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Antifungal activity of some non-fluorescent *Pseudomonas* bacteria isolated from *Phaseolus vulgaris* L. rhizosphere

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Abstract
Biological control is an alternative of synthetic chemicals that have secondary and harmful effects on environment. In this context, 10 non-fluorescent *Pseudomonas* isolated from bean rhizosphere were characterized for PGP traits and antifungal activities against various phytopathogenic fungi. Characterizations of biosurfactants produced by the non-fluorescent *Pseudomonas* revealed particularly Rhamnolipid production by (P2, P3, P5, P6 and P7). They showed a surface tension reduction varying from 35.04 % to 64.75%. All strains produced salicylic acid in quantities of 3.1 mg/ml to 16.9 mg/ml. Only two strains P10 *Pseudomonas cepacia* and P7 *Pseudomonas plantarii* produced HCN (134.64 ppm and 87.12 ppm), respectively. Study of Antifungal activities of the non- fluorescent *Pseudomonas* showed that all isolates exerting very highly significant inhibition of mycelium growth of *Fusarium*, *Penicillium*, *Aspergillus* and *Mucor*. Percentages inhibition observed ranged from 32.42 % to 100%. The combination of *Pseudomonas pseudo mallei* and *Pseudomonas cichorii* showed the best result of fungal inhibition. The less active bacteria, was isolate P10 *Pseudomonas cepacia*. Thus, non-fluorescent *Pseudomonas* endowed with PGP traits could have a potential inhibition of various phytopathogenic fungi.

Keywords: non-fluorescent *Pseudomonas*, Phytopathogenic fungi, PGPR traits, antifungal activity

Introduction

Fungi are an important group of plant pathogens (Knogge 1996) [7, 24]. They can cause substantial losses in yield and quality of subject crops, fruits, and different edible plant material, and this will become increasingly a greater important issue to human health and the global economic system in this century, with growing human populations and local weather alternate threats to arable land (Yang *et al.* 2017) [38]. Furthermore, the use of chemical pesticides for controlling quite a number fungal plant diseases is still a common practice specially in developing countries. Although with the application of chemical fungicides, plant ailments can be managed, but the hazardous impacts of such products in human health and environment are properly known (Zaker 2016) [39].

As an alternative, biological control using bacteria called PGPR (Plant Growth Promoting Rhizobacteria) is a promising approach to avoid and/or reduce devastators effects of chemical pesticides. They are microorganisms living freely in the rhizosphere, can also have an indirect beneficial effect on the plant (van der Heijden *et al.* 2008) [35]. Common PGPR genera exhibiting plant growth promoting activity are: *Pseudomonas*, Azospirillum, Azotobacter, Bacillus, Burkholdaria, Enterobacter, Rhizobium, Erwinia, Mycobacterium, Mesorhizobium, Flavobacterium, etc (Singh 2013) [25]. Especially, *Pseudomonas spp.* strains have been reported as effective biocontrol agents of many fungi plant pathogens (Expert and Digat 1995; Elvira-Recuenco and van Vuurde 2000; Trivedi *et al.* 2008; Mokrani *et al.* 2018a; Agrillo *et al.* 2019) [20, 19, 34, 2]. They are ubiquitous bacteria in agricultural soils and have many PGPR traits that make them well appropriate as biocontrol agents of soilborne pathogens (Weller 2007) [37].

This work was focused on the characterization antifungal antagonistic activities and PGPR traits of some non-fluorescent *Pseudomonas* bacteria isolated from bean rhizosphere against phytopathogenic fungi.

Material and Methods

Source of *Pseudomonas* bacterial isolates

Ten bacterial isolates were isolated in 2010/2011 from the rhizosphere of *P. vulgaris* L from Tighennif-Mascara (35°24′ N 0°19′ E). Bacterial isolates were related to their according *Pseudomonas spp.* species by biochemical and physiological identification. Numeral identification data was released by ABIS software. The isolates were stored on ordinary nutrient agar by transplanting every 3 months (Mokrani *et al.* 2019) [16].

Identification of the *Pseudomonas* isolates were shon in Table 1.

Table 1: Species attribution of non-fluorescent *Pseudomonas* isolates.

