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Analysis of secondary metabolites produced by four antifungal bacteria from potato and strawberry plants and their rhizosphere soils

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ABSTRACT

This study was designed out in the framework of evaluating the capacity of growth improvement and protection of plants against biotic and abiotic agents by plant growth-promoting rhizobacteria (PGPR). Accordingly, we evaluated the power of four bacterial isolates that were identified as B6 (*Bacillus* amyloliquefaciens DMB3), F31 (Acinetobacter lwoffii strain HATC14), Fr43 (*Pseudomonas brassicacearum* subsp. neoaurantiaca strain IHBB13645), and B29 (*Bacillus* amyloliquefaciens strain CD2901) in producing specific metabolites, such as siderophores, indole acetic acid (IAA), phosphatases, cellulases, hydrocyanic acid (HCN). The ratio of siderophore production reaches its maximum on the 3rd day of incubation in the four bacterial strains with yellow halo diameter values varying between 1.9 and 2.15. The production of siderophores in the liquid medium is maximal at concentrations of 1 and 20 mmol/l of Fe^{3+} with a percentage between 70.8 and 90.64%. This declines at concentrations of 100 and 150 mmol/l in Fe^{3+}. The catecholate and hydroxamate siderophores are produced by the three bacterial isolates (Fr43, F31, B29). While strain B6 produces only catecholate. In addition, the Carboxylate siderophore is produced only by strain Fr43. Qualitative production of indole acetic acid was observed in all isolates tested in solid and liquid media. Strains F31, Fr43, B29 and B6 produced 51.21 µg/ml, 62.8 µg/ml, 77.29 µg/ml and 83.09 µg/ml of IAA, respectively. In addition, the results show solubilization of $Ca_3(PO_4)_2$ by the four strains with the presence of a clear halo with a diameter varying between 10 and 16 mm and a quantity between 30.15 µg/ml and 82.43 µg/ml. The cellulolytic activity of the four isolates Fr43, B6, F31 and B29 showed a halo that reached diameters of 28 mm, 25 mm, 23 mm and 21 mm, respectively. Furthermore, HCN production is illustrated in strains B6 and B29 alone.

Keywords: PGPR bacteria, AIA, siderophores, phosphatases, cellulases, HCN.

INTRODUCTION

Worldwide agricultural production is limited by a number of biotic and abiotic constraints. Given this situation, using bacteria to promote plant growth and protection remains the most promising method. A large number of microbial populations are commonly found in soil, actively colonizing plant roots and enhancing their growth and yield (Agarwal et al., 2013). Iron plays a vital role in most redox enzymes involved in the electron transport chain of intermediary metabolism. It is essential for many plant processes, photosynthesis included, and is also required for chlorophyll synthesis and the general functioning of the photosynthetic apparatus (Ganz and Briat, 2015). Moreover, iron's bioavailability remains extremely low, despite its abundance in nature. This is why most organisms have to react to these problems by several mechanisms, such as capturing and assimilating iron from their environment (Dreschsel and Winkelmann, 1997). The bacteria

have then developed complex capture systems, via the secretion of siderophores and the capture of ferri-siderophore complexes. They also beneficially influence the plant by mobilizing nutrients in soils and stimulating growth through the production of phytohormones or growth regulators (Dodd et al., 2010), and/or through protection against plant pathogens (Ahemad M et al., 2011; Welbaum et al., 2004). In parallel to their ability to suppress plant pathogens, PGPR bacteria have the capacity to degrade soil organic matter, which plays an important role in plant production (Mohamed et al., 2019), supply nutrients to plants and enhance their growth (Xiang et al., 2017). PGPRs isolated and selected from rhizospheric soils have been commercialized as pesticide alternatives, microbial bio-inoculants and bio-fertilizers (Adesemoye and Kloepper, 2008).

Considering the beneficial effects of plant growth-promoting rhizobacteria (PGPR), an experimental study was conducted in the framework of isolating bacteria as PGPR under laboratory and greenhouse conditions. As a result, 24 antifungal isolates were isolated, of which four bacterial isolates showed high antifungal activity and plant growth stimulating ability. Molecular identification of these isolates by 16S rRNA gene sequencing allowed the determination of four species; Bacillus amyloliquefaciens DMB3, Acinetobacter lwoffii strain HATC14, *Pseudomonas brassicacearum* subsp. neoaurantiaca strain IHBB13645), and *Bacillus amyloliquefaciens* strain CD2901 (Hichar, 2024). The aim of the present study is to evaluate the ability of these four bacterial isolates to produce certain metabolites such as siderophores, indole acetic acid (IAA), phosphatases, cellulases and hydrocyanic acid (HCN), in order to classify them as alternatives to pesticides.

