Investigation of some Endophytic Bacteria as Biocontrol Against of Root–rot Pathogens

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Abstract: Microbial inoculants are an essential tool for increasing arable land productivity and reducing the application of mineral fertilizers. The purpose of this work was to isolate and identify endophytic antagonistic bacteria from the roots of medicinal plants and to assess their antifungal activity, and plant growth-promoting traits. *Bacillus subtilis* and *Proteus mirabilis* were isolated from roots of two native medicinal plants (*Urginea martime* and *Atriplex lindlyi*) from flora Arish and characterized by 16S rRNA gene sequencing. *In vitro*, El antagonistic activity experiments against (*Fusarium oxysporum* and *Rhizoctonia solani*) were evaluated. Our results revealed that the percentage of growth inhibition by *B. subtilis* and *P. mirabilis* were 96% and 78.5 % against *R. solani* followed by 85.3 % and 77% against *F. oxysporum*, respectively. we investigated by Screening electron microscope (SEM). Both endophytic bacteria *B. subilits* and *P. mirabilis* produced protease and chitinase. while also producing sidrophores and ammonia, but *P. mirabilis* capable to phosphate solubilization, and *B. subtilis* is fixing N_2. That promotes plant growth and facilitates bio control. These findings imply that this bacterial strain offers excellent protection against diseases of various agriculturally significant plants via direct and indirect modes of action.

Keywords: Endophytic bacteria, *F. oxysporum*, *P. mirabilis*, medicinal plants.

INTRODUCTION

Soil-borne fungal infections are difficult to control because of their ability to live in soil for an extended period, and the casing pathogens are among the most significant challenges farmers face. We encourage integrated approaches that employ microbes as bio control agents. In recent decades, environmentally friendly control methods for soil-borne diseases have become an alternative to standard chemical treatments in many agricultural systems (Heydari and Pessarakli, 2010; Alamri et al., 2019). Endophytic bacteria are well-known for their potential growth-promoting properties in plants. Many Bacillus species isolated from varied hosts were capable of promoting plant development via the synthesis of ACC (deaminase), IAA, siderophore, and phosphate-solubilizing enzymes (Ijaz et al., 2019 and Amaresan et al., 2021).

The biochemical characterization of bacterial strains based on hydrolytic enzymes such as chitinase revealed that Bacillus strains could produce this enzyme (Sarwar et al., 2020), their essential function in phytopathogen well degradation, and plant bio protection. B. subtilis has been examined extensively as a potential biological control agent against many fungal plant diseases, and it has produced a variety of PGP traits and hydrolytic enzymes (Chen et al., 2016).

MATERIALS AND METHODS

Isolation of endophytic bacteria from plant samples: Two healthy medicinal plants species (*Urginea martime* and *Atriplex lindlyi*) were collected from Al Arish, in November 2019, recognized the plant species in the herbarium of Faculty of Science, Suez Canal University plants were gathered and put in sterilized bags then in an ice box. laboratory, and kept there for further studies at 4 ºC. Root's samples were completely washed in distilled water and then submerged for 2 mints in 70% ethanol, they underwent three rounds of sterile distilled water rinsing than cut into 3cm. According to (Patriquin et al., 1987) put cutting root in tube 10 ml Nutrient broth for 10 days at 28 ºC.at incubator shaker. Serial dilutions of the root suspension (100µl) were plated onto Nutrient agar (NA) and incubated at 28 ºC for 2 days. The individual colonies were picked up with a sterile loop and placed on fresh NA slants.

Antagonistic Activity (*In Vitro)*: All isolates tested against *R. solani* and *F. oxsporum* fungi. The phytopathogenic fungi were taken from stock reference cultures kept in the phytopathology laboratory at Suez Canal University faculty of agriculture. They were evaluated against these phytopathogenic fungi on PDA plates, which were streaked on the two edges of plates (one cm away from the edge). 0.9 cm mycelial discs from a culture of the tested fungus there were 3 replicates for each treatment and incubated at 28 ºC for fungal dishes as control covered all palate for maximum 7 days. For each set, we zone According to (Skidmore and Dickinson,1976) we recorded two distinct zone. According to (Vincent, 1927). Inhibition rate % = (C-T) ×100/C Where, C: Mycelial growth of pathogen in control. T: Mycelial growth of pathogen in dual plate.

