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Screening and Phenotypic Diversity of Amylase Producing Rhizospheric Bacteria from Some North African Plants

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Abstract

Plant Growth Promoting Rhizobacteria (PGPR) are gaining interest because of their positive effect on plant growth and defense. Among these effects some enzymes secreted by PGPR are used by the plant in nutrient uptake and in biocontrol. The aim of this work is to isolate amylase producing bacteria from some North African plants rhizospheres. Amylases are starch degrading enzymes and can play a role in plant growth promoting by degrading organic matter in soil (starch). Total rhizospheric bacterial diversity was screened and *Ceratonia ciliqua* and *Agrania spinosa* showed the best results. Also, *Caratonia ciliqua* and *Ficus carica* isolates gave the best amylase production. Finally, crude amylase produced by *Argania spinosa* and *Pistacia lentiscus* had the best activities.

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Keywords: Amylase; bacteria; rhizosphere; PGPR; diversity.

1. Introduction

In the last decades agricultural production is growing rapidly to meet world increasing demand. But to do so in a sustainable and environmentally friendly manner chemicals in agriculture is being replaced by plant growth promoting microorganisms (PGPM) especially bacteria (PGPB) [1]. Neverthless, most of the activities of PGPB

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have been studied in the rhizosphere (PGPR). These activities can affect positively the plant in different manners; directly by facilitating assimilation of nitrogen, phosphate and iron and by secreting certain hormones; or by playing a role in plant biocontrol by the production of antibiotics and sedirophores that protect plant against pathogens [2]**.** Enzymes also are synthetized by PGPR and are involved in both plant nutrition and biocontrol. The most active enzymes in soil include protease, urease, pectinase, cellulase, ehydrogenase, Catalase, amylase and phosphatase [3]**.** However, few studies about enzymes produced by PGPR especially amylases. In this context amylase producing rhizospheric bacteria were isolated from seven different plants ie: *Phoenix dactylifera, Ceratonia siliqua L*, *Eucalyptus globulus Labill* , *Pistacia lentiscus L*, *Argania Spinosa L*, *Ficus carica* , *Opuntia Ficus-indica.* The choice of these plants is due the fact that two are originally from North Africa (Cer S; ArgS) and the others widely present in the Mediterranean basin and adapted to its climate.

2. 2. Material and methods

2.1. Soil samples preparation

Soil samples were taken from eight different plant rhizospheres. Table 1 shows the details about plants and the zone of sampling. One gram of soil was collected from plant rhizosphere (attached to roots) and put in 900µl of saline water (9g NaCl per 1000ml distilled water). The solution was allowed to stand and the supernatant was used for the next tests.

Table 1. The plants used for soil sampling

Plant common name	Scientific name	Code	Sampling date	Sampling area
Palm	*Phoenix dactylifera*	Ph da	27/02/2014	Street in Bir El Djir (Oran)
Young Carob tree	*Ceratonia siliqua L*	CerS young	23 /02/2014	University of Science and Technology of Oran USTOMB.
Eucalyptus	*Eucalyptus globulus Labill*	Glob l	28/02/2014	Bani-saf (Ain timouchante)
Pistachio	*Pistacia lentiscus L.*	Pisl	04/03/2014	Street in Bir El Djir (Oran)
Argan tree	*Argania Spinosa L*	ArgS	23/02/2014	University of Science and Technology of Oran USTOMB.
Fig tree	*Ficus carica*	Fcar	28/02/2014	University of Science and Technology of Oran USTOMB.
Barbary fig tree	*Opuntia Ficus-indica*	OFind	23/02/2014	University of Science and Technology of Oran USTOMB.
Aged Carob tree	*Ceratonia siliqua L*	CerS aged	08/04/2014	University of Science and Technology of Oran USTOMB.

2.2. Culture media preparation

All culture media used in this work were sterilized by autoclaving at 121°C for 20min and pH were adjusted to pH 7:

- Nutrient Agar (NA): 1g peptone, 0.5g meat extract, 0.5g NaCl, 1.5g Agar powder.
- Starch agar (SA) : 50% Sea water (filtered) and 50% distilled water were mixed then 0.5 % technical grade starch (Sigma, USA) was added. Finally, 1.5% agar-agar was added to solidify the medium.

