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Isolation of endophytic bacteria from wild plants in dry regions and investigation of their ability to promote plant growth and inhibit pathogenic fungi

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Abstract

In the dry regions environments such as Saudi Arabia, bacterial endophytes isolated from the roots and soil of healthy wild plants can promote plant growth by developing siderophores, phosphate solubilizing, Indole 3 Acetic Acid (IAA), and inhibit the growth of some plant fungal pathogens. This study aimed to isolate bacteria from wild plants in dry regions and to investigate their ability to be used as plant growth-promoting agent and inhibit plant pathogenic fungi. More than ninety endophytic bacterial isolates associated with the leaves, roots, and soil of healthy wild plants were collected from different sites in the Qassim region, Kingdom of Saudi Arabia, with the aim of characterizing and testing for their ability to promote plant growth activities and inhibit plant pathogenic fungi. Seventy strains were shown to produce indole-3-acetic acid (IAA) and 45 isolates produced siderophores, with 16 of these exhibiting large amounts. Twelve isolates tested positive for phosphate solubilization, with two isolates (*QUSA 66* and *QUSA 91*) exhibiting greater efficiency for phosphate solubility. Twenty-five isolates produced more than 100 µg mL^{-1} of IAA, with production ranging from 100.5 to 404.7 µg mL^{-1}. Six of these bacterial isolates (*QUSA 2, 7, 10, 29, 30.* and *40)* produced more than 200 µg mL^{-1}. A wide spectrum of activities was noted within the pseudomonas strains, indicating promising plant growth-promoting potential (e.g., the isolates from *Pulicaria crispa* and *Calligonum comosum*). *In vitro* results against plant pathogenic fungi showed that fifteen of the bacterial isolates inhibited growth of fungal mycelia by producing wide antagonistic zones. The isolates *QUSA 26, 27, 28, 36,* and *87* were able to produce siderophores and IAA and to solubilize phosphate. In addition, these isolates delayed mycelium growth of some *Fusarium*, *Rhizoctonia solani*, *Botrytis*, and *Stemphylium* spp.

Keywords: endophytic bacteria, indol-3-acetic acid, fungi, siderophores, solubilization.
Abbreviations: IAA_indole-3-acetic acid, PDA_potato dextrose agar, PCA_principal component analysis, QU_Qassim University, KSA_Kingdom of Saudi Arabia.

Introduction

Beneficial microorganisms can promote plant growth through direct interaction with the host plant or indirectly via their antagonistic activity against plant diseases pathogens (Gameza et al., 2019). Internal tissues of apparently healthy plants host endophytic bacteria and endosymbiotic microorganisms (Schulz and Boyl, 2006).

Endophytic bacteria have been isolated from the disinfected surface of plant tissues or extracted from inside of plants. These bacteria apparently cause no obvious damage to the plant (Hallmann et al., 1997). Recently, research has established that bacterial endophytes provide beneficial effects for host plants that include promoting growth and biological control of pathogens (Awad et al., 2014; Hamed et al., 2015). Several studies have indicated that the plant growth-promoting feature of endophytes is greater than that of the rhizosphere microorganisms (Reiter et al., 2002) although the functions of bacterial endophytes regarding plant growth are not yet completely understood. The study of root-associated bacteria and their corresponding antagonistic potential is important for understanding the ecological roles of these bacteria in the rhizosphere, their interaction with plants, and their possible biotechnological applications, e.g., the biological control of soil-borne plant pathogens (Abo-Elyousr et al., 2019).

The mechanisms for promoting plant growth through microorganisms have been studied extensively and include nutrient uptake facilitation (e.g., phosphorus), nitrogen fixation for plant utilization, siderophores for plant iron reservation, production of plant hormones (e.g., gibberellins, cytokinins, and auxins), reduction of plant ethylene levels, secretion of plant hormones or antagonism of plant-pathogenic microorganisms by reducing iron availability to the phytopathogens in the rhizosphere, assembly of enzymes that lyse fungal cell walls, and competing with harmful microorganisms (Ryu et al., 2006; Selosse, et al., 2004). Anti-pathogenic soil-borne bacteria contribute significantly to the protection of plants against pathogenic fungi and may serve as eco-friendly alternatives to chemical pesticides in agriculture (Walsh et al., 2001).