Identification of Endophytic bacteria Isolates: Two refresh isolates were recorded the highest Inhibition rate send to National Center for Biotechnology in Cairo to exam 16s rRNA to identification isolates. and BLAST was used to match the nucleotide sequences to the Gen Bank database. (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Scanning electron microscopy: Changes in the phytopathogens have been seen using a scanning electron microscope and a gold-coated hyphae (*R. solani* and *F. oxysporum*). After being fixed, hyphae were coated in gold using an auto fine coater (JFC-1600) (Wu-Yuan et al.,1995).

Evaluation of Hydrolytic Enzyme Activity: The production of protease enzyme activity by both bacterial strains were evaluated by skim milk for protease according to (Zhou et al., 2009). Chitin-agar for observing chitinase activity (Berg et al., 2000). Using M9 medium which had been supplemented with yeast extract (1.2 g l-1) and cellulose (10 g l-1) (Gao et al., 2008) for cellulase activity. Treatments were performed in triplicate. Inoculated plates

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were incubated at 28 ± 2 °C for five to eight days, isolates show the presence of the clear areas around their colonies were determined to be positive for enzymes production.

Phosphate solubility: The ability to dissolve phosphate was determined according to (Pikovskaya, 1948). The endophytic strain was inoculated in Pikovskaya medium. The strains were evaluated for its ability to grow on Pikovskaya medium using tricalcium phosphate ($Ca_3(PO4)2$) as the sole source of phosphate. After seven days of inoculation at 28 °C, haloes were observed around the colonies, indicating the solubilization of inorganic phosphate.

Ammonia production: According to (Bakker and Schippers, 1987), two endophytes are cultured in 10 milliliters of peptone water then incubated for two days at 28°C. After incubation period, adding 1 milliliter of Nessler's reagent to each test tube, the positive results cause the changing colour from yellow to brown.

Siderophores production: On chrome azurol S (CAS) media, siderophore production by the endophytic strains were measured using a previously published method (Alexander and Zuberer, 1991). Bacterial strains were spot inoculated onto CAS plates, which were then incubated at 28°C for five days. The intensity of the yellow-orange halo that surrounded the plant indicated the full activity of siderophores.

Statistical analysis: One-way analysis of variance was used to assess (ANOVA). A post-hoc test was done using the results if there were significant differences in the results between the treatments. With a 5% error rate, the Duncan test. Using the SPSS program, version 25.0, statistical analysis of the data was performed.

RESULTS

Isolation, Identification and Selection of Endophytic bacteria: Two endophytic bacteria were isolated from (*Urginea martime* and *Atriplex lindlyi*) while, identified isolates as *Bacillus subitils* and *Proteus marbilis* by Using16s RNA gene sequence analysis. two strains had antifungal effect on the radial growth of *Rhizoctonia solani* and *Fusarium oxysporuim.* Data in Table (1) illustrated by Fig. (1) demonstrated that the isolate *P. mirabilia* was inhibited the linear growth of both *F. oxysporuim* and *R. solani* by rate 81% otherwise *B. subtilis* was inhibited the linear growth of *F. oxysporium* by rate 66% and *R. solani* by rate 97.6%.

Table (1) Antifungal activity of endophytes isolates against phytopathogenic fungi .

Isolate name	*F. oxysporuim*		*R. solani*	
	Average of linear growth(cm)	Inhibition%	Average of linear growth(cm)	inhibition%
Proteus marabilia	1.6	81%	1.6	81%
Bacillus subtilis	2.91	66%	0.2	97.6%

