Studies on the antimicrobial activity of a locally isolated lactic acid bacterium and its application on *fusarium solani*-infected plants

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Background
Lactic acid bacteria (LAB) have proven a great inhibitory effect toward phytopathogenic bacteria and fungi, giving them the importance as natural biological control agents without hazards to the surrounding eco-system.
Objective
This study aims for the local isolation of a lactic acid bacterial strain active against different microorganisms especially phytopathogens.
Materials and methods
Of the 40 isolated bacterial strains, 36 were preliminarily characterized as LAB using both Gram staining and catalase test methods. Their antimicrobial activities were then tested against different bacterial and fungal strains. The most potent isolate was molecularly identified and tested, *in vitro*, on different pathogens, and then, *in vivo*, against infected *Phaseolus vulgaris* plants.
Results and conclusion
Results revealed that 17 isolates showed antimicrobial activities against *Pectobacterium carotovorum* and four of them were solely effective against *Staphylococcus aureus* (inhibition zones of diameters ranging between 0.4 and 0.8 cm). However, only isolate 32 showed satisfactory antimicrobial activity (inhibition zones of ~0.8 cm) against both of the two aforementioned test microorganisms. Moreover, isolate 32, molecularly identified as *Enterococcus faecium*, was also found to be active against *Fusarium solani phaseoli*, a common phytopathogen fungus that affects many economically important crops. The addition of 1.5% (v/v) of the 24-h old fermentation broth of this isolate, *in vitro*, resulted in a shrinking percent of the fungus growth of more than 51% and gave very promising results when applied in the field experiment. Therefore, the use of the selected LAB culture broth as a bioagent to control some plant pathogens, as well as a plant growth stimulator, could be a promising approach to reduce the economic losses in agricultural crops.

Keywords:
bioagents, *Fusarium solani phaseoli*, lactic acid bacteria

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Introduction

There is an increasing interest to establish alternative byproducts to replace chemicals and toxic pesticides, which are unfortunately extensively used to prevent and control plant diseases around the world. They are the major cause of many health risks for humans and animals, affect the ecological equilibrium of the environment, and encourage the growth of agrochemicals resistant pathogens [1,2].

These byproducts are in fact microbial metabolites that exhibit the same inhibitory effect, on phytopathogens and plant spoilage microbes, as chemical pesticides, eventually resulting in more efficient crops yields as well as an extended storage life of the produced crops in addition to the safeguarding of the environment [3].

Reported data state that lactic acid bacteria (LAB) have the ability to produce a variety of antimicrobial compounds and effective substances such as organic acids (lactic, acetic, and probionic acids), bacteriocin antibiotic, and bacteriocin-like substances, as well as hydrogen peroxide and carbon dioxide [4–6]. These substances were proven to efficiently inhibit the growth of many phytopathogenic fungi including some *Fusarium* sp., *Penicillium expansum*, *Botrytis cinerea*, *Aspergillus niger*, and *Aspergillus flavus* [7,8], as well as that of some phytopathogenic bacteria such as

Xanthomonas campestris and *Pectobacterium carotovorum* [9]. All of these soil pathogens are known to cause different plant root and foliar diseases such as damping-off root rot, white rot, wilt, as well as downy and powdery mildew [10,11]. These plant diseases are among the most important factors that result in yield limitation of many economically important crops and eventually result in serious economic losses.

In Egypt, *Phaseolus vulgaris* L., known as common bean, is one of the most important agricultural crops, especially because it is exported to different countries. However, the common bean crop is consistently subjected to serious infections by different contaminants that cause many diseases such as stem canker, dry rot, wilt, white mold, leaf spots, Anthracnose, and others. One of the most important causes of such infections is the common plant pathogen fungus *Fusarium solani,* which mainly infects the roots of the *P. vulgaris* plants resulting in the drastic wilting and deterioration of the entire crop [12].

Materials and methods

Soil sampling and lactic acid bacteria isolation

Isolates were recovered from two soil samples originating from two different Egyptian farms, one situated in El-Fayoum Governorate region and the other found on the Cairo-Alexandria road. The samples were collected during the winter season of common beans cultivation of October–December, 2016. These samples were serially diluted and then cultured on the surface of solid de Man Rogosa and Sharpe (MRS) agar medium plates using the pour plate method [13] and incubated at 37°C for 48 h. Morphologically distinct colonies were then picked up and transferred to MRS agar slants and maintained at 4°C until needed.

Preliminary identification of lactic acid bacteria strains

According to Holt *et al.* [14], the isolated bacteria were preliminarily identified as LAB strains relying on three parameters: Gram staining, catalase production ability, as well as the morphology of the cultivated colonies.

Test strains' cultivation conditions

To investigate the antimicrobial activity of the preliminarily identified LAB isolates, different test microorganisms including both gram-positive and gram-negative bacteria, as well as some common plant pathogens, were used. These test strains were cultivated as follows: *Staphylococcus aureus* (ATCC 29213) and *Bacillus subtilis* (NRRL-B-4219), both representative of gram-positive bacteria, as well as *Escherichia coli* (ATCC 25922) and *P. carotovorum* subsp. *carotovorum* (kindly obtained from plant pathology group, University of Padova, Italy), both representative of gram-negative bacteria, were cultured on nutrient agar medium and incubated at 37°C for 24 h, whereas the tested fungi, including *Fusarium graminearum*, *B. cinerea*, *Sclerotinia sclerotiorum* (University of Padova, Italy), *F. solani* f. sp. *phaseoli,* and *Rhizoctonia solani* (CLOA, ARC, Egypt), were grown on potato dextrose agar medium (PDA) at 28–30°C for 5 days.

Antibacterial activity of preliminarily identified lactic acid bacteria strains

Agar well diffusion assay [7] was used for evaluating the antibacterial activity of the 36 isolates, preliminarily identified as LAB, as follows: triplicates of 25-ml nutrient agar medium were separately seeded with $\sim 10^5$ cells/ml of each of the 4 aforementioned bacterial test strains and poured in 9-cm large sterile Petri dishes. After medium solidification, agar medium wells of 0.5-cm diameters were cut using a sterile cork borer, and 100 µl of the culture filtrate of each tested isolate, previously incubated overnight in MRS broth medium, was pipetted into each well. Plates were incubated for 24 h at 37°C, and the antibacterial activities of the isolates were determined by measuring the diameters of the formed inhibition zones.