The soil and the rhizosphere are often an ideal environment for isolating and screening the agents with potential for use in the biological control of soil-borne plant pathogens. Because of their role in soil fertility and plant health, the bacterial species most widely used as biological control agents are *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp. The production of several antibiotics from

Bacillus spp. has been reported (Ferreira et al., 1991). The assumption is that the bacterial antagonistic effects are mainly due to the production of antifungal antibiotics, which seem to play a considerable role in biological control of plant pathogens (Phae et al., 1990).

The aim of this study was to isolate endophytic bacteria from wild plant species native to Qassim region (KSA), to screen for their plant growth-promoting potential, and to test them against plant pathogenic fungi, with the goal of applying them to facilitate the vegetation reestablishment strategy in the Qassim region.

Results and Discussion

Ability of the bacterial isolates to enhance plant growth

More than 90 bacterial isolates were obtained from the collected samples. All the bacterial isolates were tested for their ability to produce indole-3-acetic acid (IAA), siderophores and phosphate solubility in addition to testing for their antagonism against some important fungal plant diseases.

Low-molecular-mass molecules (1000 Da) with high specificity and affinity for chelating or binding Fe3+, accompanied by Fe transport and deposition within bacterial cells, are known as siderophores (Neilands, 1995). The bacterial isolates produced siderophores and Halo zones which were detected surrounding bacterial colonies in 45 of the tested isolates (Fig. 1), while 16 of them exhibiting large halo zones. As a result, based on media amendment using succinic-containing chrom azurol S dye, the segregation in distinct quadrants of the halo zones encircling the colonies was clearly revealed in the biplot based on principal component analysis (PCA) (Fig. 2), which accounted for 100 percent of the observed variability. Sixteen bacterial isolates produced the largest amount of siderophores (Fig. 2). It was noted that a number of isolates, such as QUSA 5, 6, 10, 19, 25, 31, 35, 38, 56, 58, and 85, had the ability to produce siderophores, whereas the isolates QUSA 27, 51, and 52 could produce both siderophores and phosphate solubility (Fig. 2). This assay showed no reaction by other bacterial isolates on the medium, meaning that they did not have the ability to produce siderophores.

Plant-bacteria interactions take place through, endophytic and symbiotic processes, with varying degrees of proximity to the roots and around the soil. Since they colonize roots and establish a favorable environment for development, endophytic plant growth-promoting bacteria (PGPB) are perfect inoculant candidates. Endophytic relationships that are not symbiotic take place in the intercellular space of the plant cells (Danhorn and Fuqua, 2007).

Microorganisms have evolved successful Fe absorption strategies. Bacteria can overcome Fe nutritional deficiencies by using siderophores as chelating agents. In this study, a number of strains, such as QUSA 5, 6, 10, 19, 25, and 27, had the ability to produce siderophores in order to promote plant growth by building up direct ion viability in cases of iron deficiency or to restrain growth in plant pathogens (Ahmed et al., 2008).

Screening for phosphate solubilizing bacteria

Twelve isolates (*QUSA 26, 27, 28, 36, 45, 51, 52, 59, 60, 66, 87,* and *91*) were considered to be positive for phosphate solubilization activity, as distinct halos were formed surrounding their colonies on the Petri dishes (Fig. 3). The two isolates *C 66*, and *91* were more efficient in phosphate solubility as they formed larger halo zones. The isolate *QUSA 66,* from a root sample of the Al-Arfaj plant (*Convolvulus dorycnium*), and *QUSA 91,* from a root sample of the Al-Ramram (*Heliotropium bacciferum*) plant, exhibited wide halo zones surrounding their colonies in both the siderophore and phosphate assays (Fig 2). Phosphorus is one of the main nutrients indispensable for the biological evolution and growth of plants. Many reports indicate the ability of different bacterial species to solubilize indissoluble mineral phosphate components including tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986). In this study, all the tested bacterial isolates that were capable of solubilizing phosphate also produced iron carriers, but in different efficiencies (Fig. 2). Phosphate-solubilizing bacteria have been isolated from soils and rhizospheres in many studies. Chen et al. (2014) found that the endophyte *Pantoea dispersa* isolated from the roots of cassava (*Manihot esculenta* C.) effectively dissolved Ca_3 $(PO4)_2$, $FePO_4$, and $AlPO_4$, producing salicylate and benzene-acetic acid, confirming that endophytes are essential for phosphate solubilization.