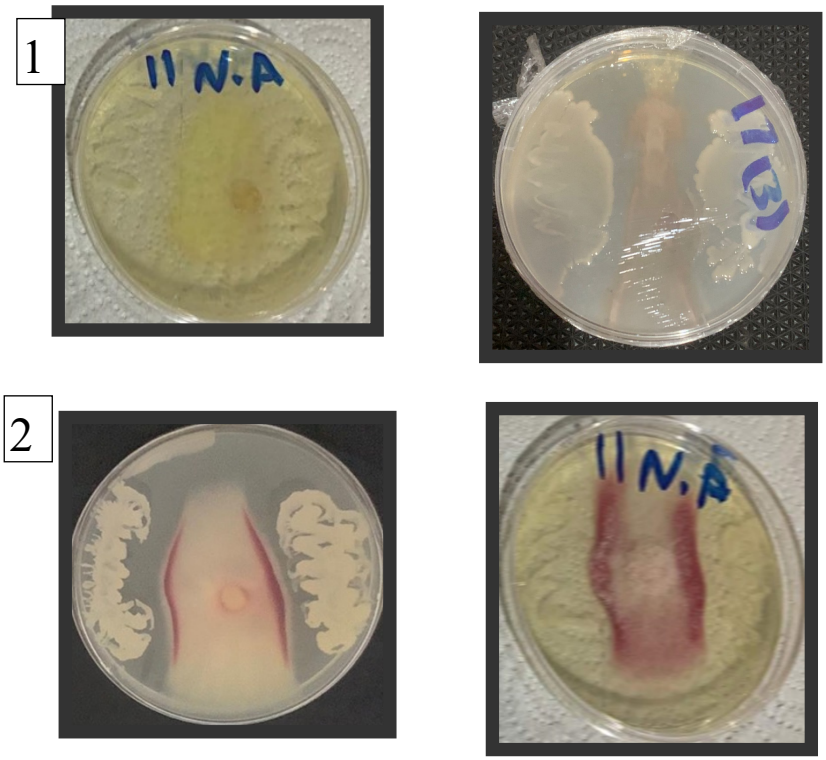

Fig. (1): Antagonism of *Bacillus subtilis* and *Proteus mirabilis* NAW7 against fungal phytopathogens: (1) *Rhizoctonia solani*; (2) *Fusarium oxysporum*

Scanning electron microscope (SEM): SEM results demonstrated that the complicated interaction between pathogens and endophytes. Hyphae of *R. solani* and *F. oxysporum* were seen to become turgid and lysis cell wall (Figure2: C, D, E and F) in comparison to manage (Figure2: A and B). Additionally, the hyphal tips' development changed and became abnormally shaped.

