ISOLATION OF FLUORESCENT *PSEUDOMONAS* SPP. STRAINS FROM RHIZOSPHERE AGRICULTURAL SOILS AND ASSESSMENT OF THEIR ROLE IN PLANT GROWTH AND PHYTOPATHOGEN BIOCONTROL

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Abstract :*Plant Growth Promoting Rhizobacteria , PGPR are the rhizosphere bacteria that can enhance plant growth. The exploitation of the PGPR in sustainable agriculture as biopesticides / biofertilizers has became a real alternative for agrochemicals used as environment friendly agriculture. In the rhizosphere, Pseudomonas spp. are an important and abundant group affecting plant growth. From diverse agro-ecosystems of Algeria. In this study, 19 fluorescent strains of Pseudomonas spp were isolated from the rhizosphere roots and soils of durum wheat and barley from different biotopes, these strains were screened in vitro for their plant growth promoting activity and the biocontrol of phytopathogenic fungi. From an in vitro bioassay of Lactuca sativa, 8 strains were selected as PGPR. According to phenotypic identification, the strains were affiliated with P.aeruginosa, P.fluorescens and P.putida species. These strains were tested for their ability to produce enzymes of agricultural interest of which OD11, FR14, FR24, FR13 have been producing proteases, amylases, lipases. The 8 isolates were studied for their antagonistic power against Fusarium oxysporum. FR14 and FR15 showed significant inhibition of mycelial growth with a percentage inhibition of 52.72% and 41.81% respectively. Two antifungal metabolites (phenazines, pyrrolnitrins) were extracted from cultures of strains FR14 and FR15 by thin layer chromatography. The R_f values obtained ranged from 0.6 to 0.62 for PHZ and absence of PLN. The phenazic extracts were re-tested for their antifungal activities against Fusarium.oxysporum and Aspergillus.niger, the results showed an important inhibition of pathogens by the extract of the strain FR14 with a percentage of inhibition of 11.66% to 23, 63%. These results allow FR14 and FR15 strains to be potential biocontrol agents for a future application. Understanding on the diversity of a most important genus of PGPR in the rhizosphere and mechanism of action should facilitate their application as a reliable component in the management of sustainable agricultural system.*

Keywords : *Biocontrol, Biofertilizer, PGPR, Phytopathogenic, Sustainable agriculture.*

INTRODUCTION

The mechanization and the modern means of agronomy (fertilization, genetic biology, etc) have become more criticized because of the environmental degradation of which they are responsible in a large part (Pretty and Bharucha, 2014). In addition, the use of chemicals in the form of fertilizers and pesticides or resistant plants, are the basis for improving yields of various crops, but in the other side these chemical inputs have caused serious problems on biodiversity, human health, and the development of resistance in pathogens (insects, nematodes, fungi, bacteria) (BHARDWAJ *ET AL.*, 2014). These constraints prompted the scientific community to look for alternatives to ensure the sustainability of agriculture through the use of beneficial microorganisms by increasing yield and safeguarding natural resources and plant health (ALTIERI, 2009).

Soil is the mainstay of most agricultural products. It is also the life support of a very large variety of living organisms in continuous interactions (plants, earthworms, nematodes, mites, protozoa, algae, fungi, eubacteria, Archaea, etc.) (BARRIOS, 2007). The rhizosphere; the interface zone between the plant and the soil; is home to several microorganisms that interact with the plant and influence its growth. Some have deleterious effects, others are beneficial. Plant Growth Promoting Rhizospheria, PGPR establish an associative symbiosis

with plants by stimulating their growth and inducing resistance against various phytopathogenic microorganisms (DORJEY *ET AL.*, 2017; SINGH *ET AL.*, 2017A; SINGH *ET AL.*, 2017B; VERMA *ET AL.*, 2018).