Antifungal activity of preliminarily identified lactic acid bacteria strains

The preliminarily identified LAB isolates that showed some positive antibacterial activities were also tested against the previously mentioned phytopathogenic fungi according to the method of Gebily [15] as follows: the five tested fungal strains were first individually grown, on PDA medium, in sterile Petri dishes at 28°C. After 5–7 days of incubation, discs of 0.5 cm in diameter were then cut out of each of the formed cultures and carefully transferred to the surface of sterile nutrient agar medium, previously poured in another set of sterile Petri dishes. Each disc was placed at about 2 cm of the Petri dish edge and a streak of each of the tested LAB isolate was performed at 2 cm of the opposite edge of the same Petri dish, so that all the isolated strains, preliminarily identified as LAB, were subjected to all of the fungal strains under test. The plates were then incubated at 30°C for 5–7 days, and the growth pattern of both microorganisms was investigated.

Molecular identification of the selected lactic acid bacteria isolate 32

Selected LAB isolate 32 was further identified via 16 S rDNA sequencing. PCR amplification was carried out

using universal primers and conditions described by Weisburg *et al*. [16]. PCR products were then purified using the QIAquick PCR purification kit according to the supplier's instructions (Qiagen, Hilden, Germany). DNA sequences were determined by the dideoxy chain termination method [17]. Finally, DNA similarity with preindentified DNA standards was determined by BLAST search tool at the National Centre of Biotechnology Information (NCBI), and the genetically identified isolate was consequently registered at the GenBank [18].

Safety assessment of *Enterococcus faecium* MN32

Two different experiments were performed to evaluate the safety of the selected isolate for human beings and animal use and eventually assess its safe use in different applications that directly affect their life.

Susceptibility to antibiotics

The antibiotic susceptibility of the selected LAB isolate was determined against six commonly used antibiotics, namely, ampicillin, vancomycin, kanamycin, tetracycline, erythromycin, and fusidic acid. Antibiotic susceptibility discs were obtained from bioMerieux (Marcy L'Etoile, France) in different concentrations ranging from 10 to 30 µl. Discs were stored in sealed containers with a desiccant at 4°C. Antibiotic susceptibility was determined semiquantitatively using a modification of the agar overlay diffusion methods of the National Committee for Clinical Laboratory Standards [19]. The susceptibility to antibiotic was graded as either resistant or susceptible according to Kim *et al.* [20].

Hemolytic activity

To perform the hemolytic activity test, the selected LAB strain was cultivated by its streaking on Columbia blood agar plates containing 5% of fresh sheep blood. The plates were incubated for 48 h at 37°C. As suggested by Maragkoudakis *et al.* [21], the production of green-hued zones around the colonies or the absence of any effect on the blood plates are both considered as a negative result, revealing that the cultivated strain is nonhemolytic. However, the formation of blood-lysis zones around the colonies are considered as positive results, and the cultivated strain is therefore classified as hemolytic, or having the ability to perform β-hemolysis.

Chromatographic identification of the produced biological agent

An attempt to partially identify the produced biological agent responsible for the antimicrobial effect, exhibited by the isolated *E. faecium* MN32 culture, was performed using the reversed phase high-performance liquid chromatography (RP-HPLC) analytical method, described by Zamanova *et al.* [22] as well as Kim *et al.* [23], as follows: aliquots of the fermentation broth (FB) of 24-h old culture of the selected isolate were centrifuged at 6000 rpm for 10 min at 4°C. The collected supernatants were filtered using a membrane filter (pore size 0.45 µm; Sartorius, Goettingen, Germany) to obtain a cell-free supernatant (CFS). The latter was injected at room temperature into a RP-HPLC system (Dionex Ultimate 3000, Sciencix, Brunsville, USA) coupled with ultimate 3000 variable wavelength ultrasound–visible detector (Dionex Corporation, Sunnyvale, California, USA), and equipped with a C18 150 mm×4.6 mm with particle size 5 µmol/l, using 0.01 mol/l phosphoric acid solution (pH 2.5) as the mobile phase at a flow rate of 1.0 ml/min, and an injection volume of 10 µl.

Studies of some physiological parameters on growth and antifungal activity of *E. faecium* MN32

(1) Effect of different incubation temperatures and salinity percentages on the growth of *E. faecium* MN32:

The effect of different incubation temperatures on the *E. faecium* MN32 growth rate was determined by incubating triplicate Petri dishes of the tested isolate, cultivated by streaking on the surface of MRS medium, for 24 h, at different degrees of temperature ranging between 15 and 45°C. The growth ability of the tested isolate, for this incubation period, at each of these incubation temperatures was recorded.

Moreover, the ability of the tested isolate to sustain different percentages of salinity was also spectrophotometrically determined by monitoring its growth in MRS broth supplemented with different concentrations of NaCl ranging between 4 and 8%, at 600 nm (Lambda EZ 201; Perkin Elmer, Houston, Texas, USA) over a period of 48 h. The viability of the cells was additionally tested each 24 h by their subculturing on MRS plates and their subsequent cultivation for 24 h at 37°C.

(2) Effect of different inocula types and ages on the antifungal activity of *E. faecium* MN32:

The antifungal activity of the selected isolate, *E. faecium* MN32, of different culture ages against the common phytopathogen *F. solani* was evaluated using the Poison Food Technique, previously described by Wang *et al.* [24], and Bousonet *et al.* [25], as follows:

the growth of the *E. faecium* MN32 cells was first initiated by their cultivation in MRS broth medium (Difco) at 37°C for three different incubation periods of 24, 48, and 72 h. At the end of each incubation period, the obtained FB and the CFS, obtained by cooling centrifugation of the culture at 6000 rpm for 10 mn (Sigma, laborzentrifugen, Germany), were tested by their separate addition to triplicates of sterile, melted aliquots of 25 ml PDA medium, in the final concentrations that ranged from 0.5 to 1.5% (v/v) and 1 to 10% (v/v), respectively. The aliquots of seeded PDA medium were then individually poured in 9 cm large sterile Petri dishes and left to solidify. After that, discs of 0.5 cm in diameter were cut from 5- to 7-day-old cultures of *F. solani*, previously cultivated in PDA at 30°C, and transferred to the center of each of these plates. Moreover, control plates, containing the same volume of unseeded PDA medium, were also provided with central *F. solani* discs. The plates were incubated at 30°C for a period of 2–6 days, until the fungus growth in the control plates almost reached the external walls of the Petri dish. At this stage, the radial growths of the tested fungus in both treated and control Petri dishes were measured diametrically in perpendicular directions. The percentage of growth inhibition (I%) was calculated using the following formula: $I(\%) = [(C - T)/C] \times 100$. Accordingly, the corrected inhibition (IC%) was then calculated as follows:

$$IC(\%) = [(C - T)/(C - C_0)] \times 100.$$

Where:

C: diameter of the fungal radial growth in the control Petri dishes.