Code of the isolate	ID
P1	*Pseudomonas pseudomallei*
P2	*Pseudomonas plantarii*
P3	*Pseudomonas cichorii*
P4	*Pseudomonas corrugota*
P5	*Pseudomonas alcaligenes*
P6	*Pseudomonas stutzeri*
P7	*Pseudomonas plantarii*
P8	*Pseudomonas cepacia*
P9	*Pseudomonas pseudomallei*
P10	*Pseudomonas cepacia*

ID: Identification.

Source of phytopathogenic fungi and examination

Eight strains of phytopathogenic fungi were provided by the Laboratory of Microbial Systems Biology, ENS Kouba-Algeria. The strains belong to the species: *Mucor ramannianus* NRRL 1829*; Penicillium glabru ; Aspergillus parasiticus* CBS 100926; *Aspergillus ochraceus* ATCC 3174; *Aspergillus niger ; Aspergillus flavus* NRRL 3251*; Fusarium oxysporum f. sp. albedinis* et *Fusarium oxysporum f. sp. cecereae.* In order to confirm the purity and aspect of the fungi, macroscopic (Su *et al.* 2012) [32] and microscopic (Thilagam *et al.* 2018) [32] observations were carried out.

Biosurfactants production

Production of biosurfactant by non-fluorescent *Pseudomonas* isolates was determined by the modified method reported by (Morikawa *et al.* 1992) [17]. Fresh colonies were transferred into a 250 ml Erlenmeyer containing 50 ml of TSB broth supplemented with 2% of olive oil. The cultures were then incubated in a stirred incubator at 37°C/24h/200rpm. The bacterial cells were removed by centrifugation and the samples were subjected to measurement of surface tension with a ten siometer (PHYWE 02416.00, Germany). For the calibration of the instrument, the surface tension of the pure water was measured.

The surface tension of the bio surfactants produced by the isolates bacteria was expressed as the percentage of surface tension reduction (Pornsunthorntawee *et al.* 2008) [28] using the following equation:

$$STR = (ym - yc)/ym \times 100$$

Where: STR: surface tension reduction (%); ym: is the surface tension of the prepared medium solution; yc: is the surface tension of the centrifuged culture medium.

Salicylic acid synthesis

Bacterial isolates were cultured in conical flasks of 250 rpm containing 50 ml of TSB broth on a rotary shaker at room temperature 28±2°C/48 h. The cells were then collected by centrifugation at 10000 rpm for 10 min and 4 ml of the free cell culture filtrate was acidified with HCl (1N) at pH 2.0. Salicylic acid was then extracted with chloroform (2x2 ml). For the chloroform extracts collected, 4 ml of distilled water and 5 ml FeCl3 (2M) were added. The absorbance of the salicylic acid-iron complex developed in the aqueous phase was deterimined at 527 nm (Meyer *et al.* 1992). A standard curve was prepared with salicylic acid (0.101 mg/ml-1.625 mg/ml).

HCN determination

To quantify HCN amount, the method described by Bakker and Schippers (1987) was performed. Thus, King's B broth-containing tubes, amended with 0.44% filter sterilized L-glycine, were inoculated with bacterial isolate. Whatman paper strips (10 cm×1 cm) were soaked in an alkaline picrate solution and introduced at the tube tops. Tubes were then sealed with parafilm and incubated at 28 °C/72 h. Color changes from yellow to brown or reddish brown indicates moderate or high HCN production, respectively.

The total cyanide content (TCC) in Whatman paper was estimated by using the following equation:

$$TCC\ (ppm) = 396 \times OD\ (510\ nm)$$

Where: OD is the absorbance of the medium after incubation.

Antifungal activity

Study of antifungal activity was carried out by dual culture technique using the method described by (Landa *et al.* 1997) [8], which consisted of inoculating pathogenic pellet fungus (6 mm in diameter) in the center of the Petri plate containing PDA medium and simultaneously four agar pellets of the isolate bacteria at a distance of 4 cm from the fungus. Then, Petri plates were incubated at 25°C for 6 days.

The results were scored by measuring percentage of mycelium growth inhibition (Idris *et al.* 2007) [22]:

$$IP = (R\ control - R\ test)/R\ control \times 100$$

Where: IP: Inhibition percentage; R test: Maximum radial distance growth of fungus on a line towards the antagonist bacteria; R control: Maximum radial distance growth of fungus inoculated alone in the center of Petri plate).