Among these bacteria that have an agricultural interest, fluorescents *Pseudomonas* spp. are the subject of particular attention. Their use in agriculture as biofertilizers or biopesticides offers a good yield and could reduce the input of fertilizers or chemical pesticides (BHATTACHARYYA AND JHA, 2012). Thus, these bacteria are known for their effect of improving health and stimulating plant growth (Rabhi, 2018). These bacteria are a good biological control tool; because they can also increase the level of resistance of plants to various diseases because of their antagonistic activity towards phytopathogens by the production of secondary metabolites with antimicrobial effect (cell lysis, stop of DNA replication, antioxidant power ...) (ABDELWAHAB, 2017; BASET *ET AL.*, 2010; GOSAL *ET AL.*, 2017; GRAY AND SMITH, 2005).

Herein is presented and evaluated the contribution of fluorescent *Pseudomonas* spp. strains isolated from Algerian farm fields.

MATERIAL AND METHODS

1. Isolation of fluorescent *Pseudomonas* spp from the rhizosphere

Six samples of rhizospheric soils were collected from Béni-Saf, Northwest Algeria, in March 2018 from the three separate sites of the cereal fields: durum wheat (*Triticum turgidum L. subsp. durum*) and barley (*Hordeum vulgare*). Each soil sample is composed of seedlings and adhering rhizospheric soil. Samples were taken from two random plots. In each plot, whole plants from the same crops were taken with their root systems. Sufficient soil has been kept around the root system to avoid a rapid loss of moisture. Bacterial isolation was performed using the suspension-dilutions method. In sterile flasks with a capacity of 250 ml, 10 g of each soil sample was added to 100 ml of sterile physiological water and agitated for 20 minutes at room temperature. Test tubes containing 9 ml of sterile distilled water were prepared; 1 ml of the parent suspension (10^{-1}) was sterilely removed and added to the first test tube containing 9 ml of sterile distilled water, constituting the second dilution (10^{-2}). Successive decimal dilutions (up to 10^{-6}) were prepared in the same way. From each dilution (10^{-3} 10^{-4} and 10^{-6}), a volume of 100 µl was spread over two Petri dishes containing the King B agar medium. The Petri dishes were incubated in an oven at a temperature of 28°C for 48 hours. After incubation, the 25 most representative colonies were selected based on morphological criteria (colour, external appearance, appearance of the border, fluorescence under UV). After incubation, nineteen colonies of different appearances, fluorescent developed on the King's B medium, were purified on new petri dishes containing the King's B agar medium and then coded to 28°C for 48 hours. the purified strains were conserved in the refrigerator at 4°C.

2. Screening of fluorescent *Pseudomonas* spp. rhizospheric phytobenefic strains

The selected fluorescent *Pseudomonas* spp. strains were grown individually on King's B medium. after 48 h of incubation at 22°C, bacterial cultures were collected by adding sterilised water to each petri dish and scraping with a sterile spread. The optical density " O.D " of the suspensions was adjusted by appropriate dilutions of the suspensions to O.D = 2.0, equivalent to a concentration of 10^9 to 10^{10} CFU/ml. For the selection of phytobenefic strains, a biotest was carried out on *Lactuca sativa in vitro*. This test allows the selection of plant growth-promoting fluorescent *Pseudomonas* spp.strains for seed susceptibility in lettuce. Lettuce seeds were surface disinfected by soaking twice in 2% sodium hypochlorite for 2 min, and rinsed extensively at sterilised water after each soaking. Under aseptic conditions, the disinfected seeds were dried in sterile blotting paper and dispersed in petri dishes containing water Agar for 20 seeds per petri dish and incubated for 24 hours at room temperature. This test consists of inoculation of the seeds of this plant, which are pregermed on a petri dish by fluorescent *Pseudomonas* spp. strains and then measurement of the germination percentage and radicle length after 48 h of incubation.

3. Morpho-physicochemical characterization of selected fluorescent phytobenefic *Pseudomonas* spp. strains

All eight isolates coded (FR15, OD11, OD21, FR13, FR14, OD13, GB13, FR24) have been identified according to their morphological and biochemical characters. Morphological characteristics of the colony of each isolate were examined after culture on Nutrient agar and King's B medium.Cultural characterization of isolates observed by different characteristics of colonies such as shape, size, elevation, surface, margin, colour, odour, pigmentation etc were recorded as per Bergey's Manual of Determinative Bacteriology. The cell shape and gram reaction were also realized. Different biochemical tests performed such as Catalase test and oxidase test. Finally, The eight selected strains were tested for their ability to grow at 4°C and 41°C temperatures, respectively.