T: diameter of the fungal radial growth in Petri dishes containing PDA medium seeded with the tested bacterial cells.

C_0: diameter of the fungal agar disc placed in the center of each Petri dish (0.5 cm).

Application of the *in vitro* experimental results on the greenhouse scale

The effect of *E. faecium* MN32 culture broth as a bioagent and/or a growth stimulator was tested on the growth of *P. vulgaris* L. plants, either free of or intentionally infected by *F. solani* cells, a phytopathogen that commonly infects the roots of common beans. The experiment was carried out over the period of 40 days during the two successive common bean winter agricultural seasons of October–December in 2018 and 2019 on 'Polista' cultivar of common bean, *P. vulgaris* L., plants in a greenhouse, at the Central Lab of Organic Agriculture, Agricultural Research Center, Giza, Egypt.

Cultivation conditions and investigated treatments

The experiment was conducted in pots of 25 cm in diameter and 20-cm depth. The pots were maintained inside a greenhouse, where temperature and humidity conditions were controlled. Approximately 10 kg of a sterile mixture of soil and peat moss in equal ratio was added in each pot and then sowed with five seeds of common bean (var. Polista). The pots were then divided into eight groups, designated as T1–T8, and each group was treated differently according to the investigated treatments as follows:

T1: free of *F. solani* and irrigated with pure water (negative control).
T2: inoculated with *F. solani* and irrigated with pure water (positive control).
T3: inoculated with *F. solani* and treated with *E. faecium* MN32 culture broth in the concentration of 5 l/Fed.
T4: free of *F. solani* and treated with *E. faecium* MN32 culture broth in the concentration of 5 l/fed.
T5: inoculated with *F. solani* and treated with *E. faecium* MN32 culture broth in the concentration of 10 l/fed.
T6: free of *F. solani* and treated with *E. faecium* MN32 culture broth in the concentration of 10 l/fed.
T7: inoculated with *F. solani* and treated with *E. faecium* MN32 culture broth in the concentration of 15 l/fed.
T8: free of *F. solani* and treated with *E. faecium* MN32 culture broth in the concentration of 15 l/fed.

Three replicates were used for each treatment. All the pots received the standard agricultural practices that are normally used for common bean cultivation including soil fertilization, irrigation, and pest control, except for the tested pathogen, *F. solani*, which was inoculated in some of the tested groups as previously mentioned. Approximately 5 g of sterile corn sand meal (CSM) medium was added to each of the pots not inoculated with *F. solani*.

Preparation of *Enterococcus faecium MN32* treatment suspensions

E. faecium MN32 cells were cultivated for 24 h on a large scale, by their scaling up inoculation, in large flasks containing MRS broth medium. The culture broth, containing 10^9 CFU/l, was added to of the irrigation water that was used to irrigate the tested

common bean pots, a single time at the beginning of the experiment, in three different concentrations of 0.5, 1, and 1.5 ml/pot corresponding to 5, 10, and 15 l/fed.

Preparation of *Fusarium solani* inocula

Inocula of *F. solani* cells were prepared according to the method of Abd El-Moity [26] as follows: conical flasks, each containing 50 g of CSM medium, composed of 25 g corn and 7 kg sand moisturized using 1 l of 0.2% peptone solution, were sterilized by autoclaving and then inoculated with 0.5-cm discs, cut from 5- to 7-day-old cultures of *F. solani*, previously cultivated in PDA at 30°C. The inoculated flasks were then incubated at 28°C for 15 days, after which, their cell concentrations were adjusted at 30×10^6 CFU/g, only by adding sterile CSM medium and mixed thoroughly. The resulting powder was eventually used as inocula of *F. solani*.

Inoculation of pots with *Fusarium solani* inocula

Pot's inoculation was performed by mixing about 5 g of the previously prepared *F. solani* inoculum powder, containing ~30×10^6 CFU/g, to the soil content of each pot followed by the sowing of the common bean seeds and cultivation as previously described. As previously mentioned, the pots not inoculated with *F. solani* were provided with approximately 5 g of sterile CSM medium.

Disease assessments

Disease scale

Depending on visible symptoms, scale from 0, which refers to healthy roots, to 5, which refers to severely infected roots, was recorded to measure the degree of plant infection in all the treated plants in the greenhouse (Fig. 1).

Disease incidence

Common bean plants were rated for disease incidence, that is, presence or absence of *F. solani* infection. The disease percentage of each treated plant was measured using the following formula:.

$$\mathrm{DI}\% = n/N \times 100$$

Where n=number of infected plants and N=total number of examined plants.

Disease severity index

Root rot severity was assessed 40 days after planting using a rating scale from 0 to 5, where 0 = no visible symptoms and 5 = dead plants. For each replicate, disease severity percentage was calculated as described by Liu *et al*. [27] using the following formula:

Disease severity index (DSI %)=$\sum$ (rating number×number of plants in the rating) total number of plants×highest rating×100.

Agricultural characteristics

At the end of each growing season, different plant agricultural characteristics were recorded to assess the plant vigor before and after its treatment with different concentrations of *E. faecium* MN32 culture broth in the presence or absence of the *Fusarium* sp. infection. These characteristics include plant height (cm), branches number, as well as the total fresh and dry weights (g) of the plant.

Results and discussion

Isolation and preliminary identification of lactic acid bacteria

A total of 40 isolates were recovered from the two soil samples collected from the two Egyptian farms, as

Figure 1

Disease scale of *Fusarium solani* f. sp. *phaseoli* infection of common bean roots.

previously described in the materials and methods section. These isolates were grown on the MRS agar medium [13]. It was reported that MRS is the best medium for the secretion of antifungal secondary metabolites [28], a semispecific medium for LAB forming colonies of more or less identical morphological shapes that could be described as raised, convex, smooth, and shiny. Gram staining followed by microscopic examination revealed that only four isolates were Gram negative and 36 isolates were Gram positive but genotypically different as they appeared as rods, short rods, or cocci in shape. However, these 36 isolates also showed negative catalase activities and were therefore primarily identified as LAB.