***Indole-3-acetic acid (IAA) produced by the bacterial isolates*:**

Twenty-five isolates produced more than 100 µg mL^{-1} of IAA, with production ranging between 100.5 and 404.7 µg, with six bacterial isolates (*QUSA 2, 7, 10, 29, 30,* and *40*) producing more than 200 µg mL^{-1} (Table 1). In the present study, the isolate *QUSA 29* belonging to the soil sample of *Chenopodiaceae* grass from Rawdat Al-Tanhat yielded the highest production of IAA (404.7 µg mL^{-1}). This isolate produced large amounts of siderophores, but it was unable to solubilize phosphate.

All the bacterial isolates with the ability to solubilize phosphate also produced IAA, testing positive in the three previous assays although in different efficiencies.

Nine isolates (*QUSA 26, 27, 28, 36, 40, 51, 52, 67,* and *87)* produced siderophores (showing wide to large halo zones) and more than 70 µg mL^{-1} IAA and solubilized phosphate.

Among the many phytohormones, IAA is widely regarded as the most essential native auxin and most rhizobacteria are known to have the biological structure of the IAA. Approximately 80% of root bacteria have the ability to excrete IAA (Leinhos, 1994).

Fungal inhibition growth assay

Many of the bacterial isolates were able to inhibit one or more of the tested plant pathogenic fungi in this assay, and the mycelium growth of all of the fourteen fungi were affected by one or more of the bacterial isolates in different degrees. Results are shown in Table 2. Each of the isolates *QUSA 32, 37,* and *40* showed large ranges of antagonism to fungi and inhibited the growth of 13 out of the 14 tested fungi.

The bacterial isolates antagonized different fungi in a variety of ways. Some, such as isolates *QUSA 26, 27, 51, 54, 58, 60, 61,* and *65,* delayed fungal mycelium growth compared with the control. The isolates *QUSA 26, 27, 28, 36,* and *87* were able to produce siderophores and IAA and solubilize phosphate in addition to delaying the mycelium growth of some *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis ceniria*, and *Stemphylium* spp.

Table 1. Quantitative estimation of indole-3-acetic acid (IAA) produced by some of the tested bacterial isolates (*QUSA*).

QUSA	IAA	*QUSA*	IAA	*QUSA*	IAA
1	124.3	26	75.2	53	90.8
2	263.5	27	86.9	54	83.6
4	86.8	28	130.6	55	86.0
5	177.8	29	404.7	57	99.9
6	119.6	30	233.5	58	47.2
7	238.9	33	82.5	59	60.7
8	143.2	34	103.5	60	54.1
9	140.5	36	82.1	66	57.6
10	246.9	37	80.1	67	104.4
11	78.8	38	163.9	69	156.8
13	82.7	40	215.9	70	81.0
14	133.9	41	169.3	73	94.6
15	100.5	45	84.3	77	61.9
16	162.6	46	139.3	83	116.3
17	174.5	47	122.6	86	71.1
19	83.3	50	92.1	87	96.4
23	92	51	71	89	120.9
25	120.8	52	74.3	91	51.8
F pr.	<.001				
cv%	10.9				
L.S.D. (5%)	26.20				

Quantitative estimation of IAA production as µg/mL according to the equation y = 185.8 x + 41.05, where x is the spectrophotometer reading at 530 nm.

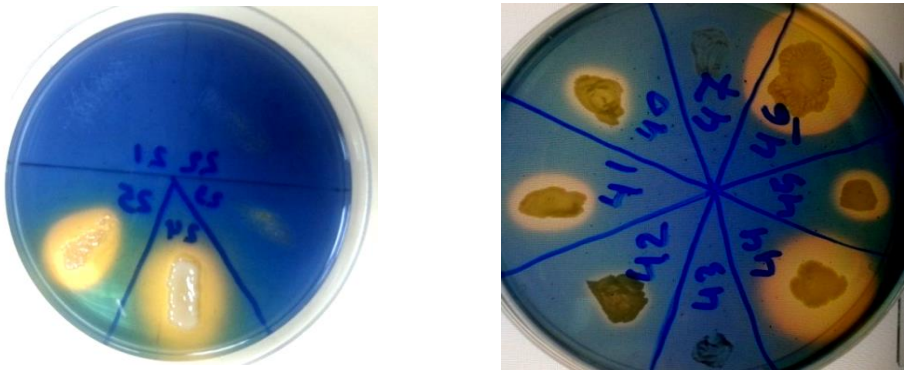


Figure 1. Siderophore assay showing different halo zones surrounding bacterial colonies.