4. Production of enzymes of agricultural interest

Some enzymes produced by soil bacteria (cellulase, chitinase, protease etc.) play an important role in the degradation of organic matter in the soil by contributing to its fertility and the availability of mineral elements necessary for plant nutrition. In addition, these enzymes can be involved in the suppression of plant diseases caused by phytopathogens by participating in the degradation of cell walls. in this study, four enzymes were investigated (cellulases, amylases, proteases, lipases) in the eight isolates and the protocols followed are briefly outlined below.

a. Cellulases : To reveal the expression of enzymes with cellulase activity, an Nutritive broth (BN) was prepared by adding cellulose as the only carbon source, then the broth thus prepared is autoclaved at 121°C/20 min. The eight strains were seeded in tubes containing 15ml of BN, then incubated at 28°C for 24 hours. After 24 hours of incubation, the Fehling reagent (A and B) is added and heated, the appearance of a red brick pellet indicates a positive reaction.

b. Amylases : The ability of the strains studied to degrade starch is demonstrated on a starch-based culture medium (starch agar).The eight strains were seeded by touch (spots) in a petri dish, then incubated at 28°C for 24 hours. A positive reaction is marked by the appearance of a clear halo around the colonies after flooding all the petri dishes with a lugol solution.

c. Proteases : The protease activity of the isolates is revealed on the milk agar prepared as follows: to 100 ml of ordinary liquefied agar cooled to 45°C, 5 ml of sterilized milk is added. The strains studied were seeded by touch (spots) in a petri dish, then incubated at 28°C for 24 hours. A positive reaction is revealed by the appearance of a clear halo around the colonies.

d. Lipases : The search for lipases is carried out by culturing isolates on GN with 1% oleic acid added. A positive reaction is marked by the appearance of a halo around the colonies.

5. Evaluation of the biocontrol activity of selected strains and their secondary metabolites against a phytopathogenic fungus *Fusarium oxysporum*

The antimicrobial potency of isolates is determined by culturing the 18-hour-old bacterial suspensions studied, with the phytopathogenic fungus using the "Dual plate assay" method (Vincent et al., 1991). The eight bacterial isolates are spread 1cm from the edge of a petri dish containing PDA (Potatoes Dextrose Agar) and incubated overnight at 28°C, while a 8mm fungal disc from a 7-day culture is placed in the centre of the dish. The boxes are then incubated for one week at room temperature. The inhibition percentage is calculated according to the following relationship:

$$I= [(T-C)/T] \text{ X } 100$$

I: percentage of inhibition of the fungi tested (%).

T: mycelium diameter in the control dish (mm).

C: mycelium diameter in bacterially inoculated petri dishes (mm).

The search for metabolites with antibiotic effect was carried out in strains that showed an antagonistic power against the fungal pathogenic strain "*Fusarium oxysporum*". The production of phenazine antibiotics is manifested by PDA pigmented colonies which, when examined under UV light at 365nm, reveal dark areas around the phenazine producing colonies (Thomashow and Weller, 1988). The production of phenazines and pyrrolnitrins, *in vitro*, was investigated in FR14 and FR15 strains that showed a greater antagonistic power (52.72% and 41.81%) respectively against the pathogenic fungus

Fusarium oxysporum. The fresh 18-hour inoculum of the FR14 and FR15 strains was inoculated into 250 ml capacity Erlenmeyers containing 100 ml of BN with yeast extract and incubated for 5 days at 28°C under permanent agitation (180 rpm). For the extraction of phenazines, the fermentation broths were acidified (pH2) with HCl and then centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was extracted with the same volume of ethyl acetate, the organic phase containing the metabolites was first filtered through anhydrous ammonium sulphate and then vacuum evaporated in a rotavapor at 55°C. The dry extract is resuspended in 1ml of methanol. For the extraction of pyrrolnitrins, the pellets were suspended in 5ml of acetone and mixed then the acetone phase is evaporated and the residual aqueous phase was extracted with 15ml of ethyl acetate then evaporated at 55°C, the dry residue was retained in 25µl of methanol. The fractionation of the extract was carried out by TLC on silica gel. The deposition volume is 30 µl and the mobile phase consists of chloroform/ethyl acetate (1:1). After migration and evaporation of the solvent, the bands have been delimited and the frontal ratios are calculated (Rf).