Antibacterial activity of preliminarily identified lactic acid bacteria strains

The selected 36 isolates, preliminarily identified as LAB, were tested using the agar well diffusion method against different gram positive and negative bacterial test strains as previously mentioned in the materials and methods section. The results illustrated in Table 1 showed that 17 isolates exhibited satisfactory antimicrobial activities only against the Gram negative, most common bacterial phytopathogen, *P. carotovorum* (Fig. 2), and that four isolates were solely effective against the Gram positive, *S. aureus*, as they resulted in clear inhibition zones of diameters ranging between 0.4 and 0.8 cm. However, only one isolate, designated as isolate 32, showed relatively high antimicrobial activity

Table 1 Antibacterial activity of the 36 isolates preliminarily identified as lactic acid bacteria

	Diameter of inhibition zone (cm)			
Isolate number	*Bacillus subtilis*	*Staphylococcus aureus*	*Pectobacterium carotovorum*	*Escherichia coli*
3	ND	ND	0.6±0.1	ND
4	ND	ND	0.6±0.1	ND
5	ND	0.4±0.2	ND	ND
6	ND	ND	ND	ND
7	ND	ND	ND	ND
8	ND	ND	ND	ND
9	ND	0.4±0.2	ND	ND
10	ND	ND	ND	ND
11	ND	ND	ND	ND
12	ND	ND	ND	ND
13	ND	ND	ND	ND
14	ND	ND	ND	ND
15	ND	ND	ND	ND
16	ND	ND	ND	ND
17	ND	0.6±0.1	ND	ND
18	ND	0.6±0.1	ND	ND
19	ND	ND	ND	ND
20	ND	ND	ND	ND
21	ND	ND	0.6±0.1	ND
22	ND	ND	0.6±0.2	ND
23	ND	ND	0.6±0.1	ND
24	ND	ND	ND	ND
25	ND	ND	0.8±0.1	ND
26	ND	ND	0.8±0.1	ND
28	ND	ND	0.6±0.1	ND
29	ND	ND	0.4±0.1	ND
30	ND	ND	0.6±0.2	ND
31	ND	ND	0.4±0.2	ND
32	ND	0.8±0.2	0.8±0.2	ND
33	ND	ND	0.4±0.2	ND
34	ND	ND	0.4±0.2	ND
35	ND	ND	0.6±0.2	ND
36	ND	ND	0.4±0.2	ND
37	ND	ND	ND	ND
38	ND	ND	0.8±0.1	ND
39	ND	ND	0.4±0.1	ND

Mean±SD (n=3). ND, not detected.

as clear inhibition zones of about 0.8 cm were formed when the isolate was tested against both of the two aforementioned test microorganisms.

Antifungal activity of preliminarily identified lactic acid bacteria strains

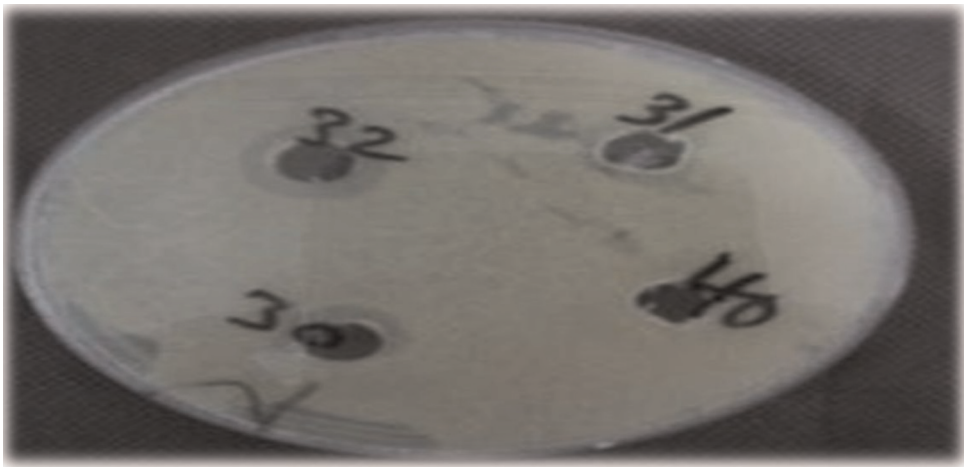
The 18 isolates, which showed positive activities against the plant bacterial phytopathogen *P. carotovorum,* were again tested against the five different most common phytopathogenic fungi previously mentioned in the material and method section. The test was conducted as previously described, and the results showed that only isolate 32 was able to obviously inhibit the growth of both *F. solani and F. graminearum,* showing a visible antagonistic effect between the growth of the bacterial isolate and that of the tested fungi (Fig. 3). On the contrary, all the 18 tested bacterial isolates failed to affect the growth of the other three tested fungal strains. Isolate 32 was therefore selected for further studies.

Molecular identification of the selected lactic acid bacteria isolate 32

The selected LAB isolate 32 was genetically identified using the 16 S rDNA sequencing technique described previously. As stated before, after genomic DNA isolation, the 16 S rDNA region was amplified using PCR, and then 20 µl of PCR product was visualized by agarose gel electrophoresis under ultrasound light. Figure 4 obviously shows the length of amplified product, visualized at 1500 bp.

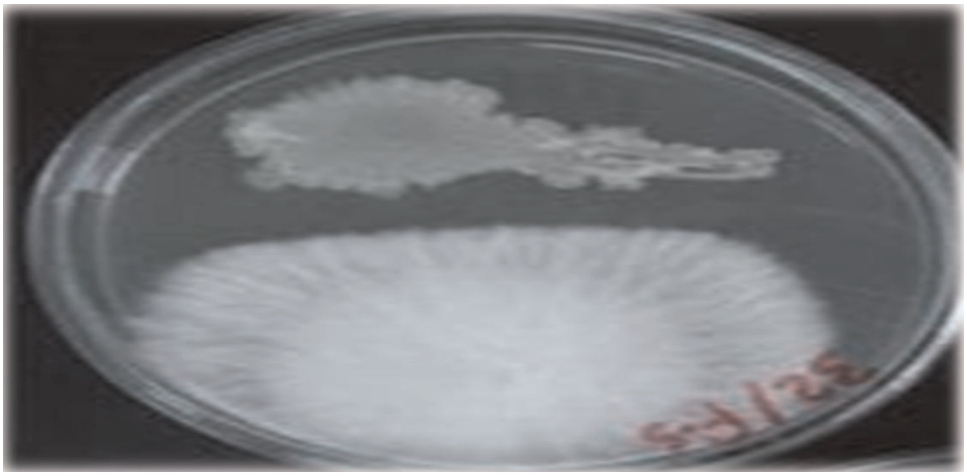
Moreover, the preliminarily bioinformatics analysis of the partial gene sequence showed that when matched with the nucleotides sequences, present in the database,

