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Screening of soil rhizobacteria isolated from wheat plants grown in the Marrakech region (Morocco, North Africa) for plant growth promoting activities

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and improve plant growth using a variety of direct and indirect mechanisms. These mechanisms can affect hormone production, solubilization of different minerals and nutrients and even protection from phytopathogens. The use of PGPR thus offers a powerful eco-friendly alternative to chemical fertilizers and pesticides and constitutes a great tool for not only improving the yield and quality of various crops but also maintaining the sustainability of agro-ecosystems. In this study, we have isolated, screened and characterized bacteria from the rhizospheric soil of wheat plants in the region of Saada, Marrakech, Morocco. The plant growth promoting activities of 8 isolated strains were evaluated through in vitro screening for IAA production, phosphate solublization, ammonia production, N-Fixation, HCN production as well as cellulolytic and pectinase activities. Additionally, the PGPR isolates were also tested for antagonistic activities against phytopathogenic fungi such as *Fusarium oxysporum*, *Fusarium graminearum*, and *Verticillium dahliae*..

1. Introduction

Soil microbe – plant interactions are complex and known to influence plant health and productivity. In recent years, the use of these microorganisms to improve plant growth has been extensively investigated [1] and the most widely used techniques involve the use of PGPR (Plant Growth Promoting Rhizobacteria).The term PGPR was first introduced in 1978 by Kloepper and Schrothto [2] to describe beneficial microbial populations in the rhizosphere, capable of colonizing the roots of plants. In the context of sustainable agriculture and environmental protection, these microorganisms presented great potential thanks, in part, to their ability to reduce the use of chemicals [3].

PGPR use a variety of direct and indirect mechanisms to stimulate and improve plant growth and development [4]. Direct mechanisms include the solubilization of dicalcium phosphate, tri-calcium phosphate and other nutrients [5,6], fixation of atmospheric nitrogen [7] and hormone production such as zeatin, gibberellic acid, auxins and cytokinins [8].Direct mechanisms also involve the production ACC deaminase which helps reduce the level of ethylene in the roots of the developing plants thereby increasing root length and growth [9], and the production of peptides that act as biostimulants [10] Indirect mechanisms include the biological control of pathogens [11], production of chitinase, Hydrogen cyanide (HCN) as well as siderophores which chelate iron and make it available to the plant roots [12]. However, there is no real boundary separating direct and indirect mechanisms of growth promotion by PGPR [1]. With such a wide variety of ways by which PGPR can positively impact plant growth and development, the screening and selection of efficient PGPRs and their utilization in integrated practices is of great importance for enhancing the growth and yield of agricultural crops while maintaining the sustainability of agro-ecosystems. In Morocco, several studies were conducted in order to investigate the effect of PGPR on different plant species such as argan [13], chickpeas [14] and fava beans [15].

The aim of this study is the in-vitro screening for plant growth promoting activities in bacteria isolated from wheat rhizosphere.

2. Experimental details

2.1. Study area, soil sampling and rhizobacteria isolation

Rhizosphere samples surrounding the roots of wheat plants were collected in March 2013 from the Saada reservoir in Marrakesh, Morocco. The climate in the study area is arid to semi-arid and the mean temperatures vary between 37.7 °C in July and 4.9°C in January, the mean annual rainfall is low, less than 400 mm per year in the plain [16]. The soil was collected at depths of about 0 and 15 cm and the physical and chemical characteristics of the soil were as follows: pH (H2O)=8.3 ; clay (%)=20.3 ; fine silt (%)= 33.7, ; coarse silt (%)=14.0, ; fine sand (%)=22.6, ; coarse sand (%)=8.2, ; carbon (%)=0.98 ; Olsen phosphorus (P) =20 mg/kg. The rhizobacteria were isolated using a dilution plate technique [17] and the pure culture isolates were preserved at -20°C in 50% glycerol before they were used in the conducted experiments.