The antifungal activity of the crude extracts obtained from bacterial strains FR14 and FR15 was tested against two phytopathogenic fungi *Fusarium oxysporum* and *Aspergillus niger* using the disc method (Vincent, 1947). The sterile discs were impregnated in a solution of crude extract of the metabolites and then placed in petri dishes containing a fungal explant in the centre, then incubated at 28°C for one week. The antifungal activity is expressed according to the percentage of inhibition (I%).

RESULTS AND DICUSSIONS

1. Isolation of fluorescent Pseudomonas spp. from the rhizosphere

Isolation from the rhizosphere part of the six durum wheat and barley plots for the three sampling sites allowed us to obtain 25 strains of *Pseudomonas*, of which 10 strains (40%) were isolated from the Ghar El Baroud region, 9 strains (36%) were isolated from the REMINI Farm and 6 strains (24%) were from the Oued DIRIS region. On the other hand, 19 strains (76%) originate from durum wheat root fragments (S1 and S2), 6 strains (24%) have been isolated from barley root fragments (S3).

After the UV fluorescence test (365nm), only 19 strains revealed the presence of a fluorescent green pigment (Pyoverdine) (**Figure 1**). The latter were transplanted onto new petri dishes containing King's B medium for purification.

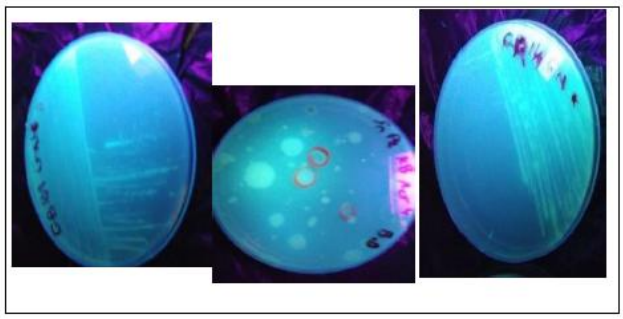

Fig. 1. Appearance of Pseudomonas spp colonies fluorescent under UV light (365nm)

2. Screening of strains of *Pseudomonas* spp fluorescent rhizospheric phytobeneficial strains

The screening of phytobeneficial fluorescent Pseudomonas strains reveals an important test by means of an *in vitro* test; bio-test on lettuce seeds.

The bioassay allowed us to classify the 19 strains of fluorescent *Pseudomonas* in three categories compared to the uninoculated control (**Figure 2.2**): *Lactuca sativa L* seedling growth inhibitory strains. (4 strains, or 21.04%), growth promoting strains (8 strains, or 42.10%) (**Figure 2.1**) and strains with no effect on plant growth (7 strains, or 36.84%). In addition, we have found that some inhibitory strains have caused not only total inhibition of sprouted seed growth but also necrosis of part of the radicles (**Figure 2.3**).

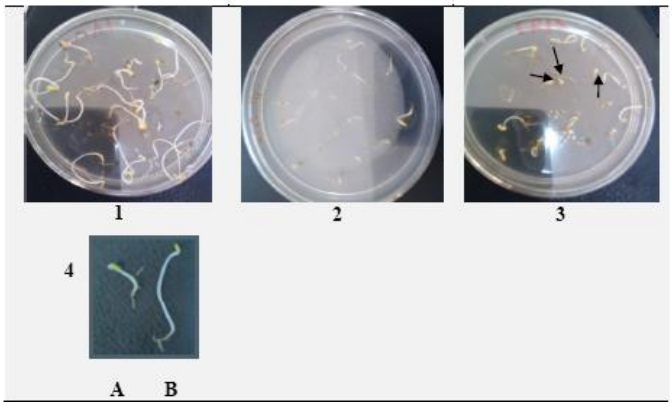

Fig. 2. Effect of fluorescent Pseudomonas spp strains in lettuce bioassay
1. Stimulation of growth, 2 control, 3. growth inhibition,
4. A/ control seedling, 4. B/ stimulated seedling, The arrows indicate a necrosis