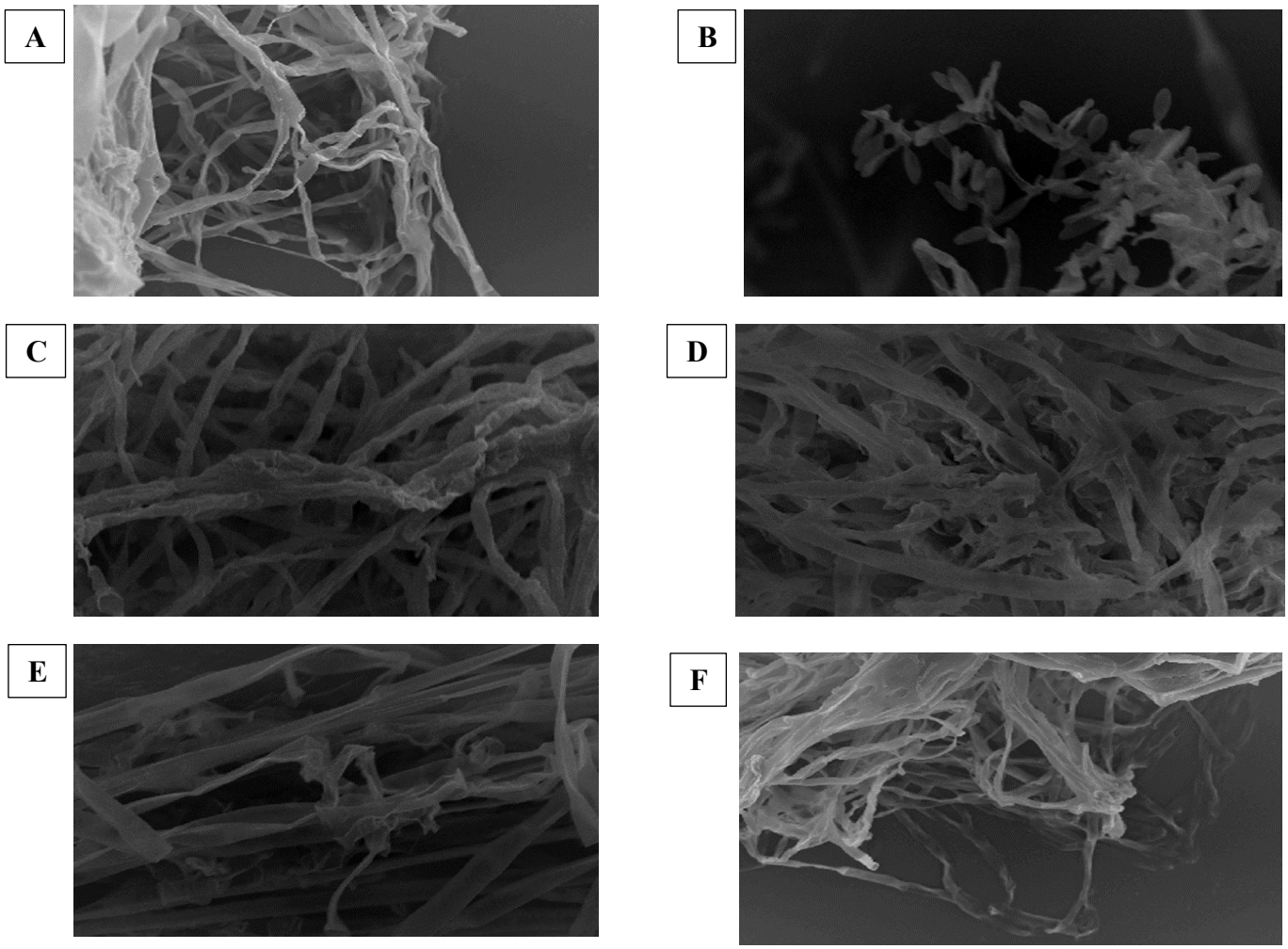

Fig. (2): Effect of *Bacillus subtilis* and *Proteus mirabilis* on *R. solani* and *F. oxysporum* growth, using scanning electron microscopy. (A) *R. solani*. (B*) F. oxysporum.* (C) *R. solani* and *B. subtilis*. (E) *R. solani* and *P. mirabilis*. (D) *F. oxysporum* and *B. subtilis*. (F) *F. oxysporum* and *P. mirabilis*

Characterization of selected bacterial strains: Many qualitative tests, such as the detection of indole acetic acid (IAA), siderophores, phosphate solubilization, and Ammonia production, were conducted to identify plant growth-promoting characteristics. In a solid PVK medium enriched with $Ca_3(PO_4)_2$ as the sole source of inorganic phosphate, the phosphate-solubilizing potential of the isolated strains *B. subtilis* and *P. mirabilis* were tested. The endophytic strains showed continued growth on media for a longer incubation time. In addition, they produced halos around the colonies, indicating the ability of the strain to utilize inorganic phosphate in the medium Figure (3, b) and Table (3). Both strains were confirmed siderophore production by using a qualitative test that showed siderophore production by a color change from blue to orange Figure (2, c) and Table (3). *P. mirabilis* and *B. subtilis* showed the ability to produce ammonia Figure (2, B). Enzymes detection of endophytic bacterial strains showed that both endophytic bacterial strains produced protease Figure (2, D) and chitinase but could not produce cellulase (Table 2).

Table (2): Production of fungal cell wall degrading enzymes by endophytic bacteria.

Isolates	Protease	Chitinase	Cellulase
B. subtilis	++	Trace (+)	-
P. mirabilis	++	Trace (+)	-

Positive weak;(++) positive strong;(-) negative(+)

Table (3): Plant growth promoting activities of antagonistic endophytic bacteria

Isolates	Phosphate Solubilization	Ammonia Production	N_2- Fixing	Siderophores
Bacillus subtilis	-	+	+	+
Proteus marabilia	+	+	-	+

(+) positive; (-) negative.

