ANTAGONISTIC EFFECT OF PLANT GROWTH PROMOTING RHIZOBACTERIA ASSOCIATED WITH *Rhus tripartitus* ON GRAM POSITIVE AND NEGATIVE BACTERIA

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Abstract. Antagonistic rhizobacteria play an important role in biological control by producing lytic enzymes and antibiotics and then inhibiting the growth of a large number of pathogenic agents. The present work is in a perspective of antagonists' strains exploration among sixty Plant Growth Promoting Rhizobacteria isolated from *Rhus tripartita's* rhizosphere, characterized in a previous study. Therefore, six bacterial strains are tested: *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Enterococcus faecalis* and *Klebsiella pneumoniae*. Different techniques were used: direct inoculation of bacterial cultures, diffusion discs impregnated with the supernatant and the use of bioactive substances extracted. The hydrolytic activity of carbohydrates, lipids and proteins of the positives strains was evaluated. In the present study, the antagonism activity proved to be more relevant for bacterial products than for bacterial culture. Moreover, out of the 60 PGPR strains utilized, 12 showed antagonistic potential against Gram positive and negative bacterial strains tested. Furthermore, the majority (66.66%) of the isolates assayed in our experiment were Gram-positive and belonged to *Bacillus* genera, compared to only 33.33% Gram-negative. The maximum zone inhibition was 20 mm, and the minimum zone inhibition was 12 mm. In the same way, the tested strains could produce at least two hydrolytic enzymes. The antagonistic effect of selected PGPR suggests the possibility of directly including these microorganisms in preventive control program against plant microbial infections or indirectly through the application of active substances as a curative treatment.

Keywords: PGPR; antagonism; antibiosis; hydrolytic enzymes.

INTRODUCTION

The rhizosphere is an important ecological niche of microbial biodiversity, which interact with each other's and with host plants roots. Therefore, Rhizospheric microorganisms exert various effects on plants by influencing their development [16]. Moreover, some of these microorganisms can hinder the good development of plant while others benefit it.

Furthermore, beneficial rhizobacteria can secrete substances to inhibit the growth of phytopathogenic microorganisms, called antagonist bacteria. Consequently, antagonism is the ability of one germ to inhibit the growth of another germ when they are in the same micro-biotope. Similarly, it expressed in laboratory when they are grown together in the same Petri dish. However, majority of studies showed that only 1 to 10% of soil isolates may have some antagonist potential in vitro, and a small number are able to inhibit a broad spectrum of pathogenic species [19]. On the other hand, antagonists' rhizobacteria play a role in biological control by inhibiting the growth of a large number of phytopathogenic agents [13]. Indeed, the various biocontrol mechanisms include secretion of extracellular metabolites like hydrogen cyanide, siderophores, antibiotics, hydrolytic enzymes and / or competition for nutrients [13-23].

Beneficial Rhizobacteria to plant are called Plant Growth Promoting Rhizobacteria (PGPR), they can also play a role in crop protection and soil improvement. Some consideration of the PGPR relationship to biocontrol is worth studying. In the same way, PGPR strains increase plant development indirectly by suppressing diseases caused by known pathogens or by reducing the deleterious effects of minor pathogens (which do not produce obvious symptomes) [29]. Moreover, some species are well known such as *Pseudomonas, Bacillus, Azospirillum, Rhizobium* and *Serratia* [13]. Furthermore, Bashan and Holguin (1998) [4] suggested that bacteria with PGP and protective effects at the same time could be reclassified into one category: Plant-Growth Promoting Bacteria Biocontrol (PGPB Biocontrol).

The arid Algerian soil remains unexplored, in particular the Ahaggar. The present work aims to select PGP strain isolated from *Rhus tripartitus* rhizosphere characterized in our previous study [6] endowed with antagonistic effect. Therefore, the likely role of PGPR in biocontrol is demonstrated through the antagonistic potential reflected in the screening of hydrolytic enzymes and the extraction of inhibitory substances against Gram negative and positive bacteria.