Taking into consideration the geographical origin of the eight strains stimulating plant growth, 1 strain (12.5%) is native to the first site (Ghar El Baroud), four strains (50%) are natives to the second site (Ferme REMINI) of which two strains have induced the greatest stimulation of the length of the roots of *Lactuca sativa*. Finally, three strains originated from the third site (Oued DIRIS) (37.5%), one of which induced maximum stimulation of lettuce seeds. Maximum stimulation of seed growth with an average length of 23mm was obtained following the application of the OD11 coded strain originating from the third site (Oued DIRIS).

3. Morpho-physicochemical characterization of selected phytobeneficial fluorescent *Pseudomonas* spp strains

After 48 hours of incubation, the strains were observed with the naked eye (Table 1), all strains developed small or medium, smooth, round or irregular colonies. three strains showed yellow-green pigmentation, while the other five did not develop pigmentation. Under UV_{365nm} light, the strains showed a greenish-yellow pigment on both culture media, with very intense fluorescence in King's B medium.

Table 1

Cultural characteristics of fluorescent *Pseudomonas* spp isolates on different culture media

Strains	Aspects of the colonies on NA (Nutrient Agar)	Aspect of the colonies on King's B
OD11	Yellow, round, convex, brilliant	Round, yellow green, convex
FR24	Yellow, round, convex	Round, white, convex
OD21	White, irregular, smooth, flat, opaque	Irregular, white, convex
OD13	Yellow, round, convex	Round, yellow green, convex
GB13	Yellow, round, convex	Round, white, convex
FR14	Yellow, round, convex, brilliant	Irregular, yellow-green, convex
FR15	Pale white, convex	Round, white, flat
FR13	White, irregular, smooth, flat, flat, opaque	Round, white, flat

Microscopic observation in the fresh state revealed highly mobile and asporulated bacilli. Gram staining revealed straight or slightly curved Gram negative bacilli with rounded tips. The cells are isolated or grouped in pairs.

- **Physico-chemical characteristics**

The positive reaction of cytochrome oxidase is an essential feature in the identification of *Pseudomonas* species. All strains are oxidase (+). All strains studied are Gram-, mobile, catalase (+), citrate (+), ADH (+). In addition, they give a negative answer to LDC, ODC, TDA, H_2S, Urease, VP. All strains produce a water-soluble pigment, pyoverdine, on King's B medium. Four strains (FR14, FR24, OD11, OD21) are unable to synthesize pyocyanin on King'A, and unable to grow at 41°C. These characters are oriented towards the species *P. fluorescens* or *P. putida*. In addition, the growth of FR14 and OD11 strains at 4°C confirms their belonging to the *P. fluorescens* species. FR24 and OD21 belong to the species *P. putida* due to the absence of gelatinase. The other species (FR13, FR15, GB13, GB13, OD13) capable of synthesizing pyocyanin and growing at 41°C and hydrolyzing gelatin (Gel+) belong to the *P.aeruginosa* spp.

4. Production of enzymes of agricultural interest

The strains studied were tested for their ability to produce enzymes involved in the degradation of organic matter and in the suppression of fungal diseases by degrading fungal walls (proteases, cellulases, amylases, etc). After 24 hours of incubation, a total absence of cellulases in all strains is noted, while fungal wall degrading enzymes such as proteases are produced by six strains, amylases and lipases are produced by five strains (Figure 3). The OD11, FR14, FR24 and FR13 strains are all producers of proteases, amylases, lipases.