2.2. Phenotypic and biochemical characterization

The eight isolates showing the highest PGPR activity were characterized morphologically: shape, size, margin, elevation, surface and color, they were further characterized through gram staining and endospore testing as well as biochemical tests using the methodology described by Krieg and Holf [18]: breathing bacteria, casein, hydrolysis of starch, reduction of nitrate, mannose, arabinose, fructose, maltose, citrate, degradation of arginine, gelatinase and catalase activity.

2.3. Molecular identification

Genomic DNA was extracted from the bacterial isolates using the PureLink genomic DNA extraction kit (In-vitrogen, Carlsbad, CA, USA). Polymerase chain reaction was used to amplify 450bp fragments using primers U968 (AACGCGAAGAACCTTAC) and L1401 (CGGTGTGTACAAGACCC) [19].The amplification of 16S rDNA was conducted following the procedure described by Arruda et al. [20] and the amplified DNA products were sequenced at CNRST Laboratory (National Centre for Scientific Research and Technology in Rabat, Morocco). The sequences were then compared using BLAST to find regions of local similarity.

2.4 Screening of Rhizobacteria isolated from wheat plants

2.4.1 Qualitative and quantitative estimation of indole acetic acid production

Indole-3-acetic acid production (IAA, auxin) was evaluated using the method described by Loper and Scroth [21]. Bacterial cultures were grown in LB medium supplemented with 1-tryptophan (1,02g/l) and incubated at 28°C for 48h. The cultures were then centrifuged at 7000 rpm for 3 min, 1ml of the supernatant was added to 2ml of Salkowski reagent (60% of perchloric acid, 3ml 0.5 M $FeCl_3$ solution). The development of a pink coloration indicates the production of IAA, the absorbance was measured at 530nm using a spectrophotometer [UVmini-1240; SHIMADZU] and the IAA concentration was then estimated with the help of a standard curve in the 10-100µg/ml range.

2.4.2 Qualitative and quantitative estimation of phosphate solubilization.

The qualitative evaluation of tricalcium phosphate solubilization in the isolated strains was conducted using Pikovskaya's agar. All bacterial isolated were streaked on the surface of Pikovskaya agar medium and phosphate solubilizing activity was estimated after 5 days of incubation at 28°C, the formation of a clear zone around the colony indicates inorganic phosphate solubilization [22]. The quantitative estimation of tricalcium phosphate solubilization was conducted in liquid medium using the method described by Olsen and Sommers [23].

2.4.3 Qualitative and quantitative estimation of ammonia production

All the bacterial isolates were tested for ammonia production using the method described by Cappuccino and Sherman [24]. The bacterial cultures were inoculated in 10ml peptone water broth and incubated at 30±0.1°C for 72 hour, after incubation, Nessler's reagent (0.5 ml) was added in each tube. The development of a brown to yellow coloration is a positive test for ammonia production. Absorbance was measured at 450 nm using a spectrophotometer and the concentration of ammonia was estimated using the standard curve for ammonium sulphate for concentrations in 0.1-1 mol/ml range.

2.4.4 Production of hydrogen cyanide

Hydrogen cyanide production was evaluated using the qualitative method developed by Kremer and Souissi [25]. Bacterial isolates are streaked on nutrient broth supplemented with 4.4 g/l glycine, Whatman filter paper no.1 (soaked in 2% Na_2CO_3 in 0.05% picric acid) is placed on top of the plate which is then sealed with parafilm and incubated at 28±2 °C for 96 h. A color change of the filter paper from yellow to orange-brown indicates HCN production.