MATERIALS AND METHODS

Highlighting the synthesis of siderophores

The production of siderophores was demonstrated by the chromium azurol S (CAS) test in agar medium. 100 ml of final solution (dark blue) was autoclaved, cooled to 50 °C and mixed with 900 ml of sterile LPG medium containing 15 g/l agar. After inoculation of bacterial isolates, siderophore secretion was indicated by the formation of a clear halo around the colonies, showing a visual color change from dark blue to yellow (Lacava et al., 2008). The ratio (halo diameter/bacterial colony diameter) was calculated daily to compare siderophore production between bacterial strains.

Kinetics of siderophore production

A 24-h bacterial culture was transferred into 50 ml of liquid medium (LPG). Incubation was carried out at 26 ºC for 7 days at neutral pH. After centrifugation of the bacterial cultures, the supernatant was transferred into tubes containing CAS reagent with different iron concentrations (1, 20, 30, 40, 50, 100, 150 mmol/L). After 30 min of incubation in the dark at room temperature, the absorbance was measured at 630 nm. Siderophore units were calculated using the following formula :

$$Siderophore\ unit\ (\%) = (A_C - A_S/A_C) \times 100$$

where: A_c: absorbance of CAS solution, A_S: absorbance of CAS solution with bacterial supernatant.

Chemical determination of produced siderophores

To determine the types of siderophores produced, bacterial isolates were subjected to various specific tests, including the Arnow test for catecholates (Arnow, 1937), the $FeCl_3$ and tetrazolium test for hydroxamates (Neilands, 1981) and the Shenker test for carboxylates (Shenker et al., 1992). These tests are based on specific staining reactions.

Synthesis of indole acetic acid (IAA)

Demonstration of IAA synthesis

The bacterial strains were grown in LPGA medium with 5 mM L-tryptophan. The agar was covered with Whatman paper and incubated at 26 °C. After 48 h, the paper was collected and treated with Salkowski reagent. After 10 to 30 min, AIA production was manifested by the formation of a pink-red halo around the colonies (Naik and Sakthivel, 2006).

Quantification of AIA

AIA quantification was assessed by a colorimetric method. A colony of the bacterial isolate was inoculated into 100 ml of LPG diluted to half strength with or without the addition of 200 µg/ml L-tryptophan under constant agitation. 1ml supernatant

is added to 2 ml Salkowski reagent. After 30 min incubation in the dark, absorbance is measured at 530 nm (Gravel et al., 2007).

Phosphate solubilization on solid and liquid media

On solid medium: A bacterial culture is inoculated on the surface of Pikovskaya agar (PVK) which contains $Ca_3(PO_4)^2$ as the only source of phosphate. The diameter of the phosphate solubilization zone around the colony was measured after 7 days of incubation at 26 °C. On liquid medium: 0.1 ml of each 24-hour bacterial culture is inoculated into liquid PVK medium for 4 days at 26 °C. After centrifugation, 1 ml of the bacterial supernatant is added to 10 ml of chloromolybdic acid (12 mM) and 1 ml of tin chloride SnCl2 (5 mM) to obtain a volume of 50 ml adjusted with distilled water (Olsen and Sommers, 1982). The blue coloration of the medium indicates the production of soluble phosphates. Phosphate concentration is determined by measuring OD at 610 nm.

Cellulase production

Cellulase production was determined according to the method described by Cattelan et al. (1999). Mueller Hinton agar supplemented with 10 g of cellulose and 1.2 g of yeast extract was used to test the hydrolytic power by cellulase production. The isolates were plated and incubated for 8 days at 28 °C. The cellulolytic activity was demonstrated by adding a Congo red solution for 15 minutes. The development of a clear halo around the colonies indicates a positive reaction (Verma et al., 2007).

RESULTS AND DISCUSSION

Production and kinetics of siderophores

The qualitative assessment of siderophores is reflected in the yellow halo formed around each bacterial colony. For each strain, the diameters of the colonies and halos formed by the siderophores were measured. Figure 1 shows siderophore secretion by the four bacterial isolates Fr43, F31, B6 and B29 in solid CAS medium as a function of time. Table 1 shows the ratio of siderophore production by the four bacterial isolates (Fr43, F31, B6 and B29) recorded daily. Siderophore production starts after 24 h incubation at 26 °C for the different bacterial strains. The CAS ratio peaked on day 3 of incubation for all four bacterial strains, with values of 2.15 for Fr43, 2.00 for F31 and 1.9 for B6 and B29.