Table 2. Antagonism between bacterial isolates and the tested plant pathogenic fungi.

QUSA	Tested Fungi *														Total
	F. mon	F. so	F. ox	F. gra	F. o.c	F. lis	F. (w)	F. (w1)	F. (p)	Rhiz.	Collet	Bot.	Alt.	Stem.	
26	-	-	+	-	-	-	+	-	-	+	-	+	-	+	5
27	-	+	+	-	-	-	+	+	-	+	-	+	-	+	7
28	-	+	++	-	+	-	+	+	-	+	-	-	+	+	8
29	-	-	-	++	-	+	+	+	-	+	+	++	-	+	8
30	-	-	-	++	-	-	+	+	-	+	-	+	-	+	6
32	+	++	++	+	+	+	-	++	+	+	+	+	+	+	13
33	+	++	+	+	++	+	-	++	+	+	-	+	+	+	12
34	-	++	-	+	+	-	-	+++	++	+	+	-	-	++	8
35	+	+++	-	+	+	+	-	+++	++	+	-	+	+	++	11
36	-	-	-	-	-	-	-	++	+	-	-	+	-	+	4
37	++	+++	++	+++	++	++	-	+	+	+	+++	+++	+++	+++	13
40	+	++	+	+	+	+	+	+++	++	+	++	++	-	++	13
41	-	+	+	-	+	+	-	++	+	+	-	-	-	+++	8
42	+	+	+	-	+	+	-	+	+	-	+	-	-	++	9
45	+	+	-	-	+	+	-	+	-	+++	+++	+++	+++	+	10
51	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1
52	++	-	-	-	-	-	-	-	-	+++	+++	++	+++	+	6
54	+	+	+	+	+	-	-	-	-	+	+	+	+	+	10
58	+	+	+	+	+	-	-	+	-	+	+	+	+	+	11
60	+	+	+	+	+	-	+	+	+	+	+	+	-	+	12
61	+	-	+	+	+	-	+	-	+	-	-	+	-	+	8
65	+	-	+	+	+	-	+	-	+	-	-	+	-	+	8
66	+	++	++	+	+	-	+	++	+	+	-	-	+	+++	11
67	+	+	+++	+	+	-	-	++	-	+	-	+	+	-	9
70	+	+	+++	-	-	-	-	+	-	-	+	-	-	-	5
74	-	-	-	+++	-	-	+	-	+	-	-	++	+++	++	6
77	-	++	+++	++	+++	-	++	++	+	-	-	++	+++	++	10
78	+	-	+	-	-	+	+	-	+	-	+++	+	+++	++	9
86	+++	-	-	+++	+++	-	+++	+	+	-	+++	-	+++	++	9
87	-	-	-	-	-	-	-	-	+	++	-	-	-	+	3
91	-	+	+	++	+	+	-	+	-	+	-	-	+	++	9

* F. mon = *Fusarium moniliform*, F. so = *Fusarium solani*, F. ox = *Fusarium oxysporum*, F. gra = *Fusarium graminiarum*, F. o.c = *Fusarium oxysporum* c., F. lis = *Fusarium* sp. (purple colony), F. (w) = *Fusarium* sp. (white colony), F. (w1) = *Fusarium* sp. (white colony), F. (p) = *Fusarium* sp. (pink colony), Rhiz.= *Rhizoctonia solani*, Collet.= *Colletotrichum* sp., Bot = *Botrytis* sp., Alt =*Alternaria* sp., Stem.= *Stemphylium* sp. + Delay of mycelia growth compared with control, ++ Mycelia in contact with bacterial colonies stopped growing, +++ Presence of antagonistic area free of fungal mycelia. Data presented in this table refer to the bacterial isolates which showed antagonizing effects against more than 4 fungi.

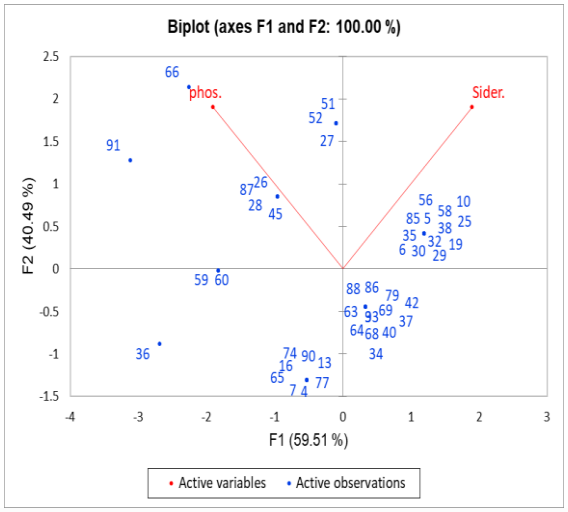


Figure 2. Principal component analysis (PCA) biplot showing siderophore production (Sider.) and phosphate solubility (Phos.) in the distribution of 46 bacterial isolates (*QUSA*) and their relationship.