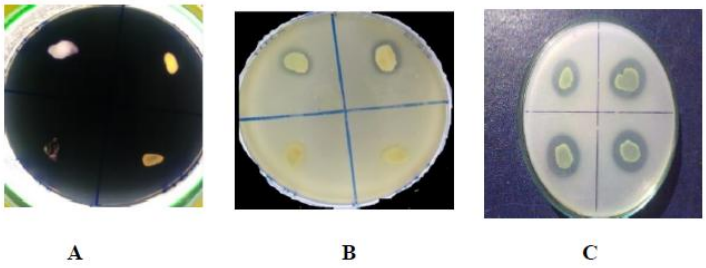

Fig. 3. Enzyme production by the strains studied
A/Amylases , **B**/Proteases, **C**/lipases

5. Evaluation of the biocontrol activity of selected strains

a. *In vitro* antagonism test against *Fusarium oxysporum*

Through antagonism test against *Fusarium oxysporum*, the inhibition zones around the fungus developed by fluorescent *Pseudomonas* strains is estimated. Four strains (OD11, FR24, FR15, FR14) among the eight strains exhibit an inhibitory activity against the phytopathogenic fungus (Figure 4). The diameter of *F.oxysporum* mycelial colonies was significantly reduced in the presence of fluorescent *Pseudomonas* antagonist strains compared to the negative control. The strongest inhibitory activity is estimated at 52.72% for strain FR14, 41.81% for strain FR15 and 34.54% for strain FR24. The OD11 strain shows the lowest inhibitory activity with an inhibition percentage of 12.72%.

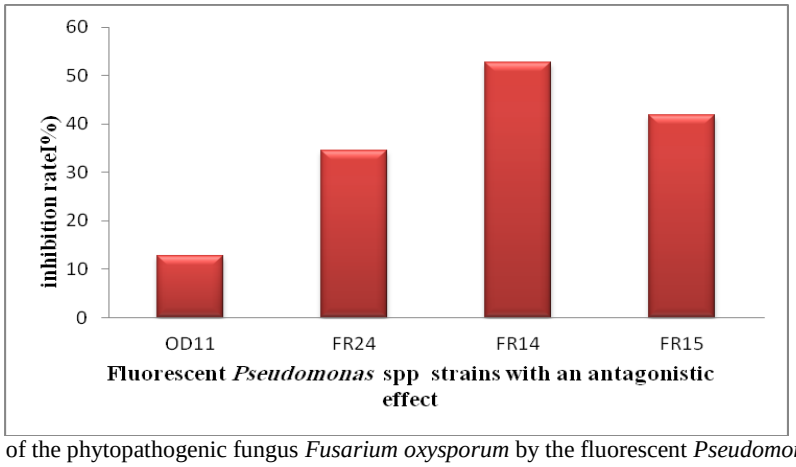

Fig. 4. Inhibition rate of the phytopathogenic fungus *Fusarium oxysporum* by the fluorescent *Pseudomonas* spp strains tested

b. Extraction of secondary metabolites with an antifungal effect

Crude extracts from the cultures of bacterial strains FR14 and FR15 were evaluated for the R_f values and spot color on the TLC plate. The phenazine extract obtained by ethyl acetate gave a single yellow spot on the TLC plate (**Figure 5**) with an equal frontal ratio of 0.62 for strain FR14 and 0.6 for strain FR15.

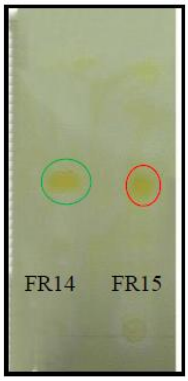

Fig. 5. Identification of compounds of phenazic extracts of strains FR14 and FR15

2. Evaluation of the antifungal activity of crude extracts of two strains FR14 and FR15

After one week of incubation at room temperature, phenolic extracts of two strains showed a percentage of inhibition against the two pathogenic strains *Aspergillus niger* and *Fusarium oxysporum* that varies from one strain to another. Figure 6 shows the inhibition rate by the two strains tested. The results of the rate of inhibition of pathogenic fungi by phenazic extracts from the strains tested showed that the phenazic extract of strain FR14 inhibits the mycelial growth of both fungi more than that of strain FR15. This difference can be explained by the effectiveness of the extract itself or the variability in the sensitivity of the fungi.