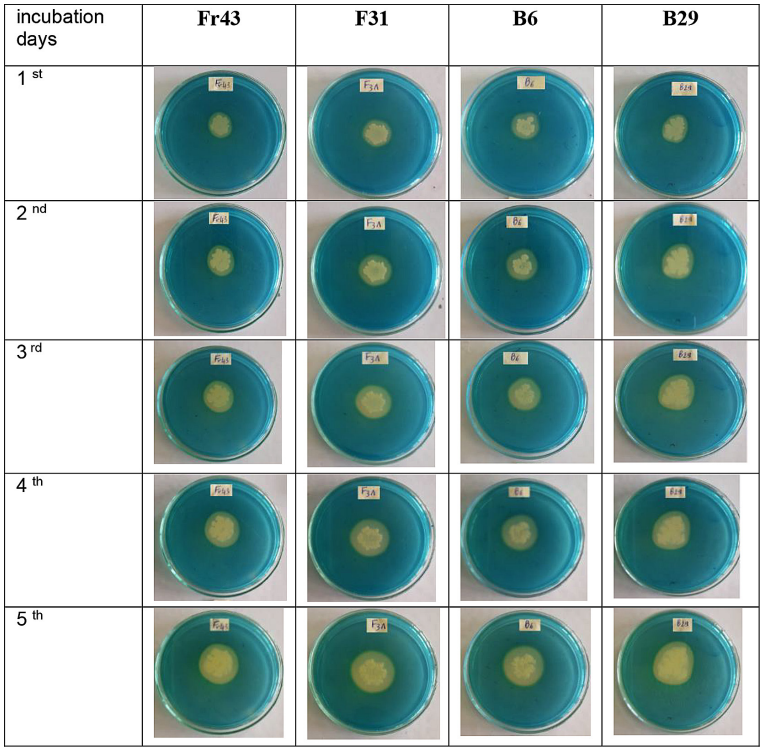

Figure 1. Results of siderophore production on CAS agar during 5 days of incubation

Table 1. Siderophore production according to the ratio of halo diameter to bacterial colony diameter

Day	Rapport CAS			
	Strains			
	Fr43	F31	B6	B29
1st	2.00±0.2	1.75±1.3	2±0.6	1.625±0.1
2nd	2.12±2.1	1.88±0.1	1.87±0.4	1.66±0.0
3rd	2.15±2.3	2±0.6	1.9±0.4	1.9±0.9
4th	1.83±0.5	1.83±1.0	1.78±2.8	1.26±1.6
5th	1.66±1.4	1.78±3.3	1.46±2.8	1.23±0.2

The results recorded in Table 2 indicate the effect of incorporating different concentrations of iron ($FeCl_3$) on siderophore production. The four strains studied showed maximum siderophore production at concentrations of 1 mmol/l and 20 mmol/l of Fe^{3+} with a percentage varying between 70.8 and 90.64%. While it declines significantly at concentrations of 100 and 150 mmol/l in Fe^{3+} with a percentage varying between 2.6 and 16.02%. However, the four isolates Fr43, F31, B6 and B29 show an average production of 58.73%, 61.32%, 54.09% and 56.39% respectively at 40 mmol/l of Fe^{3+}.

The results recorded in Table 3 show that all three bacterial isolates (Fr43, F31, B29) produce catecholate and hydroxamate type siderophores. Whereas strain B6 produces only catecholate. Moreover, Carboxylate type siderophore is produced only by strain Fr43. The results of siderophore production by our bacterial isolates on solid CAS medium are in agreement with the universal tests described by Schwyn and Neilands (1987). In liquid medium, the four strains studied showed significant levels of siderophore production at concentrations of 1 mmol/l and 20 mmol/l Fe^{3+}, but it was low at concentrations of 100 and 150 mmol/l Fe^{3+}. These results are in agreement with those of Cabaj et al. (2009) who revealed that siderophore secretion is carried out at a higher iron concentration (50 mmol/l).

All four bacterial isolates produce catecholate and hydroxamate siderophores, except isolate B6, which produces only catecholates. Moreover, the Carboxylate siderophore is produced only by strain Fr43. In another study, hydroxamates are produced by both bacteria and fungi, while catecholates are produced exclusively by bacteria, and comprise two groups, one catechol and the other hydroxyl (Baakza et al., 2004). According to Drechsel et al. 1995, carboxylates are produced by fungi belonging to the Zygomycota (Mucorales) and some bacteria (*Rhizobium meliloti* and *Staphylococcus hyicus*).