MATERIALS AND METHODS

In our experiment, the bacterial strains include 60 PGPR isolates of *Rhus tripartitus* rhizosphere from arid area of Algerian Sahara [6]. The strains used to antagonism test were: *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Enterococcus faecalis* and *Klebsiella pneumoniae* (procured by Laboratory of Science and Environment Research, Universitary Center of Tamanrasset, Algeria).

Bacterial strains were cultured on nutrient broth (NB), at 30 °C for PGPR and 37 °C for tested strains.

The optical density of each suspension is adjusted to 0.5 at 650 nm. Then, the bacterial solutions of positive strains from the first experience will be centrifuged at 4000 rpm / 20 min and the supernatants were recovered.

Direct inoculation technique (submerged culture) was performed according to Shomurat et all. (1979) [25], inoculum spots (0.1 ml) of PGPR strains were inoculated on Plat Count Agar (PCA) and incubated at 30 °C / 24h. After incubation, dishes are first inverted above the chloroform for 40 min and a thin layer of the same medium above the colonies, which will be used to seed the strains to be tested. Dishes are then incubated a second time at 37 °C / 24h. This technique is supposed to be a preliminary indicator of the antagonist potential.

Diffusion disc method was performed as described previously [11]**.** Wahtman (no. 1) paper disks of 6 mm diameter were prepared and sterilized in autoclave at 115 °C / 20 min. Disks were then impregnated with the supernatants for 1 hour in a sterile Petri dish. With aid of a metal clamp, discs are deposited on surface of Muller Hinton agar (MH agar) inoculated beforehand by the strains to be tested. After incubation at 37 °C / 24 h the inhibition halos are measured using a vernier caliper.

The technique performed to determine antibacterial activity of extracts was direct diffusion on agar [20]. It consists of cutting wells (6 mm diameter) in MH agar inoculated beforehand by tested strains. On the other hand, a few drops of agar are deposited at the bottom of the wells to prevent the leakage of the deposited extracts (200 µl). For a better diffusion of the bioactive substance, dishes are first placed in the refrigerator for 10 min before incubating at 37 °C / 24h. Therefore, the extraction and purification of bioactive molecules present in the supernatant were carried out by using two organic solvents (chloroform and ethyl acetate). According to Belter [5], supernatant was mixed with an equal volume of solvent and stirred until two phases (organic and aqueous) were obtained. Organic phase is recovered and its antibacterial activity is tested against pathogenic strains (following experience).

Actives PGPR strains, whose extracts showed antibacterial effects, were selected to test their hydrolytic capacity of organics matters. Hydrolysis test of gelatin was inspired by the technique of [12], using in our experiment a nutrient broth supplemented with 50 g / l of gelatin powder which will used as a solidifying agent, previously filtered by a filter paper (0.22 µl). Then, the liquefaction of the medium after incubation at 20 °C / 7h reflects a gelatinolytic activity. Ureaolytic activity is revealed on Christensen's Urea Agar medium (peptone 1 g, dextrose 1 g, Sodium Chloride 5 g, Potassium phosphate monobasic 2 g, urea 20 g, phenol red 0.012 g, agar 15 g) and the medium color change from yellow to fuchsia pink indicates a positive reaction. Hydrolysis of casein is tested on MH agar supplemented with 10% of skimmed milk by inoculation of 10 µl of bacterial suspension and incubated at 37 °C/ 24 h; a positive reaction is reflected by appearance of a transparent halo around the colony [8].

Amylolytic activity was detected on Tryptic Soja Agar (TSA 1/10) supplemented with 1 % starch, after a spot inoculating, dishes are incubated and revelation is done by flooding dishes with an iodine solution (Lugol). Starch hydrolysis results in a clear halo around the colony, unlike blue-starched areas [10]. Hydrolysis of cellulose is demonstrated on Agar medium supplemented with ground pulp as a source of cellulose and cellulosic activity is demonstrated by the appearance of a clear halo around the colony [18].