Figure 2



Antibacterial activity of isolates 30, 31, 32, and 40 tested against *Pectobacterium carotovorum.*

Figure 3



Antagonistic effect between the growth of isolate 32 and that of *Fusarium solani.*

isolate 32 was found to have very high genetic similarities, of 99%, with *E. faecium* strain DSM 20477 (Accession number: MH544640.1), *E. faecium* strain LM1.6. (Accession number: MH517442.1), *E. faecium* strain HPRTGL206 (Accession number: MH393916.1), *E. faecium* strain HBUAS54105 (Accession number: MH473334.1) and *E. faecium* strain HBUAS54092 (Accession number: MH473321.1). The isolate 32 was therefore named *E. faecium* strain MN32 and granted the accession number of MH507191 on the Gene Bank site.

Safety assessment of *Enterococcus faecium* MN32

Susceptibility to antibiotics

Antibiotic susceptibility profiles of the tested strain of *E. faecium* MN32, as illustrated in Table 2 and in Fig. 5, revealed that this strain is susceptible to all of the six tested antibiotics to a different extent, as clear inhibition zones of diameters ranging between 0.9 and 2 were detected. This result indicates that this strain can be safely used by human beings and animals and can consequently be used in agricultural or any other applications that deal with their welfare.

Hemolytic activity

No changes were detected in the color surrounding the colonies of *E. faecium* MN32 when grown on blood agar medium, which indicates the absence of hemolytic activity and consequently proves that the tested bacterium does not have any blood-lysis ability. According to that, this result also confirms the safety of this strain to be used in different applications involving human beings and animals.

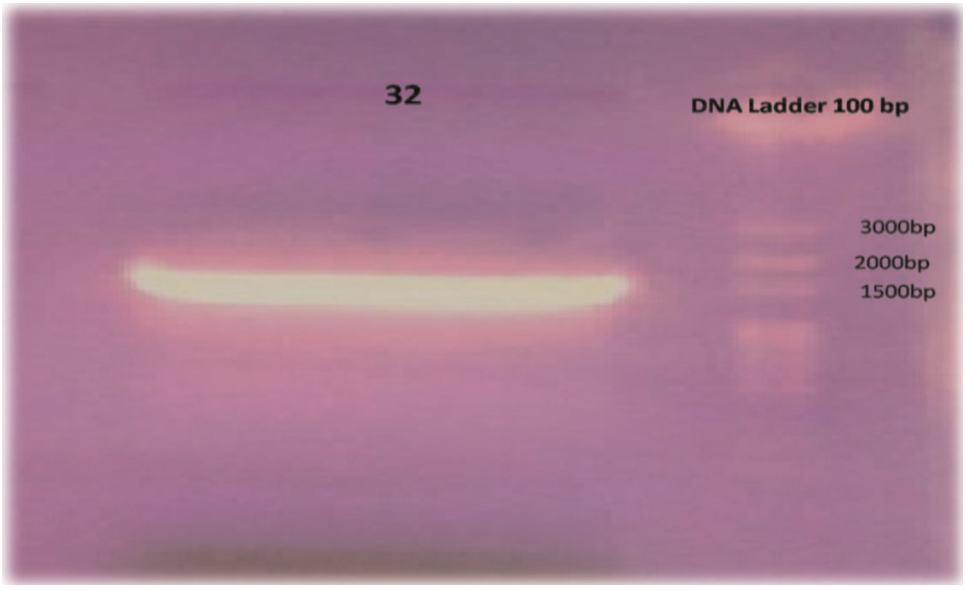
Safety assessment of the produced biological agents via reversed phase high-performance liquid chromatography analysis

The result of the chromatographic analysis proved the presence of different organic acids, mainly including lactic and acetic acids in the concentrations of 4.5 and 6.5 g/l, respectively, in the FB. The concentrations of both of these acids were calculated using calibration curves performed using their standard solutions [29]. However, the analysis also revealed the absence of fumaric acid as it was not detected in the FB. As both lactic and acetic acids are safely consumed by most human beings in moderate concentrations, as they are the major constituents of vinegar and dairy products, the RP-HPLC analysis results were considered as an additional verification for the safe use of *E. faecium* MN32 culture filtrate in any human being-related applications.

Table 2 Antibiotic susceptibility profile of *Enterococcus faecium* MN32

Antibiotic	Inhibition zone diameter (cm)
Vancomycin	1.9
Fusidic acid	2
Ampicillin	1.5
Kanamycin	0.7
Erythromycin	1.5
Tetracycline	0.9

Figure 4



The length of amplified product at 1500 bp.

Studies of some physiological parameters on growth and antifungal activity of *E. faecium* MN32:

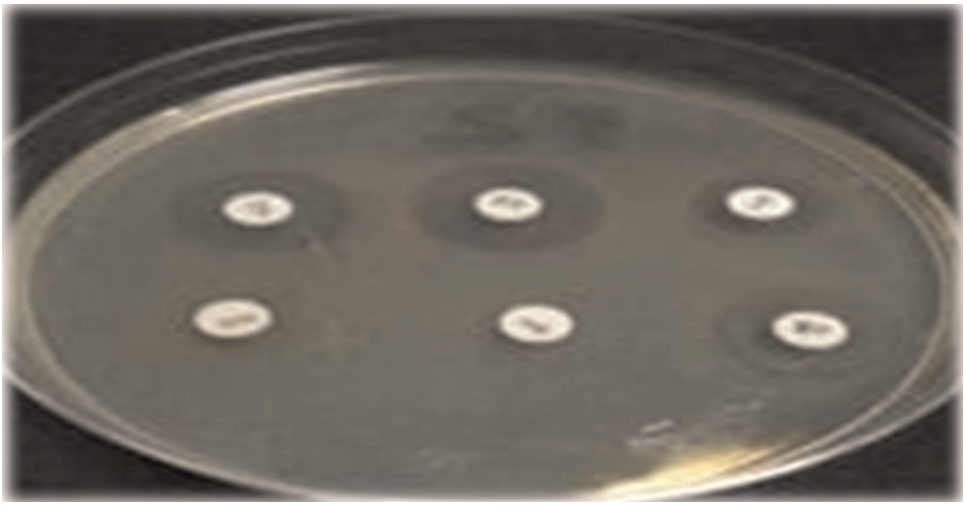
(1) Effect of different incubation temperatures and salinity percentages on the growth of *E. faecium* MN32:

When cultivated in MRS medium for 24 h, *E. faecium* MN32 was found to have the ability to grow on a wide range of temperatures, ranging from 15 to 45°C. Moreover, this isolate was also found to sustain high percentages of salinity AS significant growth was detected when the tested microorganism was cultivated for both 24 and 48 h, at 37°C, in MRS broth medium containing different NaCl concentrations ranging from 4 to 8% (w/v). These results confirm the effective use of this strain in different applications involving possible changes in both temperature and salinity, such as in the agricultural field.