Statistical analysis

Results of the antifungal activities were presented as mean ± SD (standard deviation) for three replicates. Data obtained were analyzed statistically to determine the degree of significance between isolates using two way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test at $P \leq 0.01$ and $P \leq 0.001$ levels of significance.

Results

Macroscopic examination of fungal cultures

The Figure 1 represented the macroscopic observations of target phytopathogenic fungi after 6 days of incubation at 25°C on PDA medium.

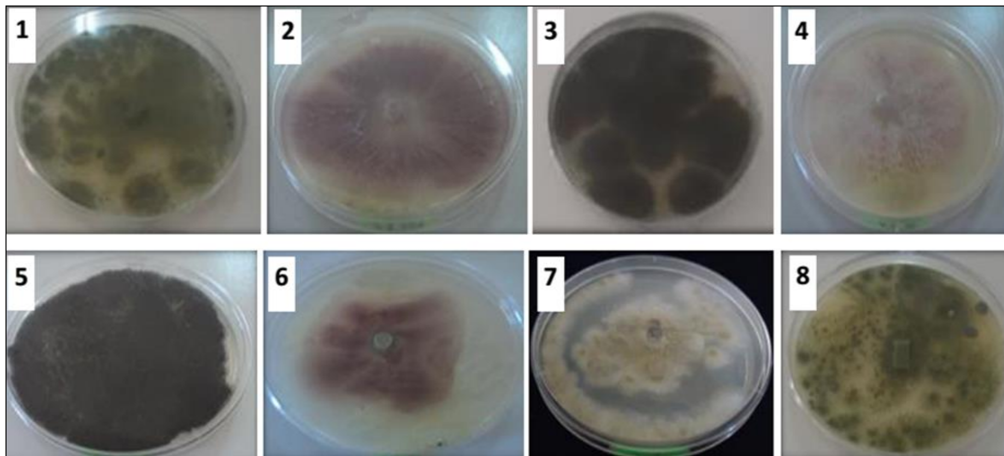

Fig 1: Macroscopic observation of the phytopathogenic fungi (1: *Mucor ramannianus* NRRL 1829*;* 2: *Penicillium glabrum;* 3: *Aspergillus parasiticus* CBS 100926; 4: *Aspergillus ochraceus* ATCC 3174; 5: *Aspergillus niger;* 6: *Aspergillus flavus* NRRL 3251*;* 7: *Fusarium oxysporum f. sp. albedinis* and 8: *Fusarium oxysporum f. sp. cecereae*).

Microscopic Examination of Fungi

The Figure 2 showed microscopic observations of the phytopathogenic fungi.

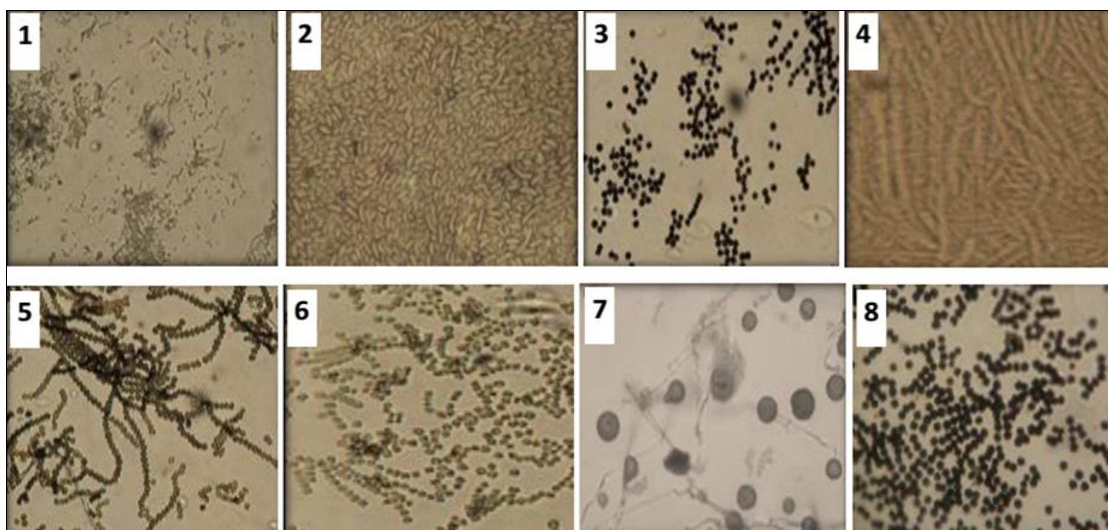

Fig 2: Microscopic observation of the phytopathogenic fungi (1: *Mucor ramannianus* NRRL 1829*;* 2: *Penicillium glabrum;* 3: *Aspergillus parasiticus* CBS 100926; 4: *Aspergillus ochraceus* ATCC 3174; 5: *Aspergillus niger;* 6: *Aspergillus flavus* NRRL 3251*;* 7: *Fusarium oxysporum f. sp. albedinis* and 8: *Fusarium oxysporum f. sp. cecereae*).