2.4.5 Nitrogen fixing activity

The qualitative estimation of Nitrogen fixation was conducted using the method described by Rajasankar and Ramalingam [26]. A nitrogen free semi-solid medium is used with the following composition: 5 g Malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$ $7H_2O$, 0.1 g NaCl, 0.02g $CaCl_2$, and 0.5% bromothymol blue in 0.2 N KOH 2 ml, 4ml of 1.64 % Fe -EDTA solution and 2g agar, distilled water was then added for a final pH of 7. The cultures were then incubated at 30°C for 5 days and the formation of a pellicle at the sub surface level was considered to be a positive test for N fixation. To confirm the positive test, the cultures were grown in NFb medium, the plates were incubated at 28°C for 7 days, and bacterial growth was used as qualitative evidence of atmospheric nitrogen fixation

2.4.6 Cellulolytic and pectinase activities

The cellulolytic potential of the isolates was assessed using carboxymethylcellulose (CMC) agar plate. After incubation, the plates were stained with 0.2% congo red for 15min, then unstained with 1 M NaCl. The formation of a clear zone around the bacterial colony constitutes a positive cellulase test [27]. For the qualitative estimation of pectinase activity, isolates were inoculated on pectin agar and incubated at 28 ± 2 °C for one week. The formation of halos around the bacterial colonies was considered a positive test for pectinase activity [28].

2.4.7 Antifungal effect assay

Inhibitory effect of isolated bacterial cultures was tested on the mycelium growth of *Fusarium oxysporum*, *Fusarium graminearum*, and *Verticillium dahlia.* A 4 mm agar disc from fresh PDA cultures of fungal phytopathogens was placed at the center of plates of (PDA and Czapek media for each bacterial isolate; four 10 µl drops from the 10^8cfu/ml suspension were used. Plates with rhizobacteria served as a control. The diameter of the fungal colony was measured after 7 days of incubation at 28°C. The percent inhibition was calculated using the formula:

Percent inhibition= (R-r)/ R×100 where r is the radius of the fungal colony opposite the bacterial colony and R is the diameter of the control fungal mycelium [29].

2.5 Statistical analyzes

Data were subjected to one-way analysis of variance. The means values were compared using the Newman & Keuls test ($p < 0.05$).

3. Results and Discussion

3.1.PGPR isolation and morphological characteristics of the strains

Eight bacterial strains were successfully isolated from the rhizospheric soil of wheat fields in Saada, Marrakech. The morphological characteristics of the PGPR isolates varied considerably on the LB agar plates. Although all the isolates were odorless, they all differed in margin, elevation, surface, color and diameter of the colonies, which varied from 1 to 4 mm (Table 1). Numerous genera, including *Achromobacter, Arthrobacter, Azospirillum, Burkholderia, Enterobacter, Citrobacter, Klebsiella, Pseudomonas, Rhizobium, Bacillus,* and others, have been found in the rhizosphere of *gramineous* plants [20,30,31,32,33]. *Bacillus* represents a significant fraction of soil microbial communities and their ability to form spores promotes their ubiquity and survival in various environments which gives them a real advantage in the rhizosphere and grants diversity to their modes of action.

Table1: Morphological characteristics of bacterial isolates from wheat rhizosphere, Saada, Marrakech

Isolates	odor	Form	Color	Margin	Elevation	Surface	Size (mm)
S4	odorless	irregular	Cream	Entire	Flat	Dry	2-3
S10	odorless	irregular	Whitish- Cream	Erose	Crateriform	Dry	1-3
S18	odorless	round	White	Entire	Flat	Dry	2-3
S35	odorless	irregular	Whitish- Cream	Entire	Raised	Dry	1-3
S47	odorless	round	Whitish	Entire	convex	Dry	2-3
S48	odorless	irregular	Cream beige	Undulate	Flat	Mucoid	3-4
S50	odorless	round	Whitish	Entire	Raised	Dry	2-4
S54	odorless	irregular	Whitish	Entire	Raised	Dry	2-4

3.2. Microscopic observation, characterization and identification:

Microscopic observations were made to determine the characteristics of the rhizobacterial isolates. All the isolates were *bacillus* shaped, had endospore formation and were Gram positive. Five isolates were mobile while the other were motionless (Table 2). All the isolates tested are positive for casein hydrolysis, production of starch, mannose, and fructose and catalase. Some strains are also capable of degrading arabinose, maltose, citrate, arginine, gelatinase and nitrate (Table 3). Based on their characteristics, they were identified as bacillus as described by Bergey's manual of Determinative Bacteriology [34], the 16S rDNA gene sequence analysis further confirmed that result.