2.3. Phenotypic diversity of total rhizospheric bacteria

After decantation 100 µl of the supernatant was streaked on nutrient agar plates and incubated at 37°C for 24h and 96h.The macroscopic aspect of the colonies was studied using colony counter device. For the microscopic study of the bacteria isolates, a photonic microscope was used (x1600) for observation after GRAM straining.

2.4. Screening and phenotypic diversity of amylolytic rhizospheric bacteria

One hundred microliter of soil supernatant was spread on starch agar medium then incubated at 30°C for four days. Finally amylolytic activity was revealed using Iodine (I_2). Amylase activity detection was realized by measuring the diameter of the colony (X) and the diameter of the starch degradation zone (Y). Specific activity ratio (X/Y) was calculated. The grown colonies were subjected to macroscopic and microscopic observation.

2.5. Amylase production by amylase hyper producer isolates

The best amylase producer isolates from each plant rhizosphere were subjected to amylase production and activity revelation. In tubes containing 5ml of 0.5% starch, colonies of each bacterial sample were inoculated. After shaking with vortex, the tubes were incubated at 30°C for 48h. One milliliter of the cultures was then put in 2ml tubes and centrifuged at 14000 rpm for 15 min. Well cut diffusion method was used to reveal amylolytic activity. Two hundred microliter of the crude supernatant were put into wells punched in starch agar medium then incubated at 30°C for 24h. The amylase activity on liquid medium was also revealed by adding iodine directly and shaking with vortex. For the well cut diffusion method the revelation was done by exposing the plate to Iodine emanations. For both the methods starch degradation appeared as clear zone and non-degraded starch took a purple color.

3. 3. Results and discussion

3.1. Phenotypic Diversity of total rhizospheric bacteria

After 24h and 96h of incubation, nutrient growth plates were examined. Fig. 1 shows the effect of plant type and incubation time on total viable count of rhizospheric bacteria. Rhizospheres that showed the highest bacterial diversity were those of ArgS and CerS young, while, bacteria isolated from F.Car and O.Find showed the highest colony number. The obtained results prove that there is a clear difference in the total rhizospheric bacterial flora associated with each of the studied plants. A proportional relationship between the incubation time and the growth of the bacteria was also noticed.

The difference in total bacterial diversity between the plants rhizospheres is due to the fact that that all microorganisms are not attracted in the same way by the roots. Indeed, the roots exudates vary from a plant to another which leads to a variation in type and quantity of rhizospheric microorganisms [4].

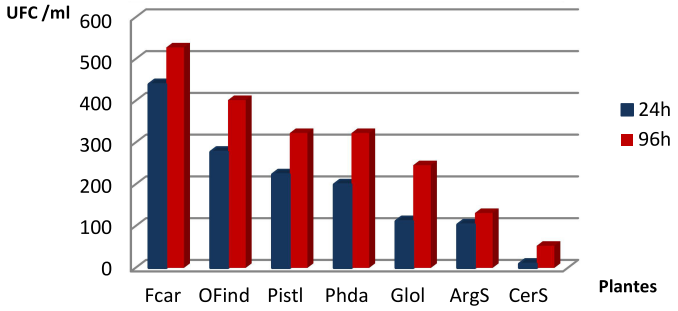

Fig. 1. Effect of plant type and incubation time on total viable count of Rhizospheric bacteria

Table 2. Phenotypic diversity of total rhizospheric bacteria according to macroscopic characterization