Biosurfactants production

Biosurfactants are surface-active molecules produced by microorganisms. There are several microorganisms capable of producing biosurfactants, the most important species are those belonging to the genus *Pseudomonas* producing rhamnolipids (Figure 3). Current findings confirmed production of biosurfactants by non-fluorescent *Pseudomonas* species by measuring of superface tension on TSB broth supplemented with 2 % of olive oil. Qualitative determination of biosurfacant production showed creamy colorloss of medium, indicating rhamnolypides biosurfacatants production by isolates P2, P3, P5, P6 and P7. For other isolates medium manifested white color, meaning other biorurfacants types production. Determination of biosurfacants synthesis revealed variation of surface tension reduction from 35.04 % to 64.75%. Maximum reduction was observed for isolates P8 *Pseudomonas cepacia* of 64.75 %. Followed by, isolates P7 *Pseudomonas plantarii* and P3 *Pseudomonas cichorii*, revealing surface tension reduction of 60.47 % and 60.42 %, respectively.

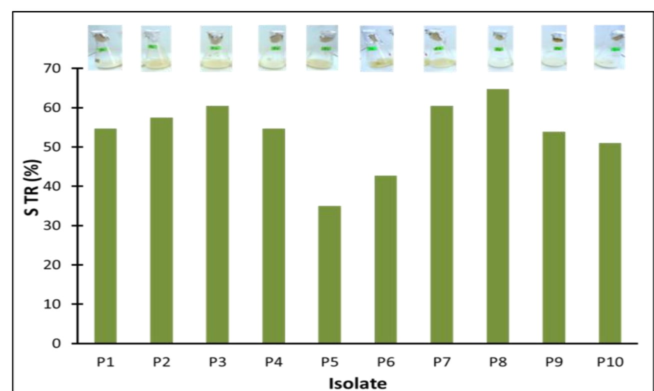

Fig 3: Production of biosurfactants by non-fluorescent *Pseudomonas* bacteria (RST: surface tension reduction).

Salicylic acid synthesis

Salicylic acid is one of the most important phytohormones involved in signaling pathways in the systemic resistance of plant pathogens. In this study characterization of non-fluorescent *Pseudomonas* bacteria for quantitative salicylic

acid synthesis revealed amounts ranging from 3.1 mg/ml to 16.9 mg/ml. Maximum production was unregistered for isolate P2 *Pseudomonas plantarii* (16.9 mg/ml). Followed by isolates P4 *Pseudomonas corrugota* and P3 *Pseudomonas cichorii* producing (16.8 mg/ml) for each isolate (Figure 4). Other isolates showed moderate salicylic acid production ranging from 3.1 mg/ml to 11.3 mg/ml.

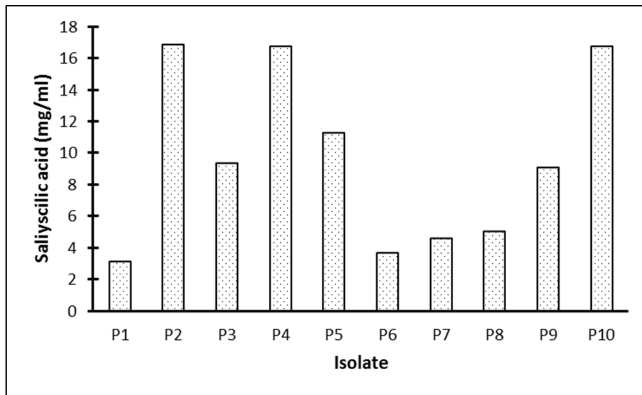

Fig 4: Quantification of salicylic production by non- fluorescent *Pseudomonas* bacteria.