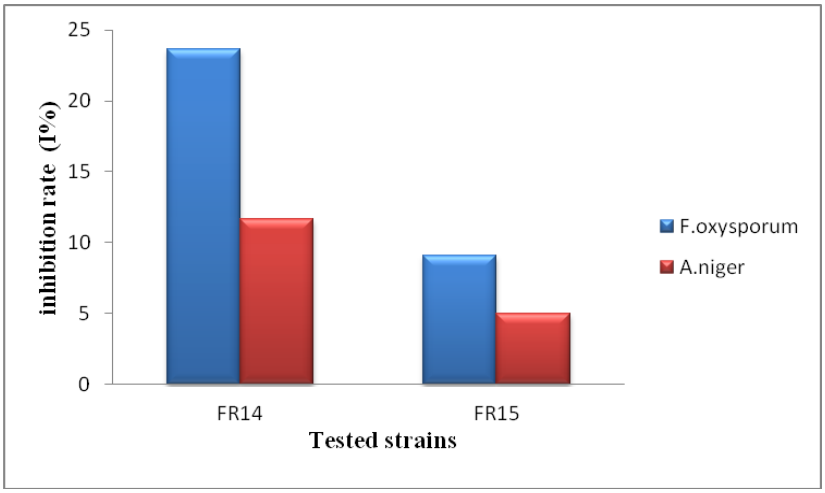

Fig. 6. Inhibition rate of pathogenic fungi by phenazic extracts from the strains tested

CONCLUSION

PGPR (in our case fluorescent *Pseudomonas* spp.) has become a very interesting alternative to the intensive and repeated use of chemical synthetic antibiotics by using compounds synthesized by these bacteria that play a role in disease suppression.

Nineteen strains of fluorescent *Pseudomonas* spp were isolated from the rhizosphere. These strains were pre-selected for their stimulating or plant growth inhibiting effect, eight strains were phytostimulatory strains with a stimulation percentage of 42.10%. This result allows these strains to be used soon as phytostimulators.

The strains thus screened were phenotypically identified. This study grouped the isolates into three groups: *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Pseudomonas putida* with a dominance of the Pseudomonas aeruginosa type species (four strains among the eight strains). However, phenotypic identification must be confirmed by genotypic identification because there is a very fine line between the two species *P. fluorescens* and *P. putida* and also the existence of biovars in the same bacterial species.

Among identified strains, four strains showed an antagonistic power *in vitro* against a pathogenic fungus *Fusarium oxysporum*. These strains have been found to produce secondary antifungal metabolites such as fungal wall degrading enzymes (proteases) and antibiotics (phenazines) as well as enzymes involved in improving soil fertility (amylases, proteases, lipases), allowing them to be biocontrol agents, biofertilization.

In order to better understand the antagonistic mechanisms existing in *Pseudomonas*, some secondary metabolites, phenazines and pyrrolnitrins have been extracted. The results showed a presence of phenazines and the absence of pyrrolnitrins. Phenazines are the main pigments that play a crucial role in the direct antagonism between these bacteria and phytopathogenic fungi. However, further studies are needed in order to better understand the biological properties of these compounds. Although the screening of phytobeneficial strains works with a view to locating strains that have combined the maximum of antagonistic activities with respect to phytopathogens, *in vitro* antagonism alone cannot be established as the fundamental selection criterion. Investigations and other studies will be able to deepen an effective screening of the most efficient strains.

From a taxonomic point of view, genotypic identification would be necessary because of the *P. fluorescens -P. putida* group is a very heterogeneous group requiring deeper genetic studies. It would also be necessary to study and identify other secondary metabolites produced by *Pseudomonas* with antimicrobial activity and to continue studies on the biological properties of these compounds and to understand their mechanisms of action.

Finally, from an agricultural application point of view, it would be necessary to advance studies to understand the complexity of the rhizospheric environment, the mechanisms of action of PGPR and to apply these organisms in sustainable agriculture either in the form of inoculants or formulations.

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