(2) Effect of different inoculum types and ages on the antifungal activity of *E. faecium* MN32:

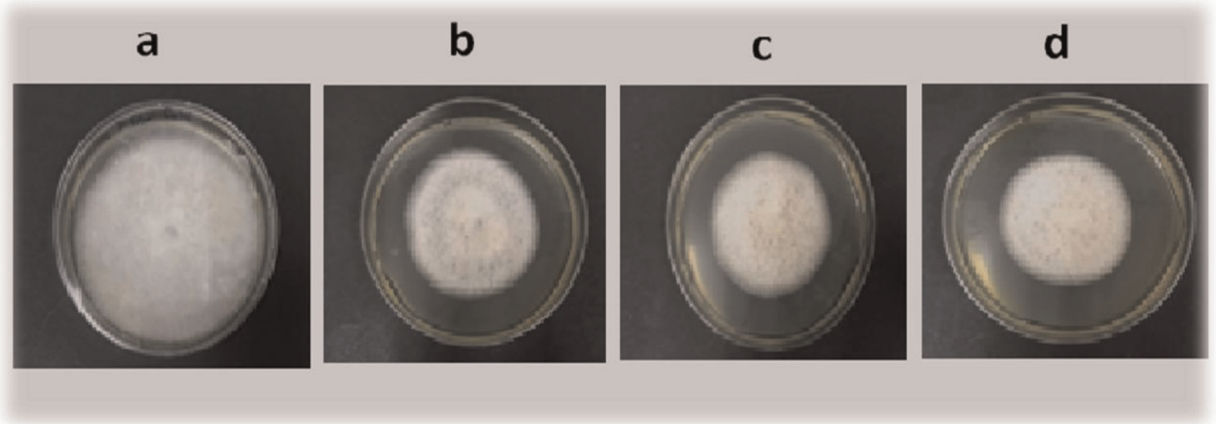
The antifungal activities of both of the CFS and FB of *E. faecium* MN32 cultivated for three different incubation periods of 24, 48, and 72 h were investigated, and the results were compared WITH the control plates in which the growth of the untreated *F. solani* fungus almost covered the entire available area of the PDA medium previously poured in the Petri dish (Fig. 6a). The experiment was performed according to the Poison Food Technique, described previously. The results illustrated in Table 3 showed that when the CFS of the 24-h-old tested isolate was incorporated in the

Figure 5



Antibiotic susceptibility profile of *Enterococcus faecium* MN32 showing the effect of Vancomycin, Fusidic acid, Ampicillin, Kanamycin, Erythromycin, and Tetracycline hemolytic activity.

Figure 6



Growth of *Fusarium solani* on: (a) control PDA, or PDA supplemented with 10% CFS of *Enterococcus faecium* MN32 previously incubated for (b) 24 h, (c) 48 h, and (d) 72 h. CFS, cell-free supernatant; PDA, potato dextrose agar.

medium in different concentrations ranging from 1 to 10%, the percent growth inhibition of *F. solani* sharply increased from about 5 to more than 31%. However, when the CFS of a 72-hr.-old culture was used, higher results of about 7 to about 35% inhibition were obtained, correspondingly (Fig. 6).

On the contrary, the addition of the 24-hr-old *E. faecium* MN32 FB in the medium in different concentrations that ranged from 0.5 to 1.5% resulted in a much higher percent of growth inhibition that reached about 31.25 to more than 51%, correspondingly. Slightly higher growth inhibition percentages, ranging from 35.75 to about 55%, correspondingly, were also recorded when the incorporated FB was 72 h old.

These results correlated with the results of the final pH of the FB, which was found to gradually shift toward the acidic range as the incubation period was increased from 24 to 72 hr, revealing the gradual increase in the organic acids production ability of the cultivated bacterial strain, and eventually explaining the increase in the antifungal activity in the same respect.

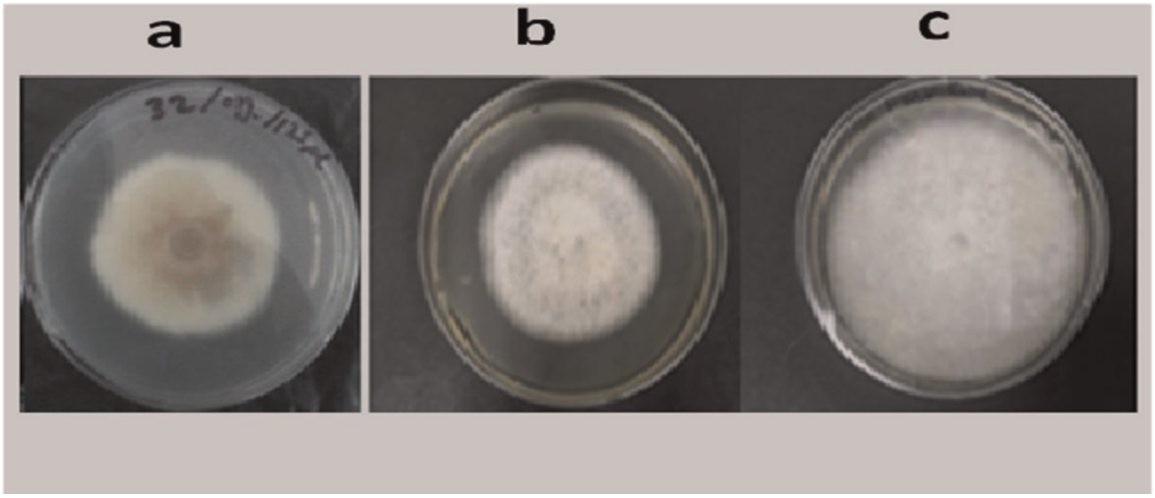
However, the increase in the antifungal activity that resulted from the use of the complete FB compared with that recorded when only the CFS of the same bacterial strain was used was apparently much greater (Fig. 7a and b), as the same fungal inhibition percent of ~31% was obtained when the PDA medium was inoculated with either 0.5% of FB or 10% of CFS. This result could be attributed to the growth of the