Revelation of lecithinase was carried out on an ordinary nutrient agar supplemented with an emulsion of egg yolk and distilled water (2 ml / 20 ml). Then, the opacification of agar around the colony reflects the presence of a lecithinase while the appearance of a white opaque halo means the presence of lipase [9].

All strains classified as positive for antagonistic potential were identified through standard catalase, oxidase and Gram assays. API 20 E, 20 Staph, 10 NH, 50 CHB and 20 NE (BioMèrieux, Lyon, France) were used for a biochemical characterization.

RESULTS

This study objective is to evaluate the antagonism potential of PGPR strains isolated from a previous work done on *Rhus tripartitus's* rhizosphere. Therefore**,** out of the 60 PGPR strains utilized, 12 showed antagonistic effect against Gram positive and negative bacteria. Furthermore, based on morphological and biochemical characteristics: 66.66% of the total antagonist PGPR strains belonged to the genus *Bacillus,* followed-up by *Escherichia* (16.66%)*, Pseudomonas* (8.33%) and *Kocuria* (8.33%).

On the other hand, our experimental results showed that the supernatant activity is slightly higher than that of the rough culture (Table 1) which means that in fact the bacterial extracellular substances were more active than the bacterium in itself. Therefore, we propose that all the former tests will have to thus be realized on the supernatant. Moreover, we have assumed that these antagonists' effects are closely related to antibiosis phenomenon. In fact, the extraction of actives molecules made it possible to clearly demonstrate an antagonistic activity against selected bacteria strains. Therefore, ethyl-acetate showed a better extraction yield than chloroform (Table 2, Fig. 1). Nevertheless, the chloroformic extract of 8/12 of PGPR strains tested showed positive effects and the inhibitory zones varied between 12 and 20 mm diameter (Table 2). However, the extracts effect didn't showed significant difference on both Gram-negative and positive bacteria.

Nevertheless, the extracts showed distinct inhibitory effects compared to the strains tested. Therefore, *Proteus mirabili*s appears to be the most sensitive strain with inhibitory zones of submerged culture (11 mm), supernatant (14 mm), chloroformic

extract (18 mm) and ethyl-acetate extract (20 mm) followed-up by *Staphylococcus aureus* and *Enterococcus faecalis* (Tables 1 and 2). In contrast, *Klebsiella pneumoniae*, seems to be the most resistant species to both extracts of all PGPR strains tested (Table 2).

In the same perspective, we have evaluated the PGPR hydrolytics activities with respect to cellulose, starch, gelatin, urea, casein and lecithin. Indeed, gelatinase is the enzyme responsible for gelatin degradation which causes the medium liquefaction. This activity is demonstrated in 66.66% strains. In addition, the urea hydrolysis was showed in 66.66% strains. This hydrolytic reaction is reflected by change of the medium red color to purple pink resulting of alkalinization, which indicates the urea degradation and the release of ammonium ions. Regarding casein hydrolysis, 50% of strains were able to produce a transparent halo around colonies which reflects a positive reaction. Furthermore, hydrolysis of starch and cellulose has been demonstrated respectively in 58.33% and 75% of positive strains. However, all strains were negative for lecithin hydrolysis, but they were endowed with lipase (Table 3).

DISCUSSION

Management of plant pathogens with pesticides has resulted in environmental pollution and resistance among pathogens [13]. Subsequently, isolation of the rhizobacteria that promote growth and protect plants at the same time; makes it possible to target a multifunctional bacterial population of great interest for agriculture corps.