Table2: Microscopic observation of rhizobacterial isolates collected from wheat rhizosphere.

Isolates	Gram reaction	Form	Motility	Sporulation
S4	+	*bacillus*	mobile	+
S10	+	*bacillus*	mobile	+
S18	+	*bacillus*	mobile	+
S35	+	*bacillus*	Motionless	+
S47	+	*bacillus*	Motionless	+
S48	+	*bacillus*	Motionless	+
S50	+	*bacillus*	mobile	+
S54	+	*bacillus*	mobile	+

3.3. Plant Growth Promoting Potential

Plant growth promotion traits are described in Table 4. The results indicate that all eight rhizobacterial isolates are capable of IAA, HCN and cellulose production as well as Nitrogen fixation. Pectinase activity was only detected in one isolate S50 while all the strains showed phosphate and ammonia activity although S4 showed no phosphate activity and S47 had no ammonia production activity .Nitrogen fixation, Hydrogene Cyanide production and cellulase activity were exhibited by all strains. Nitrogen (N) mineralization is one of the key processes of biogeochemical cycling in terrestrial ecosystem [35]. It is an important element that can directly influence plant growth. HCN indirectly promotes plant growth and plays an important role in disease suppression. In a recent study by Passari et al. [36] 15 isolates (68.1%) were positive for HCN production. Another study by Sadhu, et al. [37] investigated cellulase activity and showed that the isolated strains have the ability to metabolize cellulosic substrates for their growth. Cellulase produced by MSL2 strain was characterized and it was shown that this bacterial strain has the potential to be used in the biorefining process [38]. Pectinase catalyzes the degradation of pectin substances and has been used in the food and textile industries, pectinase from *Bacillus licheniformis* KIBGE-IB21 was immobilized on chitosan beads using formaldehyde as a crosslinking agent [39]. In this study, only one strain, S50, displayed pectinase activity.

Table3: Biochemical characterization of selected bacterial isolates from wheat rhizosphere, Saada

Isolate	S4	S10	S18	S35	S47	S48	S50	S54
Breathing bacteria	aerobics	Aerobic (microaerophilic)	Aero anaerobic	Aero anaerobic	aerobics	aerobics	Aero anaerobic	Aero anaerobic
Casein	+	+	+	+	+	+	+	+
NaCl	+	+	-	+	+	+	-	-
Hydrolysis of starch	+	+	+	+	+	+	+	+
50°C	+	+	-	+	-	+	-	-
65°C	-	-	-	-	-	-	-	-
Reduction of nitrate	+	+	+	+	-	+	-	-
Mannose	+	+	+	+	+	+	+	+
Arabinose	+	-	-	-	-	-	+	-
Fructose	+	+	+	+	+	+	+	+
Maltose	+	+	+	-	+	+	+	-
Citrate	+	-	-	-	+	+	+	-
Arginine degradation	-	+	+	+	-	+	+	+
Gelatinase	-	+	+	-	-	+	-	+
Catalase activity	+	+	+	+	+	+	+	+

Table 4: Plant growth promotion activities in Saada (Marrakech) wheat rhizosphere bacterial isolates, + : Positive, − : negative test.

strains	AIA production	PO_4 solubilization	NH_3 production	Hydrogen cyanide Production	Pectinase Activity	Cellulase Activity	N-fixation activity
S4	+	-	+	+	-	+	+
S10	+	+	+	+	-	+	+
S18	+	+	+	+	-	+	+
S35	+	+	+	+	-	+	+
S47	+	+	-	+	-	+	+
S48	+	+	+	+	-	+	+
S50	+	+	+	+	+	+	+
S54	+	+	+	+	-	+	+