Table 3 Effect of different inoculum types and ages on the antifungal activity of *Enterococcus faecium* MN32

	Inoculum age (h)								
	24			48			72		
Inoculum type	Growth diameter (cm)	I%	IC%	Growth diameter (cm)	I%	IC%	Growth diameter (cm)	I%	IC%
Control	8.0	0	0	8.0	0	0	8.0	0	0
CFS conc. (v/v)									
1%	7.65	4.97	5.30	7.55	6.21	6.62	7.5	6.83	7.28
2%	7.65	4.97	5.30	7.4	8.07	8.61	7.4	8.07	8.61
4%	7.73	4.04	4.31	7.25	9.94	10.60	7.1	11.80	12.60
6%	7.7	4.35	4.64	7.05	12.42	13.24	7.0	13.04	13.91
8%	6.9	14.28	15.23	6.5	19.25	20.53	6.3	21.74	23.18
10%	5.55	31.06	33.11	5.3	34.16	36.42	5.25	34.78	37.09
FB conc. (v/v)									
0.5%	5.5	31.25	41.2	5.33	33.38	43.2	5.14	35.75	45.4
1%	4.53	43.4	52.6	4.37	45.37	54.5	4.21	47.38	56.4
1.5%	3.91	51.12	59.9	3.73	53.37	62.0	3.61	54.88	63.4
Final pH	5.9			4.87			4.75		

CFS, cell-free supernatant; FB, fermentation broth; I, inhibition; IC, inhibition corrected.

Figure 7



(a) Growth of *Fusarium solani* in the presence of 0.5% of the FB of *Enterococcus faecium* MN 32, (b) growth of *F. solani* in the presence of 10% of the CFS of *E. faecium* MN 32, (c) growth of *F. solani* on PDA medium (control Petri dish). CFS, cell-free supernatant; FB, fermentation broth; PDA, potato dextrose agar.

different amounts of added cells included in the FB, when this type of inoculum was used to inoculate the PDA plates at different concentrations. These cells eventually had the opportunity to produce additional amounts of organic acids during the extended incubation period that followed the inoculation of the plates with the central discs of *F. solani*, as previously explained, consequently resulting in a much higher fungal inhibition results than that obtained by the addition of the CFS that contained definite amounts of the antifungal bioagents. Figure 7a obviously shows the opacity of the PDA medium surrounding the fungal colony, indicating the apparent growth of the *E. faecium* cells, as compared with the much clearer PDA medium that appears in Fig. 7b, and this observation greatly supports the aforementioned elaborated explanation.

On the contrary, it was clearly illustrated that the increase in the antifungal activity, which resulted from increasing the incubation period from 24 to 72 h, could be described as insignificant. Therefore, taking into consideration the economic cost for the application of these findings on a larger scale, the use of a 24-hr.-old culture broth of *E. faecium* MN32, for the growth inhibition of the *F. solani* phytopathogen, is recommended.

These results closely correlate with that of several other studies that have previously reported the broad and narrow inhibitory spectrum of LAB against a broad range of filamentous such as *Aspergillus nidulans*, *Penicillium commune*, *Fusarium sporotrichioides*, *B. cinerea*, *Glomerella cingulate*, *Phytophthora drechsleri Tucker*, *Penicillium citrinum*, *Penicillium digitatum*, and *Fusarium oxysporum* [27,30,31].

Application of the *in vitro* experimental results on the green house scale

Upon comparing the plants infected with *F. solani* and treated with different concentrations of *E. faecium* MN32 culture broth, equivalent to 5, 10, and 15 l/fed (groups T3, T5, and T7, respectively), in both growing seasons S1 and S2, the results illustrated in Fig. 8 showed that the use of the concentration of 10 l/fed resulted in the lowest disease incidence percentages of 40 and 53%, respectively (Fig. 8a), the lowest result of disease scale of approximately one in both seasons (Fig. 8b), as well as the lowest disease severity results of 8 and 10%, respectively (Fig. 8c).

These results were considered very promising compared with the drastic results obtained from the plants of group T2, referred as the positive control, i.e., infested with *F. solani* and untreated with the tested FB, which showed the highest result of disease incidence percentage of about 75% in both seasons (Fig. 8a), the highest results of disease scale of approximately four and five for each season, respectively, (Fig. 8b), as well as the highest disease severity result of about 65% in both seasons (Fig. 8c).

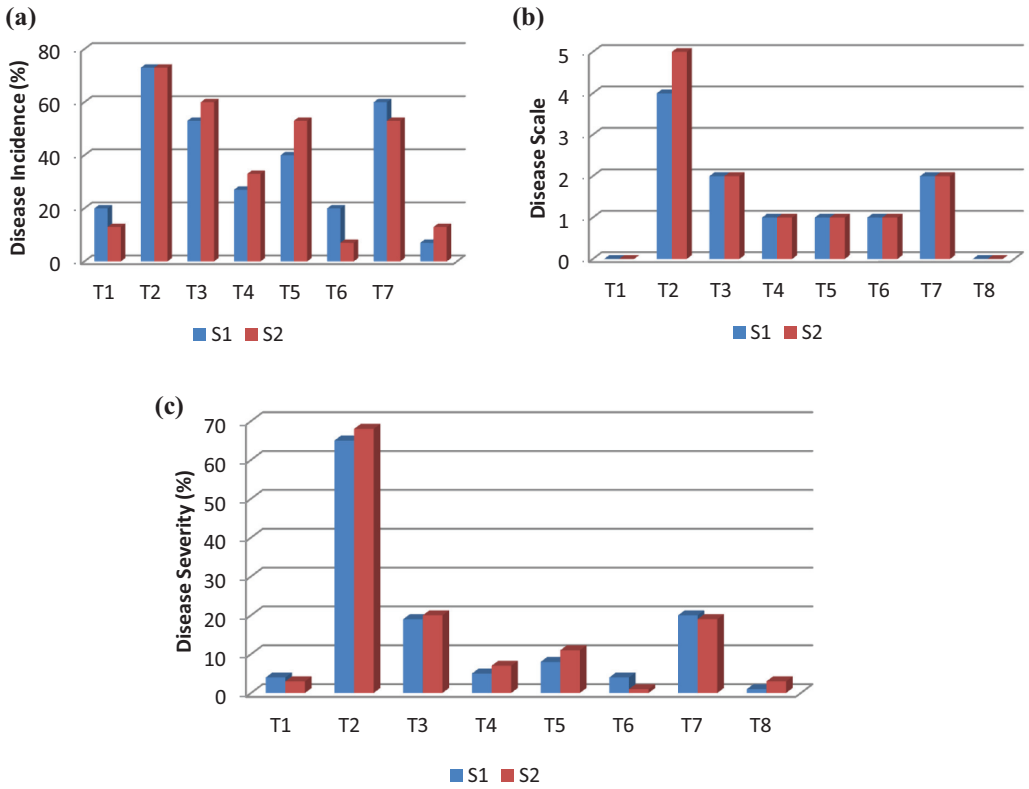
Moreover, examination of the agricultural characteristics of the infested plants of the aforementioned groups revealed obvious improvements in their growth parameters after their treatment with the same concentration of the *E. faecium* MN32 culture broth of 10 l/fed compared with the positive control T2. The results obtained from Table 4 show that the plant height increased from about 23 to more than 31 cm, the fresh weights of the plant increased by almost the double, and the dry weights increased from about 0.8 g to more than 1.65 g.

