive control treatment.

The fresh and dry root weight in infected plants with *F. solani* or *S. rolfsii* and treated with the tested bacterial strain had been enhanced significantly in comparison with other treatments by 0.5 and 0.07 g, respectively.

The highest germination percentage was recorded with infected seeds with *F. solani* 86.7% and the DI was 0 grade significantly *as the plants infected with F. oxysporum* (Table 2). These results confirm the role of treatment with *B. amyloliquefaciens as a biocontrol plant growth-promoting agent. In this context,* Srivastava *et al*. (2016) recorded that *B. amyloliquefaciens* stimulated the production of rice plants under infection of pathogenic fungi *Rhizoctonia*.

Analysis of three different treatments indicated a significant enhancement in the plant height, the number of leaves, and stem thickness (28.33 cm, 3.66, and 0.53 mm, respectively) in the treatment with the tested bacterial strain compared with positive control (19.66 cm, 3, and 0.43 mm, respectively) as shown in Figure 5.

The treatment with *B. amyloliquefaciens* MH046937 (T_2) compared with the control (PC) introduced a significant increase of germination percentage (60% and 40%, respectively) and reduced the disease incidence to be 0.44 and 1.66 for bacterial treatment and positive control, respectively. Our results agreed with Gowtham *et al*. (2018) whose work proved that out of eight PGPR isolates *B. amyloliquefaciens* provided the highest level of seed germination and also seedling vigor of chili seeds germination (84.8% and 1,423.8% respectively). Also, Hariprasad

Table 2. Effect of various treatments on biocontrol activities and plant growth-promoting properties.

Treatment	Pathogen	Plant height (cm)	Leaves no.	Stem diameter (mm)	Shoot fresh weight (g)	Shoot dry weight (g)	Fresh weight of root (g)	Dry weight of root (g)	Germination %	DI (0–4)
NC	-	17.3^{bcd}	2.33^{c}	0.5^{b}	0.665^{j}	0.058^{h}	0.179^{h}	0.02^{ef}	26.66^{b}	2.33^{a}
	FO	25^{abc}	4^{ab}	0.5^{b}	3.1^{c}	0.29^{de}	0.295^{g}	0.035^{de}	53.33^{ab}	1^{abc}
PC	FS	14.6^{cd}	2^{c}	0.5^{b}	2.888^{e}	0.315^{c}	0.42^{e}	0.056^{bc}	33.33^{b}	2.66^{a}
	Sr	19.3^{bcd}	3^{bc}	0.3^{d}	1.793^{g}	0.161^{f}	0.405^{e}	0.05^{cd}	33.33^{b}	1.33^{abc}
	FO	10.6^{d}	3.33^{abc}	0.5^{b}	0.533^{k}	0.104^{g}	0.158^{i}	0.046^{cd}	13.3^{b}	1.6^{abc}
T0	FS	11.6^{d}	2.33^{c}	0.4^{c}	1.466^{h}	0.051^{h}	0.381^{f}	0.015^{f}	33.33^{b}	2^{ab}
	Sr	20.6^{bcd}	3.33^{abc}	0.3^{d}	0.685^{i}	0.06^{h}	0.174^{hi}	0.022^{ef}	53.33^{b}	2.66^{a}
T1	-	17.3^{bcd}	3.33^{abc}	0.5^{b}	2.912^{d}	0.279^{e}	0.483^{c}	0.058^{bc}	26.66^{ab}	1.33^{abc}
	FO	27^{ab}	3^{bc}	0.4^{c}	2.555^{f}	0.297^{d}	0.44^{d}	0.069^{ab}	53.33^{ab}	0^{c}
T2	FS	24^{abc}	4.66^{a}	0.6^{a}	4.119^{b}	0.363^{b}	0.535^{a}	0.07^{ab}	86.66^{a}	0.33^{bc}
	Sr	34^{a}	3.33^{abc}	0.6^{a}	4.157^{a}	0.4^{a}	0.511^{b}	0.079^{a}	40^{b}	1^{abc}
LSD 5%		10.56	1.421	0.0166	0.0166	0.0166	0.0166	0.0166	42.47	1.695

Values in each column are mean, and means in a given column with the same letters are not significantly different ($p > 0.05$).

FO, *F. oxysporum*; FS, *F. solani*; Sr, *S. rolfsii*; NC, negative control; PC, positive control.