3.4. Quantification of IAA, ammonia production and phosphorus solubilization by PGPR isolates:

IAA production by the rhizobacterial isolates varied between 22.43 and 44.72 µg/ml, isolate S48 S35 had the highest IAA production (fig 1). Tricalcium phosphate production varied between 0.18 and 3.68 µg/ml, the highest value was measured in strain S48 (3.68µg/ml) followed by strain S47 (3.5µg/ml (fig.2). Another important PGPR mechanism that indirectly influences plant growth is ammonia production; all the isolates were able to produce ammonia and the highest value of 2,07µg/ml was measured for strains S48 and S54 (Fig 3). In the screening of the eight isolated *Bacillus* strains for various activities in vitro, the results have shown that all the isolates were able to produce IAA but had varying production rates. Similar results were obtained by Cherif-Silini et al. [40] in strains isolated from wheat rhizosphere. Bacteria that are capable of secreting indole

compounds at a rate greater than13.5 µg/mL are classified as PGPR [41] and only small amounts of IAA (10−9 to 10-12M) are required for primary root growth [42]. This phytohormone, synthesized by PGPR affects many physiological activities in plants, such as cell enlargement, cell division, root initiation, growth rate, phototropism, geotropisms and apical dominance [43]. Additionally, our results have shown that, using the qualitative test, seven strains produced ammonia, whereas when a quantitative test was employed, all eight isolates were able to produce ammonia. Similar results were obtained for ammonia production in 95% of isolates from rice rhizosphere [44] and 85% of strains from wheat rhizosphere [40]. Phosphorous is an essential mineral necessary for plant development, however, the majority of soil phosphate is found in insoluble forms, whereas plants absorb it only in two soluble forms, the monobasic and the diabasic ions [45]. An important function played by Bacillus PGPRs is the solubilization of insoluble phosphate [46] and making it available for the plant through a variety of solubilization reactions such as acidification chelation, exchange reactions, and production of gluconic acid [47]. Our study has shown that all isolated *Bacillus* strains are able to solubilize phosphate in liquid PVK medium, while only seven strains formed a white halo indicative of solubilization on solid PVK medium. Liquid media were thus more sensitive than solid media in the detection of solubilization and therefore assessing phosphate solubilization in liquid media is a more reliable technique *[48]*.

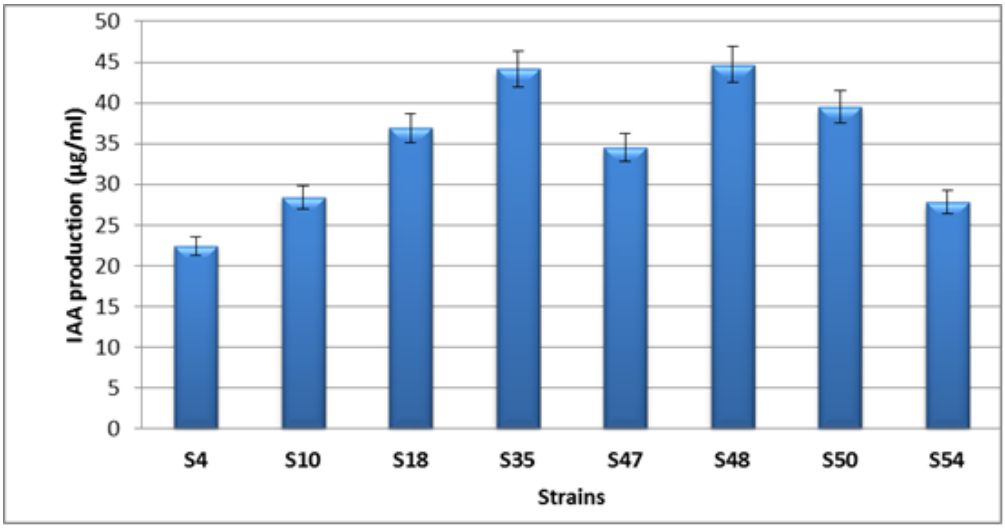

Figure 1: IAA production measured in isolated strains in LB medium supplemented with 1 % of l-tryptophan