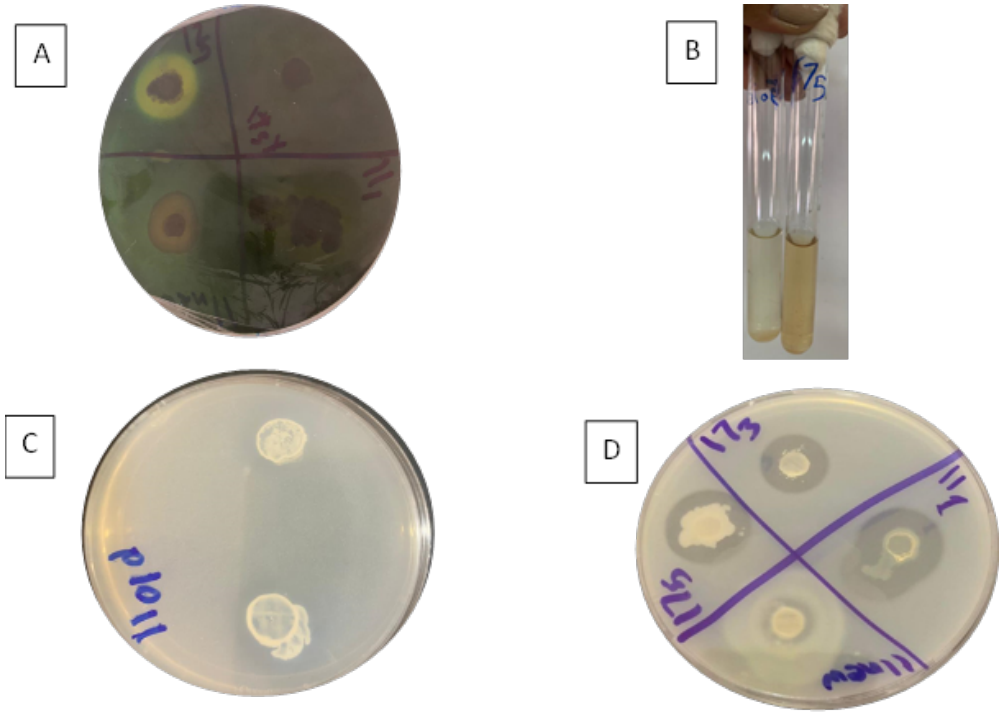

Fig. (3): Plant Growth promoting properties of the bacterial strains. **A:** Detection of siderophore production. **B: Detection** of ammonia production. **C**: Detection of phosphate solubilization **D**: Detection of protease production.

DISCUSSION

Endophytic bacteria promote host growth and enhance their ability to resist various factors, there by having a positive impact on the host, by producing bioactive metabolites with properties that fight disease and pests. They also benefit their host and prevent harm from pathogenic organisms (Gomaa, E.Z.,2021). in this study, Bacillus subtilis and Proteus mirabilis were examined to inhibit *Rhizoctonia solani* and *Fusarium oxysporuim* by reducing their radial mycelia growth. These results show that extracellular metabolites hindered the pathogen's development It is well known that bacteria produce cell wall-degrading enzymes and secondary compounds that inhibit the growth of other microorganisms. Antifungal activity of *B. subtilis* and *P. mirabilis* against various phytopathogenic fungi has been demonstrated in previous research (Durairaj et al., 2018 and A. Khan et al., 2019) investigated that *B. subtilis* strain JD-09 exhibited strong antagonism against fungal pathogen due to chitinase activity. according to Petri dish-based qualitative testing, the isolates produced protease and chitinase enzymes. The presence of two enzymes, chitinase, and protease, work as a mechanism of antibiosis against fungus. A clear zone on skim milk agar was evident for vigorous protease activity; enzyme protease possessed by two strains, in particular, play a vital role in the cell lysis process agree with, (Heydari et al., 2019). Cellulase activity was absent in the two isolates. Agree with (Beenu Shastri et al., 2020) directly linked with hyper parasitic action Mechanisms implying the beneficial plant effects. A scanning electron microscope (SEM) was employed in this investigation to determine the degree of antagonist two isolates. Interaction with fungus. The distortion and destruction of the hypha structure under SEM analysis of the fungal-antagonist interaction from dual plate antagonism. Further investigation was conducted on the effects of extracellular metabolites, including hydrolytic enzymes and secondary antifungal chemicals, which led to changes in the hypha structure. These changes included hypha swelling and mycelium distortion, as reported by (Jamali et al., in 2020). Researchers (Ijaz et al., 2019) have extensively studied the role of siderophores in bio control. In the current study, it was found that two endophytic bacteria strains produced a significant amount of siderophore, which was observed by the color change of the blue CAS agar medium around the bacterial colony to orange. It has also been demonstrated by previous studies (Carlson et al., 2020 and Heydari et al., 2019) that siderophores are