Colony characteristics	Color		Border		relief		pigmentation	
Incubation time	24h	96h	24h	96h	24h	96h	24h	96h
Fig tree	-Grey -White -Cream	-Purple -Orange -Yellow	Regular	-Regular -Indented	-plain -lobed	-plain -elevated -convex -cambered -lobed	-ab	-ab
Barbary fig tree	-Grey -Pink -Cream	-cream	-Regular -Indented	-regular -corrugated -endented	plain	-plain -convex -elevated -lobed	-ab	-ab
Pistachio	-transparent -White	-purple -brown -yellow Orange -green -beige -Pink	-regular -corugated -endented	-regular -corrugatede -filamentous -endented	-plain -elevated	-Plain -elevated -convex -lobed	-ab	-ab
Palm	-White -Cream transparent	-cream -green -orange -grey -orange	regular	-regular -Corrugatede -endented	-plain -elevated	-elevated -convex -lobed	-ab	-ab
Eucalyptus	-cream	-yellow -cream -white -grey -green	regular	-regular -corrugatede -filamentous -endented	-plain -elevated	-plain -elevated -convex	-ab	-ab
Argan tree	.beige .brown	-black -beige	regular	-regular -corrugated -filamentous -endented	-plain -elevated	-plain -elevated -convex	-ab	1 pig-ment
Carob	-cream	-orange -grey -purple	regular	-Regular -Corrugated -lobed	-plain	-elevated -convex	-ab	-ab

Results of macroscopic observation of the isolated bacteria are showed in Table 2. Concerning the phenotypic diversity it was presented in the following descending order: Pisl > ArgS > Ph da > Glob l > Fcar > Find > CerS j. An elevated microbial diversity concerning the shape and the colour on nutrient agar medium was observed. The presence of actinobacteria is mentioned by [5] who isolated and actinobacteria from the Agran rhizosphere. Actinobacteria, especially those belonging to the genus *Streptomycete*, are saprophytic bacteria known to degrade

the organic matter present in soil ie: lignocellulose, starch and chitin [6]. The presence of the organic matter as well as the root exudates can also influence the microbial population of the soil [7].

The incubation time also plays a determinant role in bacteria flora growth and diversity. In fact, after 96h incubation for almost all plant rhizospheres diversity and colony number increased and macroscopic aspect of isolates became diversified. The presence of actinobacteria appeared as well after 96h . These bacteria are numerous and widely distributed in soil. They are important in soil biodegradation and humus formation by the recycling of nutrients associated with recalcitrant polymers such as keratin, lignocelluloses and chitin [8].

3.2. Screening and phenotypic diversity of amylolytic rhizospheric Bacteria

The results of amylolytic rhizospheric bacteria screening on starch Agar are showed in Fig. 2. The amylolytic isolates appeared surrounded by a clear halo of different diameter following the isolate amylase activity. The best amylolytic isolates were isolated and purified by streaking on nutrient agar plates then they were inoculated on starch agar plates. The microscopic aspect of these isolates was examined after Gram coloration (Fig. 2). The number of colonies showing amylase activity was counted and the amylase specific activity of the best colonies was calculated and showed in Table 3.

The study of the amylolytic bacteria revealed that rhizospheric bacteria issued from OFind, CerS aged, Globl were the most numerous. Concerning the highest specific activities, they were noticed with isolates from CerS young, Fcar, ArgS rhizospheres.

The macroscopic study showed that some colonies although showing starch degradation but had a small diameter, which could be due to the absence of growth factor secreted by the plant partener. The microscopic study of the amylase producing isolates showed a dominance of Gram positive bacteria especially actinobacteria. Indeed, actinobacteria are regarded as typical inhabitants of soil environments [9].

The amylase production was noticed at all conditions of production due to the constitutive nature of the enzyme and the simplicity of the substrate (starch) and its availability in soil. Most of microorganisms produce the enzyme amylase and some are known to produce high quantities and are used in industry namely: *Aspergillus sp, Bacillus sp* and *Pseudomonas sp*.

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3.3. Amylase enzyme production and activity revelation

Results of amylase activity of the crude produced enzyme by the isolated rhizospheric are showed in Table 4. In Fig. 3 amylase activity is revealed on liquid starch medium (Fig. 3A) and on starch agar plate (Fig. 3B).

The Crude enzyme extracts obtained after production presented an amylase activity translated by clear zones around the wells. The appearance of the clear halo confirms the presence of an activated amylase in the crude produced supernatant. Amylase activity on liquid medium showed the same increasing activity order than well cut diffusion method ie: OFind< CerS aged< CerS young< Fcar < Glob l < Phda < Pisl < ArgS.