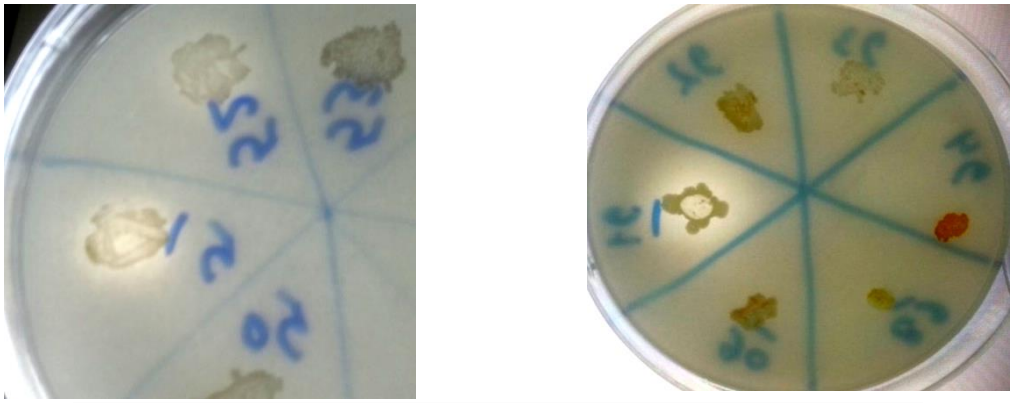


Figure 3. Phosphate assay showing halo zone formation surrounding bacterial colonies.

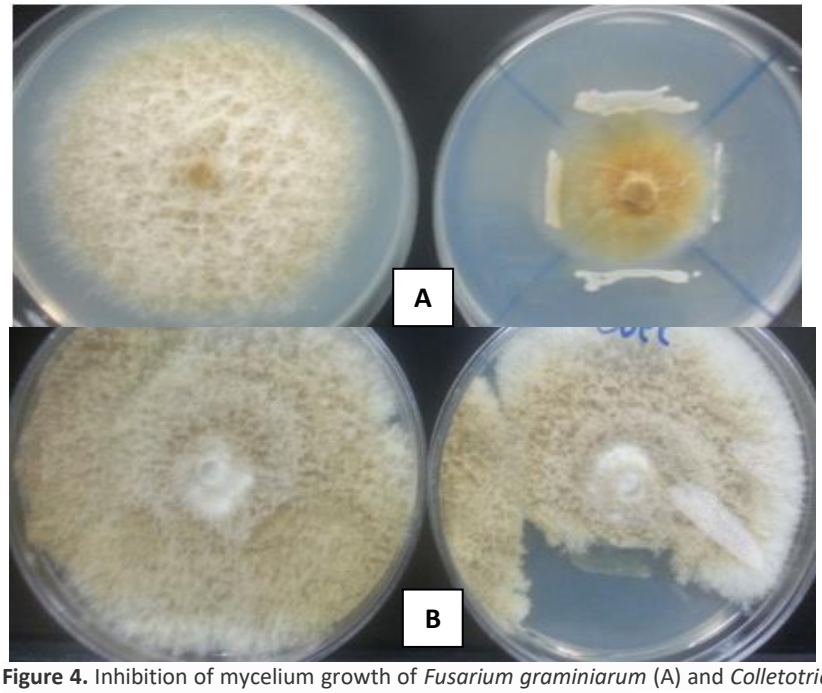
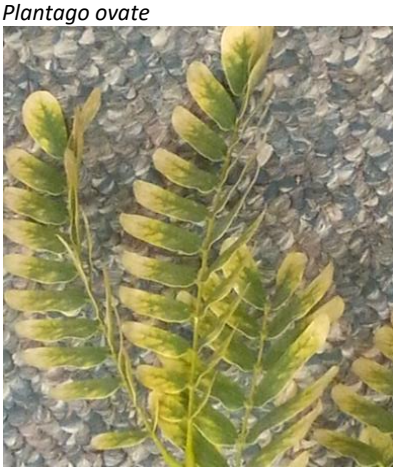


Figure 4. Inhibition of mycelium growth of *Fusarium graminiarum* (A) and *Colletotrichum* sp. (B) by different isolated bacteria compared with controls on the left.