Table 2. Siderophore production (P.S%) as a function of iron concentration (mmol/l) in liquid LPG medium

Fe^{3+} mmol/l	Strains							
	Fr43		F31		B6		B29	
	DO630 nm	P.S (%)	DO630 nm	P.S (%)	DO630 nm	P.S (%)	DO630 nm	P.S (%)
1	0.422	82.22	0.524	77.93	0.591	75.1	0.693	70.8
20	0.222	90.648	0.424	82.139	0.491	79.31	0.593	75.02
30	0.64	76.97	0.825	70.313	1.058	61.928	1.01	63.655
40	1.164	58.738	1.091	61.325	1.295	54.094	1.23	56.398
50	1.672	23.618	1.685	23.024	1.8	17.77	1.395	36.272
100	1.984	2.601	1.806	11.34	1.799	11.683	1.819	10.702
150	1.871	6.027	1.744	12.405	1.907	4.218	1.672	16.022

Table 3. Results of tests for the identification of the types of siderophores produced by bacterial isolates

Parameter	CAS agar test	Arnow test	$FeCl_3$ test	Tetrazolium test	Shenker test
Fr43	Yellow	+	–	+	+
F31	Yellow	+	–	+	–
B6	Yellow	+	–	–	–
B29	Yellow	+	–	+	–

Synthesis of indole acetic acid (IAA)

The qualitative production of indole acetic acid was observed in all isolates tested in both solid and liquid media. This production is indicated by the development of a pink coloration following the addition of Salkowski's reagent (Figure 2). The results of quantitative production of Indole acetic acid shown in Table 4, affirm that the four strains F31, Fr43, B29 and B6 exhibit significant production with concentrations of 51.21 µg/ml, 62.8 µg/ml, 77.29 µg/ml and 83.09 µg/ml respectively. According to Barazani and Friedman (1999), bacteria capable of secreting a level higher than 13.5 µg/ml of IAA are considered to be PGPR. Moreover, the variation in IAA production is also influenced by culture conditions and substrate availability (Mirza et al., 2001).

Solubilization of phosphates on solid and liquid media

$Ca_3(PO_4)^2$ solubilization was reflected by the presence of a clear zone around the bacterial colony. Strains F31, B6 and B29, with a solubilization halo of 16 mm, 14 mm in 12 mm respectively, appear to be the most effective. Strain Fr43 is less effective, with a halo of only 10 mm (Figure 3).

The amount of phosphate solubilized by our bacterial isolates in liquid medium ranges from 30.15 to 82.43 µg/ml. The maximum amount of phosphate solubilization is recorded in isolate Fr43 (*Pseudomonas brassicacearum* subsp) with 82.43 µg/ml followed by isolate B6 (*Bacillus amyloliquefaciens*) at 66.31 µg/ml. Whereas, the amount is minimum in isolate F31 and B29 with a value of 30.15 and 45.27 µg/ml, respectively (Figure 4). These results are in agreement with those obtained in several research works including Gupta et al., 2002 and Ahmad et al., 2008, which show that *Pseudomonas* sp. strain is an efficient solubilizer of phosphates. Furthermore, soil bacteria of the genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* are considered the most potent phosphate solubilizers (Hassan and Bano, 2015; Whitelaw, 1999).

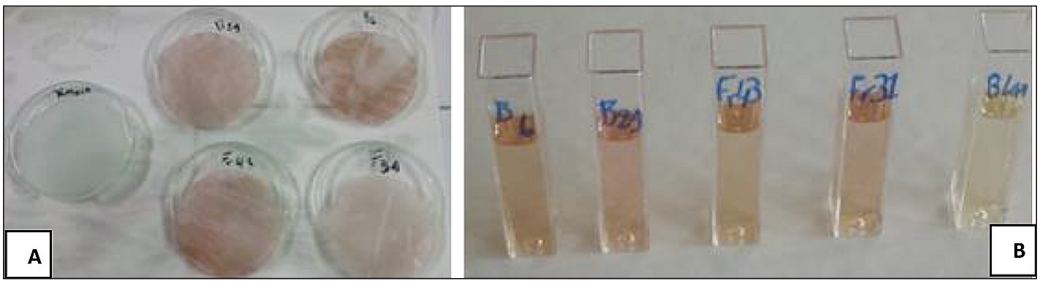

Figure 2. Production of indole acetic acid in agar medium (A) and in broth (B)

Table 4. Quantitative and qualitative production of AIA by the four bacterial isolates

Parameter	Qualitative production of AIA	Quantitative production in µg/ml
Fr43	++	62.80
F31	+	51.21
B6	++	83.09
B29	++	77.29