HCN determination

The production of volatile antibiotic molecules is largely responds in PGPR bacteria. Playing an important and direct role in the suppression of fungal plant pathogens. Determination of HCN production revealed a qualitative change of Wattman paper bands to dark brown by only two isolates P10 *Pseudomonas cepacia* and P7 *Pseudomonas plantarii* (Figure 5). Transferring of bands in tubes induced colorless of distillated water. This translated and confirmed HCN production. The amount of HCN produced were 134.64 ppm and 87.12 ppm for each isolates, respectively.

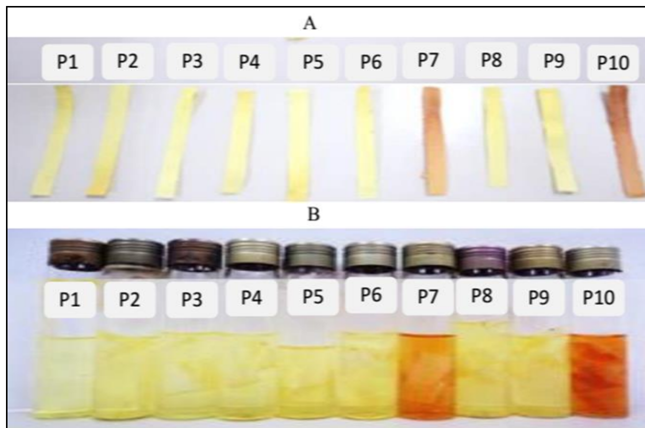

Fig 5: Production of HCN by non-fluorescent *Pseudomonas* bacteria (HCN induced dark brown color of A: Wattman paper bands; B: distillated water).

Antifungal activity

The antagonistic activities of non-florescent *Pseudomonas* isolates against the phytopathogenic fungi by dual culture technique revealed variable inhibition zones (Figure 6).

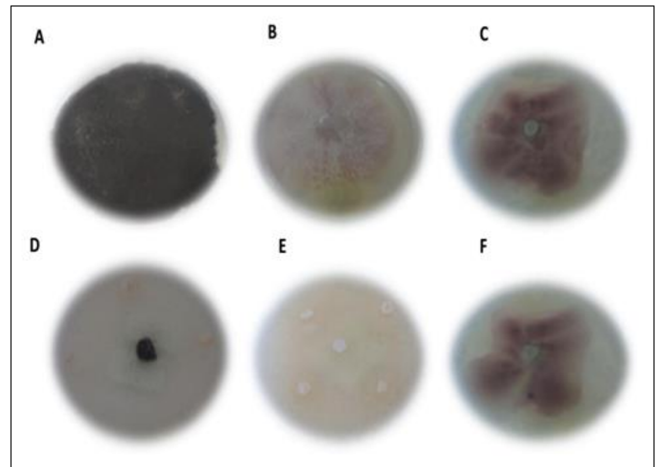

Fig 6: Antifungal activities of non-fluorescent *Pseudomonas* bacteria (A: Control *Aspergillus ochraceus* ATCC; B: Control *Fusarium oxysporum f. sp. Albedinis* C: Control *Mucor ramanniasus* 1829; D: *Aspergillus ochraceus* ATCC vs. P9; E: *Fusarium oxysporum f. sp. Albedinis* vs.P3; F: *Mucor ramanniasus* 1829 vs. P10).

After 6 days of incubation at 25°C, all non-florescent *Pseudomonas* isolates showed very highly significant inhibitory action against all the fungi species tested (Figure 7). Statistical analyses using two-way ANOVA confirmed that the variation in antifungal activities in relation to fungi and non-fluorescent *Pseudomonas* species was significant at $P \leq 0.01$ and $P \leq 0.001$.

Considered together, each non-fluorescent *Pseudomonas* isolate inhibited mycelium growth of all the eight phytopathogenic fungi, representing percentages inhibition ranging from 32.42 % to 100% (Figure 7). The most effectiveness isolate in inhibition of the fungi mycelium growth was isolate P1 *Pseudomonas pseudomallei* showing percentages inhibition of 100 % toward *Penicillium glabrum, Aspergillus niger, Mucor ramannianus* NRRL 1829, *Fusarium oxysporum f. sp. cecereae* and *Fusarium oxysporum f. sp. albedinis.* Furthermore, percentage inhibition of *Aspergillus flavus* NRRL 3251 (99.07 %), *Aspergillus parasiticus* CBS 100926 (96.77 %) and *Aspergillus ochraceus* ATCC 3174 (88.29 %).