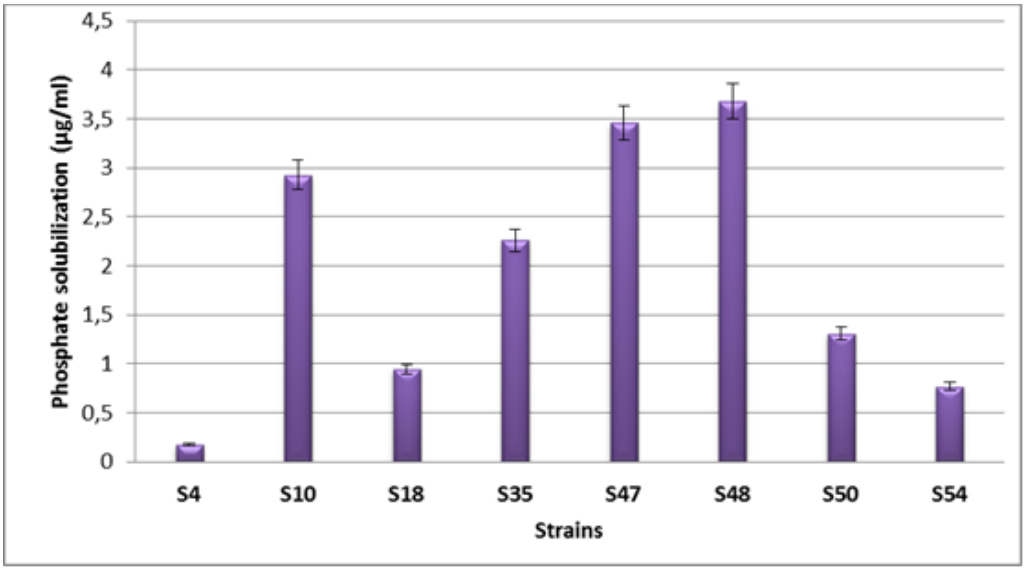

Figure 2: Phosphorus solubilization in isolated strains in liquid Pikovskaya's medium.

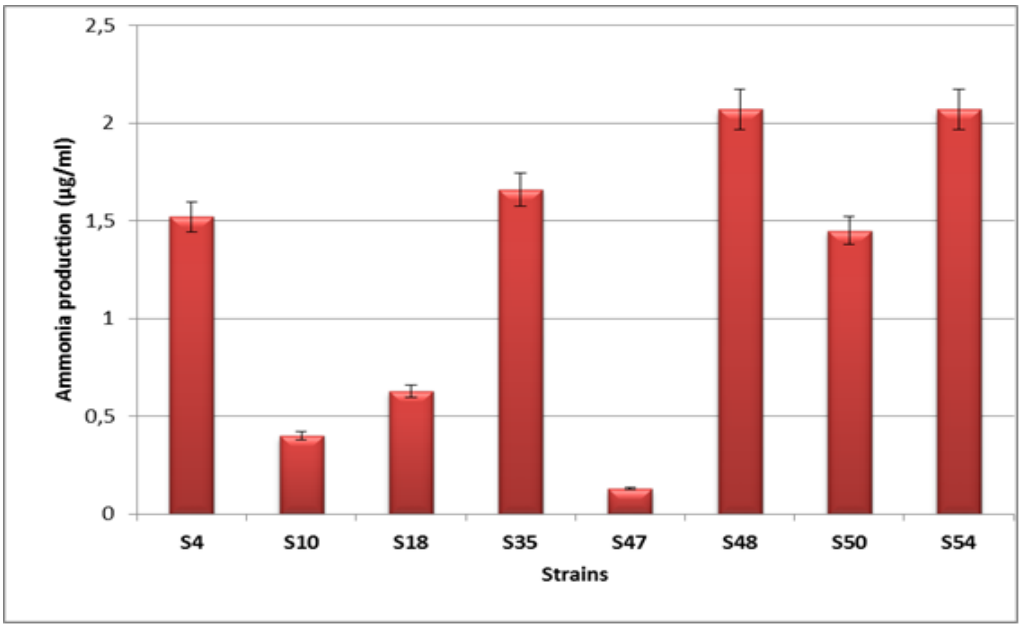

Figure 3: Ammonia production of the isolated strains in peptone water measurements