Riblaa
Plantago ovate

Al-Harmal
Rhazya stricta

Al-Bakraa
Launaea sp.



Lebbeck
Albizia lebbeck

Plant from
chenopodiaceae

Conocarpus
Conocarpus erectus

Al-Katad
Astragalus spinosus

Al-Arfaj
Pulicaria crispa

Al-Ramram
Heliotropium bacciferum

Figure 5. Some samples collected from different plants (Photos courtesy of Abdul Rahman Al-Soqair, in: Abdul Rahman Al-Soqair and Jamal Al Ghazali, *Vegetation in Wadi Al Ramah* (2013), QU Press (In Arabic).

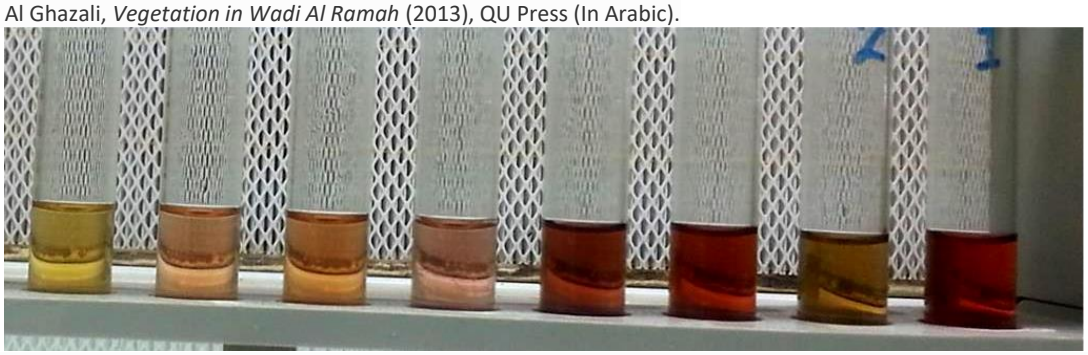


Figure 6. IAA assay showing gradation of the red color of NB medium inoculated with different bacterial isolates compared with the control (medium without bacteria) on the left.

Five isolates (*QUSA 37, 45, 52, 77,* and *86)* were considered to be the best fungal growth inhibitors. They exhibited wide antagonistic zones on at least three pathogenic fungi (Fig. 4), and all of them produced siderophores. These results suggested that the surfactant on the agar plate played an important role in inhibiting the pathogen growth (Alsohim et al., 2014).

Some bacterial isolates inhibited fungal mycelium growth when the fungal and bacterial colonies came in contact, as with isolate *QUSA 29* for both *F. graminiarum* and *Botrytis* spp., isolates *QUSA 32* and *33* for some *Fusaria* species, isolate *QUSA 42* for *Stemphylium* sp., isolate *QUSA 91* for both *Fusarium graminiarum* and *Stemphylium* spp., and *QUSA 87* for *Rhizoctonia solani*.

Fifteen bacterial isolates inhibited the growth of fungal mycelia by producing wide antagonistic zones. The isolate *QUSA 40* was able to produce siderophores and IAA and solubilize phosphate in addition to exhibiting different antagonistic effects on all fungi genera except *Alternaria*. It stopped mycelium growth of some *Fusarium* species (Fig. 4A) and *Colletotrichum* (Fig. 4B), *Botrytis,* and *Stemphylium*, whereas it produced a wide antagonistic zone for *Fusarium oxysporum* (white 1)

The isolate *QUSA 67* was able to produce siderophores and IAA and solubilize phosphate in addition to antagonizing some *Fusarium* sp., *Rhizoctonia solani*, *Botrytis*, and *Alternaria*. It also produced a wide antagonistic zone for *Fusarium oxysporum*.

Material and Methods

Sample collection and isolation of bacteria

The Qassim region, located in central KSA, is characterized as having a typical desert climate, with cold, rainy winters and hot, less humid summers. A total of 93 (leaf, root, and soil) specimens were collected from different wild trees, bushes, and desert grasses growing at different sites in the Qassim region (Fig. 5). Bacterial isolation was carried out using nutrient agar plates as the isolation media for all collected samples. The specimens were incubated in the dark for two days at 28 ± 1 °C (Alsohim et al., 2018) and maintained in 15% glycerol. For isolation of the bacteria, based on the colony characteristics, single colonies were selected and saved in 15% glycerol at -80 °C for subsequent characterization. On each plate, the colonies that could be distinguished based on their morphology were selected and subcultured.