In our experiment, the positive selected strains were mostly belonging to *Bacillus* with a high diversity of species, such as *Bacillus megaterium, B. licheniformis, B. subtilis* and *B. circulans.* Furthermore, antagonist PGPR of the genera *Bacillus* have been reported in many studies [17, 3, 24]. On the other hand, *Pseudomonas aeruginosa* performed in this study have shown antagonist effect on two bacterial species. Then, certain volatile compounds (HCN) emitted by bacteria of the genus *Pseudomonas* have antibiotic effects and play a role in the host plant protection [27] and a biocontrol agent [28]. Furthermore, previous investigations showed that *Escherichia vulneris* have an antagonistic effect [2, 21]. However, our investigation showed that the majority (66.66%) of the isolates assayed for antagonistic activity were Gram-positives compared to only 33.33% that were Gram-negative. These results are in-line with studies realized on antagonist bacteria from tobacco rhizosphere [15].
On the other hand, the results showed that extracts made with organic solvents have a higher effect than supernatant. Therefore, the antagonistic activity can be attributed to the bioactive molecules present in organic extracts. Moreover, solvents cause the molecules to detach and become soluble. Furthermore, it has been previously reported that organic extracts had shown a better antibacterial effect than aqueous extracts. Then, ethyl-acetat extract provided better antagonistic activity. Indeed, several parameters affect the effectiveness of bioactive substances, it depends on bacterial species, whether resistant or sensitive, the solvent type and the method used. Therefore, PGPR extracts showed a significant broad spectrum activity against all tested bacteria with an area size ranging from 7 to 20 mm diameter. Similary, many studies have shown an antagonistic effect of PGPR strains against bacterial pathogens such: *Ralstonia solanacearum* [1]*, xanthomonas axonopodis* [22], and *Erwinia carotovora* [7].

Moreover, secretion of microbial hydrolytics enzymes in the rhizosphere confers nutrition competitive and predation advantage to biocontrol microorganism. In soil, the antagonistic activity of telluric microorganisms beneficial to the pathogens growth, results on the antibiotics or enzymes productions [23]. Therefore, we qualitatively analysed the hydrolytic power of PGPR positive strains of cellulose, starch, gelatin, urea, casein and lecithin. The production of lytic enzymes by antagonistic bacteria can disintegrate the cells of pathogens [26]. In the same way, the tested strains could produce at least two hydrolytic enzymes in addition to their inhibitory potential.

Table 1. Phenotypical and biochemical Identification of PGPR-antagonists species

Code	Species	Division	Catalase	Oxidase	Gram	API
Rt 1	*Bacillus licheniformis*	Firmicutes	positive	negative	positive	20 E + 50 CHB
Rt 2	*Bacillus circulans*	Firmicutes	positive	negative	positive	20 E + 50 CHB
Rt 3	*Pseudomonas aeruginosa*	Proteobacteria	positive	positive	negative	20 NE
Rt 4	*Bacillus megaterium*	Firmicutes	positive	negative	positive	20 E + 50 CHB
Rt 5	*Bacillus subtilis*	Firmicutes	positive	positive	positive	20 E + 50 CHB
Rt 6	*Escherichia vulneris*	Proteobacteria	positive	negative	negative	20 E
Rt 7	*Bacillus megaterium*	Firmicutes	positive	negative	positive	20 E + 50 CHB
Rt 8	*Kocuria varians*	Actinobacteria	positive	negative	positive	20 Staph
Rt 9	*Bacillus subtilis*	Firmicutes	positive	positive	positive	20 E + 50 CHB
Rt 10	*Bacillus licheniformis*	Firmicutes	positive	negative	positive	20 E + 50 CHB
Rt 11	*Escherichia vulneris*	Proteobacteria	positive	negative	negative	20 E
Rt 12	*Bacillus licheniformis*	Firmicutes	positive	positive	positive	20 E + 50 CHB

Table 2. Supernatant and bacterial culture activity of PGPR strains exhibiting inhibition zones (mm)