one of the main antifungal metabolites in the isolates causing antagonism. *Bacillus* spp. and *P. mirabilis* produce siderophores through their antifungal activity, which can directly and indirectly promote plant growth. The microorganism's siderophore-mediated aggressive activity chelates iron, encouraging competition between strains and pathogen. *P. mirabilis* created a halo zone in the phosphate solubilization of two isolates, which was observed. To safeguard the phytopathogen, research has focused on how various phosphate-solubilizing bacteria contribute to bio-control behavior (Kafle et al., 2019). *Pseudomonas* sp. strains work against different fungicides as efficient bio-controlling agents (Sarwar et al., 2020). In prior work, (Abdel-Motaal et al., 2020) investigated if the gluconic acid produced by phosphate solubilizing Pseudomonas spp. MR11 and MR3 could reduce rice's bacterial leaf blight (BLB) pathogen. In the present work, *P. marbilis* is a bio control agent and phosphate solubilizer. It is possible to fix atmospheric nitrogen. two strains have ability to produce ammonia, which promotes plant growth and helps plants defend themselves.

CONCLUSION

The present study emphasized the importance of the wild medicinal plant, as potential sources for isolation of beneficial true endophytic bacteria acting as plant growth promoter and a bio control agent on both pathogens root rot diseases. two isolates were identified as the most effective bio-agent their plant growth-promoting abilities were confirmed via siderophores, phosphate solubilization, N_2 fixing activities and ammonia production. The two selected bacterial isolates displayed interesting enzymatic activity (protases and chitinase). So, according to our results we can use our isolates as bio inoculants and bio fertilizer and bio control agents to reduce the use of agrochemicals and support eco-friendly crop in commercial rabbit artificial insemination.

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تقيم بعض انواع البكتريا الداخلية للمكافحة الحيوية للمسببات المرضية لعفن الجذور

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اللقاحات الميكروبية هي وسيله أساسية لزيادة الأراضي الصالحة للزراعة وزيادة الإنتاجية وتقليل استخدام الأسمدة الكيميائية. الهدف من هذه الدارسة هو عزل البكتيريا الداخلية من جذور النباتات الطبية وتقييم الكفاءة المضادة للفطريات ودراسة خصائص تعزيز نمو النبات. *Bacillus subtilis* و *Proteus mirabilis* تم عزلهما من جذور النباتات الطبية التي تعتبر من اهم الفلورا التي تتواجد في العريش (*Urginea martime* و *Atriplex lindlyi*) ، وتم التعريف الجزى للعزلتين بوسطه الجين 16s rRNA . في المختبر ،تم دراسة الكفاءة المضادة للفطريات للعزلتين ضد (*Fusarium oxysporum* and *Rhizoctonia solani*). كشفت نتائجنا أن كانت النسبة المئوية لتثبيط النمو بواسطة B. subtilis و 96٪ *P. mirabilis* و78.5٪ ضد *R. solani* تليها 85.3٪ و77٪ ضد *F. oxysporum* على التوالي. ثم تم تقيم التغيرات التي طرأت على المسببات المرضيه الفطريات بواسطة العزلات البكتريه بواسطه الميكروسكوب الإلكتروني. أنتجت كل من البكتيريا *B. subilits* و *P. mirabilis* البروتياز والكيتيناز. بينما أنتجوا أيضا الأمونيا وسيدوفورز ، لكن *P. mirabilis* قادر على إذابة الفوسفات ، و *B. subtilis* تثبيت N_2 الذي يعزز نمو النبات ويسهل المكافحة الحيوية. تشير هذه النتائج إلى أن هذه السلالة البكتيرية توفر حماية ممتازة ضد الأمراض الفطرية خصوصا مرض عفن الجذور. من مختلف النباتات ذات الأهمية الزراعية من خلال طرق المكافحة المباشرة والغير مباشرة.