On the contrary, the effect of *E. faecium* MN32 culture broth as a growth stimulator was also tested on the plants uninfected with *F. solani* but treated with *E. faecium* MN32 culture broth, in the concentrations equivalent to 5, 10, and 15 l/fed (groups T4, T6, and T8) and compared with the vigor of the plants belonging to group T1, referred as the negative control, i.e., noninfested with *F. solani* and untreated with the tested culture broth. Results from Table 4 show that upon treating the aforementioned pots with the selected isolate culture broth in the concentration equivalent to 10 l/fed, the plants' heights increased from about 26 to about 31.5 cm, the number of branches increased from 5 to 9, but, more importantly, the fresh and dry weights of the plants increased from more or less 7.5 to about 18 and from 1.07 to about 2.75, thus proving that the plant vigor was significantly promoted when treated with the previously described FB.

However, although the plants of these previously mentioned groups (T1, T4, T6, and T8) were not infested with *F. solani*, they were inevitably subjected to other diseases that were obviously kept under control as a result of their treatment with *E. faecium* MN32 culture broth in concentrations of 10 or 15 l/fed (Fig. 8).

These results were in accordance with those of Hamed and colleagues and Abdel-Aziz *et al.* [6,28,32], who studied the effect of LAB on the characteristics of some plants.

Figure 8



T1 (Negative control): (-)*Fusarium solani*, (-)*E. faecium* FB;
T2 (Positive control): (+)*Fusarium solani,* (-)*E. faecium* FB;
T3: (+)*F. solani*, (+5L/Fed) *E. faecium* FB;
T4: (-)*F. solani*, (+5L/Fed)*E. faecium* FB;
T5: (+)*F. solani*, (+10L/Fed) *E. faecium* FB;
T6: (-)*F. solani*, (+10L/Fed)*E. faecium* FB;
T7: (+)*F. solani*, (+15L/Fed) *E. faecium* FB;
T8: (-)*F. solani*, (+15L/Fed)*E. faecium* FB;
S1=Season 1; S2=Season 2

Effect of the *Enterococcus faecium* MN32 fermentation broth treatment on the disease prevalence on common bean (*Phaseolus vulgaris* L.) plants, in the presence and absence of *Fusarium solani* infection, during two successive seasons S1 and S2.

Table 4 Effect of the *Enterococcus faecium* MN32 fermentation broth treatment on some growth parameters of common bean (*Phaseolus vulgaris* L.) plants, in the presence and absence of *Fusarium solani* infection, during two successive seasons S1 and S2

	Plant height (cm)		Branches number		Fresh weight (g)		Dry weight (g)	
Treatment	S1	S2	S1	S2	S1	S2	S1	S2
T1	26.11	26.34	5	5	5.39	5.63	1.07	1.07
T2	22.78	23.12	4	4	3.89	3.81	0.84	0.82
T3	25.33	25.53	4	4	4.94	5.27	0.93	0.92
T4	26.22	27.17	4	5	8.78	8.03	1.80	1.71
T5	30.00	31.74	4	6	7.11	8.00	1.67	1.64
T6	31.11	31.97	9	9	17.67	18.70	2.75	2.73
T7	27.78	28.28	5	5	6.33	7.13	1.49	1.42
T8	28.00	29.17	5	5	15.50	16.52	2.24	2.59
LSD 0.05	2.79	3.49	1.46	1.62	3.26	3.09	0.43	0.51

FB, fermentation broth. T1 (negative control): (–) *Fusarium solani*, (–) *Enterococcus faecium* FB. T2 (positive control): (+) *Fusarium solani*, (–) *Enterococcus faecium* FB. T3: (+) *Fusarium solani*, (+5 l/fed) *Enterococcus faecium* FB. T4: (–) *Fusarium solani*, (+5 l/fed) *Enterococcus faecium* FB. T5: (+) *Fusarium solani*, (+10 l/fed) *Enterococcus faecium* FB. T6: (–) *Fusarium solani*, (+10 l/fed) *Enterococcus faecium* FB. T7: (+) *Fusarium solani*, (+15 l/fed) *Enterococcus faecium* FB. T8: (–) *Fusarium solani*, (+15 l/fed) *Enterococcus faecium* FB. S1=season 2018; S2=season 2019.

Conclusion

The results of the in-vitro experiments revealed that *E. faecium* MN32, isolated from fertile Egyptian soil and molecularly identified as a LAB, is significantly active against some bacterial and fungal phytopathogens that consistently affect economically important crops.

Furthermore, the results of the in-vivo greenhouse tests demonstrated that the use of the FB of the locally isolated LAB as bioagent, to control plant pathogens, as well as an effective growth stimulator, could be a very promising approach to reduce not only the economic losses in agricultural crops but also the extensive use of hazardous chemicals and toxic pesticides.

Future experiments should target the immobilization of the lactic acid-producing bacteria for the sustainable release of the bioagent at different growth phases, and eventually the production of an eco-friendly, broad-spectrum bioagent on a commercial scale.

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Conflicts of interest

There are no conflicts of interest.

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