Tested bacteria	PGPR species																							
	Rt 1		Rt 2		Rt 3		Rt 4		Rt 5		Rt 6		Rt 7		Rt 8		Rt 9		Rt 10		Rt 11		Rt 12	
	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
a	9.66 ± 0.23	14.66 ± 0.44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
b	-	-	-	-	-	-	-	-	-	-	9.33 ± 0.44	11.33 ± 0.69	14	15.66 ± 0.69	10	13.66 ± 0.23	-	-	-	-	-	-	-	-
c	-	-	8.33 ± 0.69	9.33 ± 0.44	10	15	8.33 ± 0.44	12	7.33 ± 0.44	12.66 ± 0.46	-	-	-	-	-	-	-	-	-	-	-	-	-	-
d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
e	-	-	-	-	9	11.66 ± 0.23	-	-	13.33 ± 0.44	16	-	-	-	-	9	11.66 ± 0.44	-	-	-	-	-	-	-	-
f	-	-	7	9.66 ± 0.23	-	-	-	-	9	11	-	-	-	-	-	-	11	14	8.33 ± 0.44	11	7.66 ± 0.23	8.66 ± 0.44	9.33 ± 0.46	10

where: **S :** Supernatant**; C :** bacterial culture; **-** : negative effect; **a** : *Escherichia coli*; **b** : *Pseudomonas aeruginosa*; **c** : *Staphylococcus aureus* ; **d** : *Klebsiella pneumoniae* ; **e** : *Enterococcus faecalis* ; **f** : *Proteus mirabilis ;* **Rt 1** : *Bacillus licheniformis* ;**Rt 2**: *Bacillus circulans* ;**Rt 3**: *Pseudomonas aeruginosa* ;**Rt 4**: *Bacillus megaterium* ; **Rt 5**: *Bacillus subtilis ;* **Rt 6**: *Escherichia vulneris ;* **Rt 7**: *Bacillus megaterium* ; **Rt 8**: *Kocuria varians* ; **Rt 9***: Bacillus subtilis* ;**Rt 10**: *Bacillus licheniformis* ;**Rt 11**: *Escherichia vulneris* ;**Rt 12**: *Bacillus licheniformis.*

Table 3. Antagonism effect of positive PGPR extracts exhibiting inhibition zones (mm)

Tested bacteria	PGPR species																							
	Rt 1		Rt 2		Rt 3		Rt 4		Rt 5		Rt 6		Rt 7		Rt 8		Rt 9		Rt 10		Rt 11		Rt 12	
	CE	EE	CE	EE	CE	EE	CE	EE	CE	EE	CE	EE	CE	EE	CE	EE	CE	EE	CE	EE	CE	EE	CE	EE
a	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
b	-	-	-	-	-	-	-	-	-	-	-	13	-	16.66 ± 0.56	14.66 ± 0.44	8.66 ± 0.23	-	-	-	-	-	-	-	-
c	-	-	13.33 ± 0.78	14.66 ± 0.46	14.66 ± 0.23	-	-	16	-	15.66 ± 0.58	-	-	-	-	-	-	-	16	-	-	-	-	-	-
d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
e	-	-	-	-	13.33 ± 0.81	-	-	-	15	10	-	-	-	-	13.33 ± 0.46	16	-	-	-	-	-	-	-	-
f	-	-	13	14.66 ± 0.44	-	-	-	-	17.66 ± 0.46	13.66 ± 0.44	-	-	-	-	-	-	-	-	18 ± 0.69	13.33 ± 0.44	12	20	14.66 ± 0.23	13

where: **EE :** ethyl-acetate extract**; CE :** chloroformic extract ; **-** : negative effect; **a** : *Escherichia coli*; **b** : *Pseudomonas aeruginosa*; **c** : *Staphylococcus aureus* ; **d** : *Klebsiella pneumoniae* ; **e** : *Enterococcus faecalis* ; **f** : *Proteus mirabilis ;* **Rt 1** : *Bacillus licheniformis* ;**Rt 2**: *Bacillus circulans* ;**Rt 3**: *Pseudomonas aeruginosa* ;**Rt 4**: *Bacillus megaterium* ; **Rt 5**: *Bacillus subtilis ;* **Rt 6**: *Escherichia vulneris ;* **Rt 7**: *Bacillus megaterium* ; **Rt 8**: *Kocuria varians* ; **Rt 9***: Bacillus subtilis* ;**Rt 10**: *Bacillus licheniformis* ;**Rt 11**: *Escherichia vulneris* ;**Rt 12**: *Bacillus licheniformis.*