Note: +: light pink, ++: pink

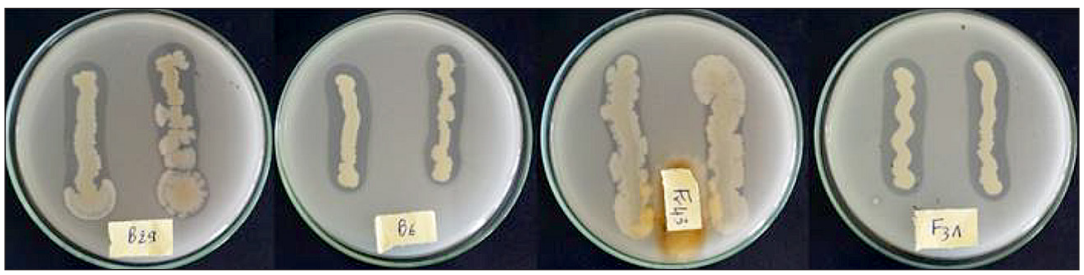

Figure 3. Solubilization of phosphates on solid PVK medium by the four bacterial isolates

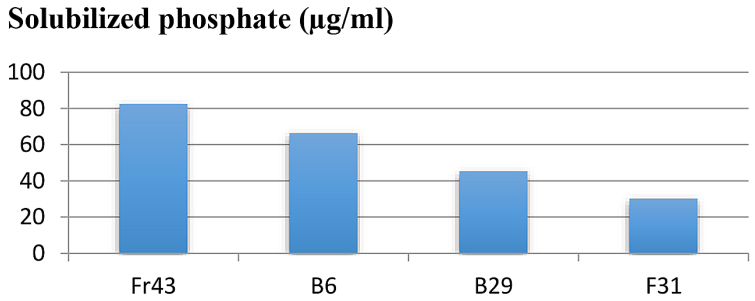

Figure 4. Quantity of phosphate solubilized on liquid medium by the four bacterial isolates

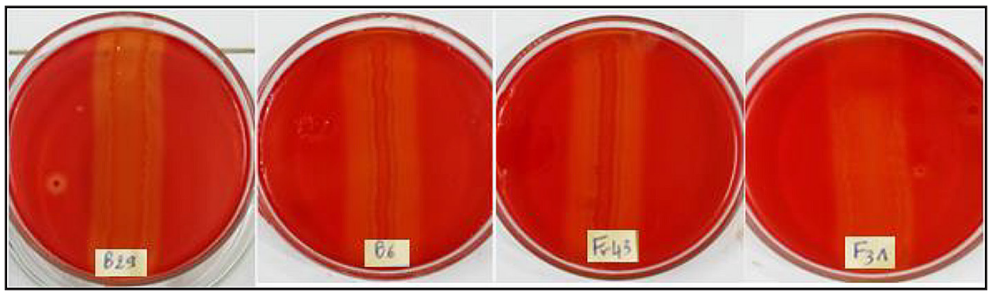

Figure 5. Demonstration of cellulase activity by the four bacterial isolates on solid medium

Among bacterial isolates, strain Fr43 does not show a significant correlation between the results of phosphate solubilization in liquid and solid media, where their solubilization of phosphate in liquid media is more important. Consequently, the ability of microorganisms to solubilize phosphate may be linked to the acidification of the culture medium through the release of organic acids into the medium (Vassilev et al., 2006, Kapri and Tewari, 2010). In addition, this difference is explained by the fact that organic acids are released with difficulty into the solid medium (Nautiyal, 1999).

Cellulolytic activity

The four bacterial isolates studied showed positive cellulase activity. This activity is indicated by the formation of a halo around the bacterial isolates on solid medium. These isolates Fr43, B6, F31 and B29 have a halo that reaches 28 mm, 25 mm, 23 mm and 21 mm, respectively (Figure 5). These results are consistent with those obtained by other previous studies, which have shown the ability of rhizobacteria to produce cellulase (Sudto et al., 2008). The excretion of enzymes that degrade fungal cell walls is frequently involved in attacks by phytopathogenic fungi (Picard et al., 2000).

CONCLUSIONS

After the metabolic tests, we can conclude that the four strains selected (*Bacillus amyloliquefaciens* DMB3 (B6), Acinetobacter lwoffii strain HATC14 (F31), *Pseudomonas brassicacearum* subsp. neoaurantiaca strain IHBB13645 (Fr43), and *Bacillus amyloliquefaciens* strain CD2901 (B29)) are capable of producing siderophores, IAA, phosphate solubilization and cellulose activity. Indeed, the majority of microbial biopesticides used worldwide are of bacterial origin. In this work, the production of these metabolites by the four bacterial isolates demonstrated their economic importance in protecting agricultural land against phytopathogens.

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