Direct growth promotion mechanisms

All the bacterial isolates were tested for their ability to produce siderophores and IAA and to solubilize phosphate, in addition to testing their antagonism against some important fungal plant diseases.

Detection of siderophores

The 24-h colonies of the bacterial isolates were inoculated in a succinic medium containing chrom azurol S dye and incubated for about five days at 28 °C to determine their ability to produce iron carriers, following the method of Schwyn and Neilandsin (1987). The results were estimated using a scale of three levels: (+) a thin yellow halo zone surrounding the colony (about 1 mm width), (++) a wide halo zone less than 5 mm width of the yellow area surrounding the colony, and (+++) a large halo zone (more than 5 mm) formed with a yellow area surrounding the bacterial colony.

Determination of phosphate solubilization

The bacterial isolates were tested for their ability of solubilize phosphate. After 24 h, colonies of each isolate were inoculated onto Petri dishes of Pikovskaya agar medium with $Ca_3(PO4)_2$ solution, then incubated for five days at 28 °C as described by Goldstein (1986). Solubilization was estimated on the bases of the diameter of the clear halo zones, using a scale of three levels: (+) a thin yellow halo zone surrounding the colony (about 1 mm width), (++) a wide halo zone less than 5 mm width of the yellow area surrounding the colony, and (+++) a large halo zone (more than 5 mm) formed with a yellow area surrounding the bacterial colony.

Determination of indole-3-acetic acid (IAA)

A 24-h culture of each bacterial isolate was grown for five days in a liquid nutrient broth (NB) medium supplemented with 4 g/L of L-tryptophan (Sigma) at 28 °C in an orbital shaker at 180 rpm. After centrifugation at 3000 rpm for 30 min, cells were collected from the liquid medium and then discarded. Orthophosphoric acid and Salkowski reagent were added to the supernatants, and if the bacteria had produced IAA in the presence of the tryptophan, after about 30 min the color of the medium changed into gradations of red (Fig. 6).

A spectrophotometer was used at 530 nm wavelength to measure IAA production via the Gordon-Weber colorimetric method (Gordon and Weber, 1951), and then the readings obtained from the standard curve were converted in order to calculate the concentration of IAA as µg per mL, using the equation : $y = 185.8 x + 41.05$.

Statistical analysis

The spectrophotometer readings for IAA production were performed twice at a wavelength of 530 nm. Analysis of variance (ANOVA) was carried out using the GenStat, 2004.2.10 statistical package (Wim et al., 2004). After testing for significant differences between treatments at $p \leq 0.05$, the least significant difference was determined. A principal component analysis (PCA) was carried out and a biplot was prepared using the XLSTAT Perpetual 2019.2.2 software to show the multiple dimensions of the distribution of siderophore production and phosphate solubility.

Fungal inhibition growth assay

To examine the antagonistic abilities of the isolates against fungal pathogens, the bacterial isolates were streaked onto four sides of a Petri dish (1 cm from the edge) containing a potato dextrose agar (PDA) medium (Difco, Detroit, MI, U.S.A.). A 6-mm mycelial disc from a 7-day PDA culture (for four fungal pathogens) was then placed on the opposite side of the Petri dish perpendicular to the bacterial streak, and the samples were incubated at 25 °C for four days. All the strains were determined in three independent replicate antagonistic assays against four common plant pathogenic fungi: *Fusaria* species, *Rhizoctonia solani*, *Botrytis* sp. and *Stemphylium*. After four days, the inhibition zones (mm) were recorded by measuring the distance between the edges of the fungal mycelium and the bacterial streak: (+) Delay of mycelia growth compared with control, (++) Mycelia in contact with bacterial colonies stopped growing,

(+++) presence of antagonistic area free of fungal mycelia (Table 2).

Conclusions

It was concluded that many of the 90 bacterial endophytes isolated from the plant rhizosphere in the desert environment of the Qassim region, KSA, have the ability to support plant growth by producing siderophores and indole-3-acetic acid (IAA) and by solubilizing phosphate, in addition to the ability to inhibit the growth of some plant fungal pathogens.

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