Table 4. Hydrolytics activities of actives PGPR strains

Enzymes Hydrolytic tests		PGPR strains											
		Rt 1	Rt 2	Rt 3	Rt 4	Rt 5	Rt 6	Rt 7	Rt 8	Rt 9	Rt 10	Rt 11	Rt 12
Hydrolysis of polyoses	Cellulose	-	+	+	+	-	+	+	-	-	+	+	+
	starch	-	-	+	+	-	+	+	-	+	-	+	+
Hydrolysis of lipids	Lecithin	-	-	-	-	-	-	-	-	-	-	-	-
	Lipid	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of proteins	Gelatin	+	+	-	+	+	+	+	+	-	-	-	+
	Urea	-	+	+	+	+	+	+	+	-	-	+	-
	Casein	-	+	-	-	-	-	+	+	+	+	-	+

where: + : positive effect; - : negative effect ; **Rt 1** : *Bacillus licheniformis* ;**Rt 2**: *Bacillus circulans* ;**Rt 3**: *Pseudomonas aeruginosa* ;**Rt 4**: *Bacillus megaterium* ; **Rt 5**: *Bacillus subtilis* ; **Rt 6**: *Escherichia vulneris* ; **Rt 7**: *Bacillus megaterium* ; **Rt 8**: *Kocuria varians* ; **Rt 9**: *Bacillus subtilis* ;**Rt 10**: *Bacillus licheniformis* ;**Rt 11**: *Escherichia vulneris* ;**Rt 12**: *Bacillus licheniformis*

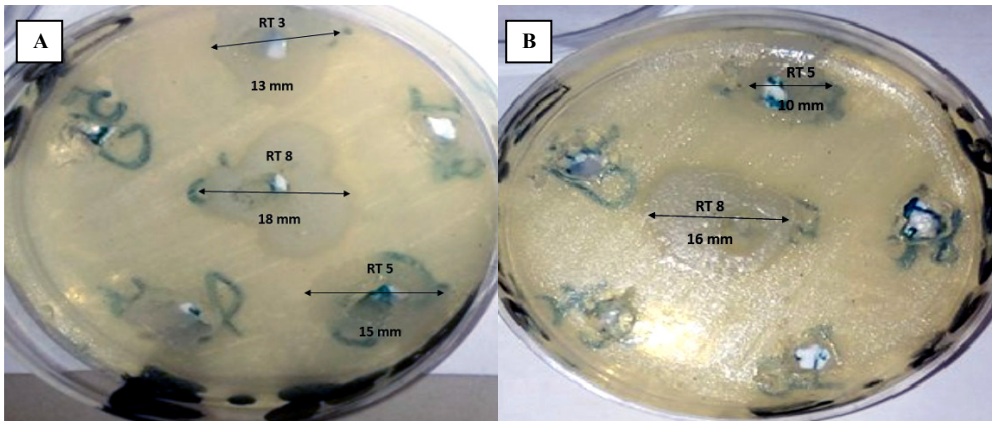

Figure. 1. Antagonistic activity of ethyl acetate (A) and chloroformic (B) extracts of Rt 3: *Pseudomonas aeruginosa,* Rt 5: *Bacillus subtilis* and Rt 8: *Kocuria varians* against *Enterococcus faecalis* using the direct diffusion method on Muller-Hinton agar. The figure clearly shows the inhibitory effect of organic extracts of bacterial supernatants from antagonists strains (Rt) on bacterial growth through the inhibition zones (mm).

Crop protection against pathogens is a major issue in agriculture. Chemical pesticides are widely used but not always in harmony with the environment. In alternative, biocontrol is proving to be a very promising strategy. In the present work, antagonistic effect of selected PGPR suggests the possibility of directly including these microorganisms in preventive control program against plant microbial infections or indirectly through the application of active substances as a curative treatment. However, further experiments are possible to test their effectiveness in vivo and optimize the production of bioactive molecules.

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