Followed by isolate P3 *Pseudomonas cichorii* exerting inhibition growth of 100 % against *Aspergillus parasiticus* CBS 100926, *Aspergillus niger, Aspergillus flavus* NRRL 3251, *Mucor ramannianus* NRRL 1829 and *Fusarium oxysporum f. sp. cecereae.* Also, inhibition of *Fusarium oxysporum f. sp. albedinis* (95.19 %), *Aspergillus ochraceus* ATCC 3174 (88.29 %) and *Penicillium glabrum* (83.48 %).

The less effectiveness isolate was P10 *Pseudomonas cepacia* revealing percentage inhibition of *Aspergillus parasiticus* CBS 100926 (40.86 %), *Aspergillus niger* (78.33 %), *Aspergillus flavus* NRRL 3251 (80.38 %), *Mucor ramannianus* NRRL 1829 (32.40 %), *Fusarium oxysporum f. sp. cecereae* (68.50 %), *Fusarium oxysporum f. sp. albedinis* (41.35 %), *Aspergillus ochraceus* ATCC 3174 (58.56 %) and *Penicillium glabrum* (100 %).

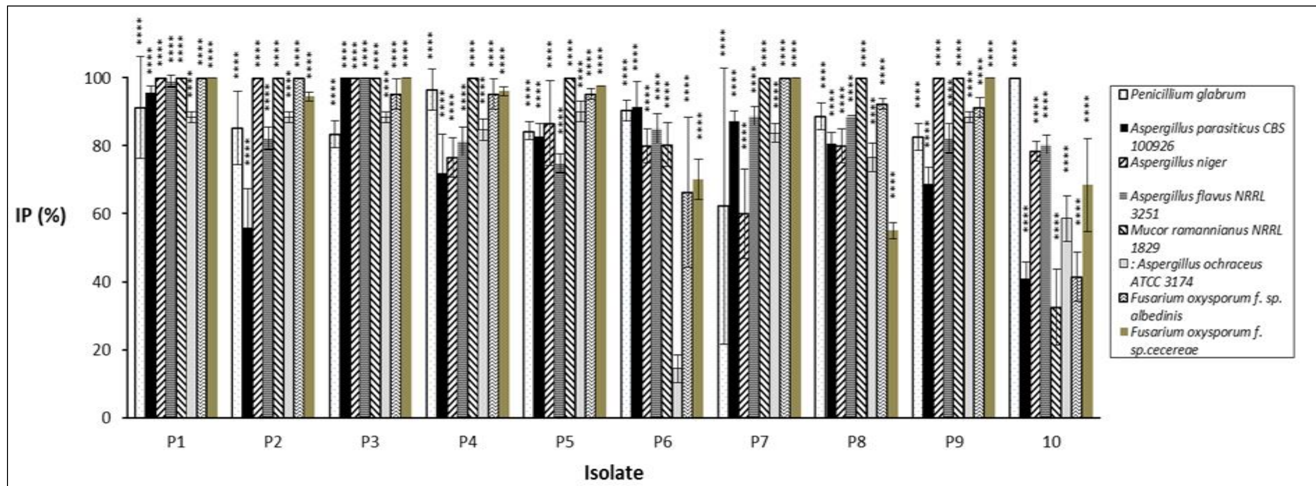

Fig 7: Inhibition percentages of non-fluorescent *Pseudomonas* bacteria against phytopathogenic fungi (****: very highly significant difference between inhibition percentages compared to the control using two way analysis of variance (ANOVA) at P ≤ 0.01 and P ≤ 0.001).

Discussion

Bacterial species belonging to the genus *Pseudomonas* are probably the best candidate bacteria in the biological control of different diseases caused by plant pathogenic fungi. These bacteria have multiple PGPR characteristics and traits which have a double beneficial effect on the suppression of pathogens and stimulation of plant growth.