3.5 .Fungal antagonism assay:

The isolates were evaluated for their antagonistic effect against F. oxysporum, F. graminearum and V. dahliae on PDA and Czapek media. All eight isolates showed some level of antagonism against the tested phytopathogenic fungi. The control plates displayed unchecked growth by the fungal pathogens and no inhibition was observed when the fungi were not exposed to the isolated rhizobacteria. The maximum inhibition of mycelial growth was observed for the S4 isolate on PDA medium with 78.03%, 68.75% and 54.25% inhibition against F. oxysporum, F. graminearum and V. dahlia respectively. Minimal inhibition was recorded for the S47 isolate against F. oxysporum on Czapek medium (table 5). Previous studies have established the inhibitory effect of soil bacteria on the growth of phytopathogens, Goswami et al. [49] found that 19 isolates inhibited F. oxysporum on PDA. In this study, all the isolated strains had an inhibitory effect on phytopathogenic fungi; however, this inhibitory effect was more prominent on PDA than on Czapek medium. This can be explained by the fact that the nutrient constituents of the medium play a significant role in influencing the production of a particular antifungal metabolite by antagonistic rhizobacteria [50]. Two different mechanisms were shown to be employed by bacillus to inhibit the growth of phytopathogens: competition and lipopeptide synthesis [51].

Table 5: Inhibition rate (IR%) of the tested PGPR against *Fusarium oxysporum*, *Fusarium graminearum* and *Verticillium dahliae*.

	Fusarium oxysporum		***Fusarium graminearum***		***Verticillium dahliae***	
strains	**PDA**	**GZAPECK**	**PDA**	**GZAPECK**	**PDA**	**GZAPECK**
S4	78,03±1,83	26,73±15,54	68,75±2,71	60±5	54,25±4,3	8,22±4,21
S10	42,77±9,1	12,83±15,07	44,58±4,71	23,12±26,56	35,6±3,92	20,6±16,56
S18	41,47±10,82	18,27±16,93	54,79±4,52	18,75±24,36	41,22±9,58	8,28±4,46
S35	59,23±5,67	7,75±7,77	45,82±4,95	11,25±8,77	40,34±9,19	15,42±18,39
S47	47,78±2,45	4,55±1,62	48,96±10,41	37,5±21,69	47,83±7,82	25,35±15,17
S48	50,52±9,69	17,83±14,16	50,54±2,61	31,25±19,73	49,48±4,75	14,28±19,04
S50	46,43±2,21	12,44±7,44	46,95±4,4	43,12±24,09	38,45±8,25	16,72±17,58
S54	42,48±5,59	8,72±9,77	43,87±5,74	31,25±17,38	46,73±6,81	35,69±2,02

Conclusions

PGPR are found in plant roots or in the adjacent soil and contribute to the plant's growth and development through multiple direct and indirect mechanisms. PGPR have been investigated in search of efficient ways to use them to improve agricultural production in a low impact ecological way [52]. All the isolated strains in this study showed varying levels of plant growth promoting activities, and all had an overwhelmingly positive effect on the plants for all the investigated bioprocesses: IAA production, Nitrogen fixation and cellulase activity in addition to their antifungal properties. Based on these results, the isolated PGPRs in this study could constitute an efficient and more eco-friendly alternative to chemical fertilizers and fungicides in the processes of bio-stimulation, bio-fertilization and biological control.

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