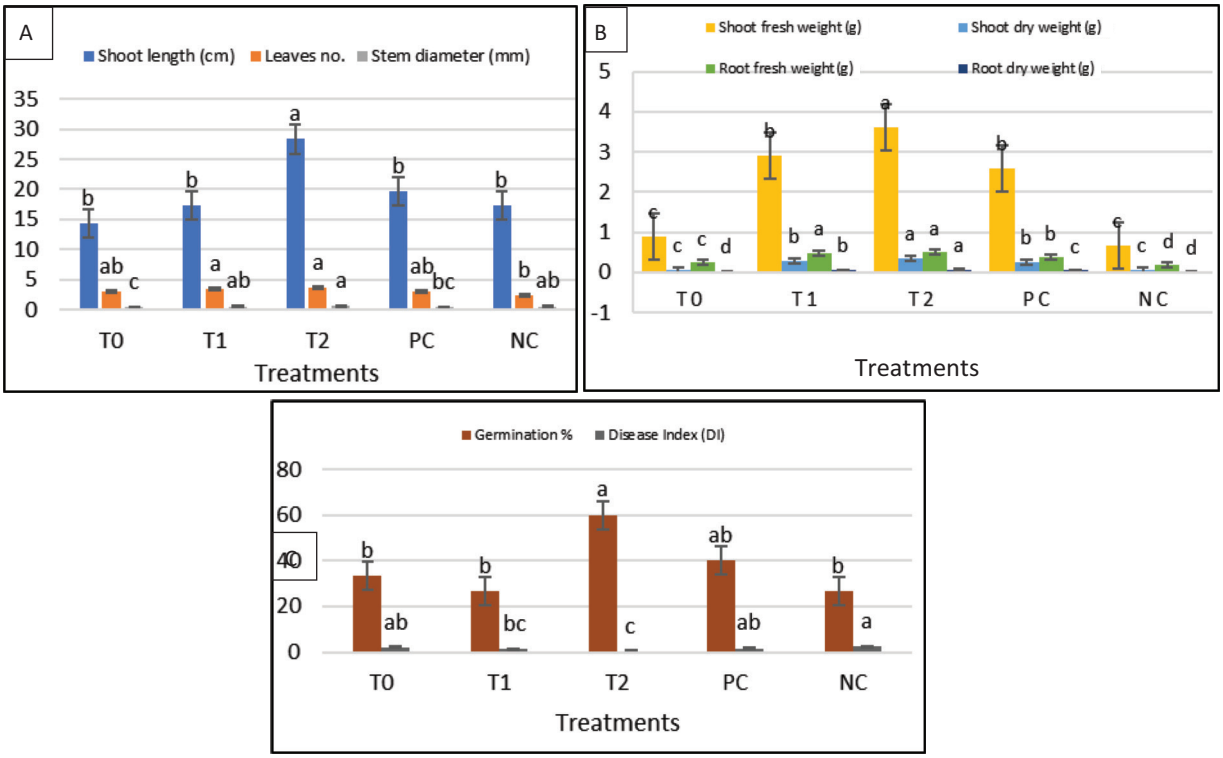

Figure 5. Effect of various treatments on plant vegetative and root growth parameters (A and B) and biocontrol activities and germination percentage (C) of *V. faba.*

Figure 6. Greenhouse application of the PGPR (*B. amyloliquefaciens* MH046937) against phytopathogens.

et al. (2011) stated that PGPR that had broad-spectrum antifungal and antibacterial activities was found to enhance the growth of many plants as well as the suppression of many phytopathogens of tomatoes by stimulating the plant's defense mechanisms. The PGPR were also recorded to eliminate both bacterial and fungal infections effectively and enhance autoresistance in some plants (Chowdappa *et al*., 2013; Ramamoorthy *et al*., 2002). Other promising findings were observed by Yuan *et al*. (2013) who found the recorded rate of *Fusarium* infection causing wilt disease in banana plants cultivated in pots containing a bioorganic fertilizer was as high as 90% while it was only 28% in ones containing a bioorganic fertilizer inoculated with *B. amyloliquefaciens* NJN-6 which could be an indication of the role of *B. amyloliquefaciens* in suppressing the growth of the pathogens.

The treatment with *B. amyloliquefaciens* MH046937 compared with the control introduced a significant stimulation of

one or more growth factors. Bacterial treatment had considerable effects on shoot and root weights, compared to control. T_2 had the highest shoot fresh weight at 3.6 g while the positive control had 2.59 g. In the same way, the treatment with bacteria had the highest significant effect on the root's fresh weight at 0.495 g as shown in Figures 5 and 6. The same findings were reported by Gowtham *et al*. (2018) who found that plants treated with *B. amyloliquefaciens* provided the highest vegetative growth parameters of chili plants such as plant height at a value 18.32 cm, dry weight recorded as 1.5 g, shoot fresh weight reaching 3.5 g, and number of leaves at a value of 15.25 leaves per plant.

CONCLUSION

In conclusion, the *B. amyloliquefaciens* MH046937 rhizospheric strain showed antagonistic activity to several plant pathogenic fungi. It demonstrated that plant growth-promoting properties depending on the ability of IAA production, ammonia production, HCN production, and solubilization of phosphate to some extent are involved in *V. faba* growth promotion. Also, the results indicated that the tested bacterial isolate has the ability to produce the chitinase enzyme and bioactive metabolite identified as 5-hydroxymethylfurfural important in the suppression of plant pathogenic fungi. Results indicated that *B. amyloliquefaciens* MH046937 had broad-spectrum plant promotion activity for *V. faba* growth in greenhouse pot experiments. Its application as a biocontrol agent can significantly contribute to some extent to the damage that occurs due to the existence of soil-borne fungi.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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