Phytothogenic fungi belong to various genera (Marin-Felix *et al.* 2017; Marin-Felix *et al.* 2019 López and Castaño 2012) [10, 11, 9]. Causing devastating epidemics of many crop cultures (Knogge 1996) [7]. They use diverse strategies to colonize plants and cause diseases. Some fungi kill their hosts and feed on dead material (necrotrophs), while others colonize the living tissue (biotrophs). Basically, all pathogens interfere with primary plant defense; necrotrophs secrete toxins to kill plant tissue. In contrast, biotrophs utilize effector molecules to suppress plant cell death and manipulate plant metabolism in favor of the pathogen (Doehlemann *et al.* 2017) [5].

In this study non-fluorescent *Pseudomonas* isolates were estimated for biosurfactants production. All isolates had reduced the surface tension on TSB broth supplemented with 2% of olive oil representing percentages ranging from 35.04 % to 64.75%. Many microorganisms produce biosurfactants of different characteristics and structures. These structures include glycolipid (rhamnolipid and trehalose lipid), lipid-protein complex (such as peptide-lipid, viscosin, and surfactin), fatty acids, and phospholipid or polymeric structures (Antunes *et al.* 2006) [1]. Rhamnolipid-biosurfactants are known to be produced by the genus *Pseudomonas* (Kiran *et al.* 2016) [23]. Furthermore, Goswami *et al.* (2015) [21] reported that the rhamnolipid bio surfactant (RL-DS9) extracted from the bacterial strain *Pseudomonas aeruginosa* DS9 was evaluated for its antifungal activity against *Colletotrichum falcatum.*

Current findings had revealed salicylic acid production by the non-fluorescent *Pseudomonas* isolates varying from 3.1 mg/ml to 16.9 mg/ml. Salicylic acid is known to play an essential role in activating the plant's defensive responses (Maurhofer *et al.* 1998) [12] after the pathogens attack (Draper 1997) [6] and induces the expression and accumulation of pathogenesis-related proteins in plants (Nandi *et al.* 2003) [18]. The production of HCN by *Pseudomonas* is involved in the suppression of various pathogens. It acts directly on the cells by blocking the cytochrome oxidase in the respiratory chain. It also contributes to the acquisition of certain metal ions by forming complexes with them (Blumer and Haas 2000) [4].

In this present work, quantitative determination of HCN production revealed that only two isolates P10 and P7 from ten were producers characterized by amounts of 134.64 ppm and 87.12 ppm, respectively. HCN synthesis appears to be confined to Proteobacteria and has been detected in several strains of *Pseudomonas fluorescens*. HCN production by *Pseudomonas* is involved in the suppression of pathogens such as *Thielaviopsis basicola*, *Septoria tritici* and *Puccinia recondita* (Ramette *et al.* 2003) [29]. According to Voisard *et al*. (1989) [36]; the production of hydrocyanic acid by the *Pseudomonas fluorescens* strain is necessary for the protection of the plant against the agent of black rot in tobacco, since a negative, CHA5 HCN mutant, has partially lost its protective properties against the disease. This mechanism is of less importance with the CHAO strain.

Non-fluorescent *Pseudomonas* isolates described in this work revealed very highly antifungal activities against phytopathogenic species belonging to of genera: *Fusarium*, *Mucor*, *Penicillium* and *Asperglillus*. *Pseudomonas spp* inhibit fungi growth by different mechanisms, including inhibition of spores (Srivastava and Shalini 2009) [31] affecting mycelium growth (Shalini and Srivastava 2008; Petatan-Sagahon *et al.* 2011; Toppo and Tiwari 2015) [27, 28, 33] or exerting deterioration of the specific reproduction forms of fungi (Mokrani *et al.,* 2018b) [15]. A particular PGPR may affect plant growth and development by using any one, or more, of these mechanisms. PGPR, as biocontrol agents, can act through various mechanisms, regardless of their role in the direct growth promotion (Patten and Glick 2002) [26].

Conclusions

The implementation of sustainable, more effective and environmentally friendly control strategies necessarily requires a more complete knowledge of the biological and biochemical processes necessary for the development of plant-bacteria interactions. In recent years, the acquisition of knowledge about beneficial rhizobacteria has been tremendously accelerated by the exploration of the metabolic arsenal. Since the "in vitro" selection of beneficial strains works with a view to locating those with the

maximum number of pathogen inhibition mechanisms, "in vitro" antagonism alone cannot be established as the fundamental selection criterion; this is due to the fact that the expression of the antagonism varies according to the culture media and target pathogens. Further investigation through further testing and characterization is required to ensure effective screening for the selection of the most efficient strains.

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