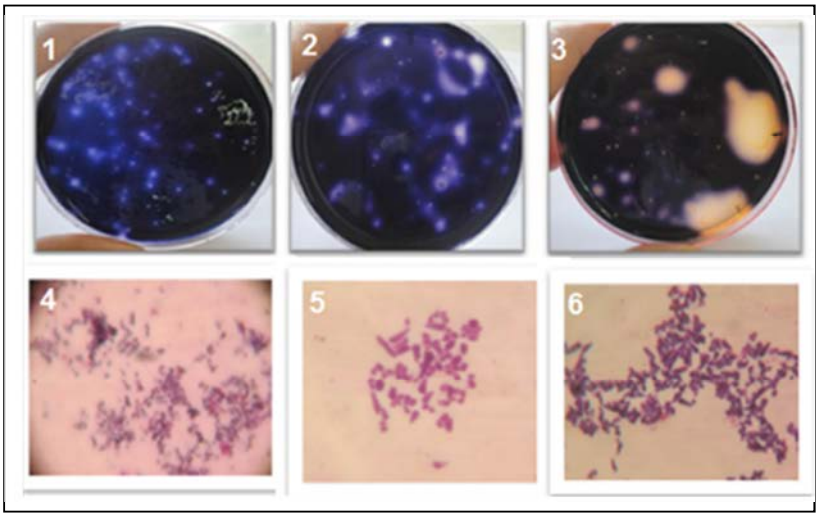

Fig 2: Results of phenotypic study of amylolytic rhizospheric bacteria.
1; 2; 3: Total bacterial diversity of starch degrading bacteria revealed on starch agar plates (1: Find, 2: Pisl, 3: CerS young).
4; 5; 6: Microscopic observation of some amylolytic rhizospheric isolates (4: CerS young, 5: OFind, 6: ArgS).

The best amylase activities were noticed with ArgS, Pisl and Phda. Argania Spinosa L and Argania Spinosa L are oleaginous fruit trees in which assimilated organic matter is transformed to fatty acids used in the synthesis of cell membranes and most notably for oil storage in seeds [10] . On the other side, for sweet fruit trees the assimilated organic matter is conserved in plant storage organs directly in form of simple sugars (glucose , fructose and saccharose) [11] that are used for (1) development and growth, (2) synthesis of most other metabolites, (3) energy provision under starvation conditions and (4) biotic and abiotic stress tolerance [12].

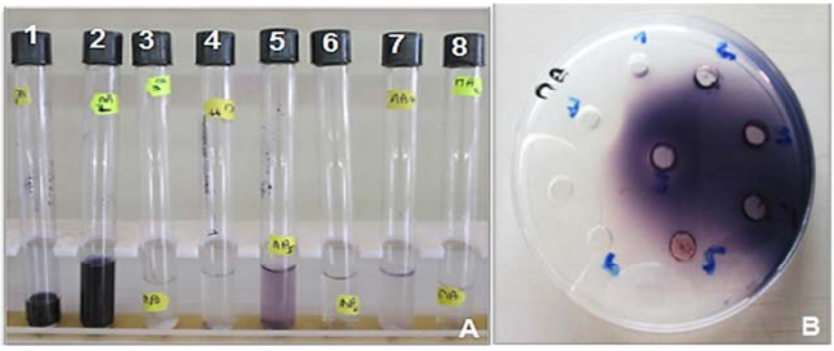

Fig 3: Amylase activity of rhizospheric isolates. (A) Amylase activity detection on starch lequid medium: 1: OFind, 2: CerS aged, 3: Ph da, 4: Glob l, 5: CerS young 6: ArgS, 7: Fcar, 8: Pisl. (B) Well cut diffusion method: 1: Ph da, 2: Fcar, 3: Glob l, 4: OFind, 5: CerS young, 6: Pisl, 7: ArgS, 8: CerS aged.

It was also noticed that bacterial strains isolated from plant rhizospheres that showed high bacterial diversity and high colony number i.e. Fcar and OFind gave low amylase activity. Beside this, the best amylase producing isolates were those isolated from plant rhizospheres that grow in arid regions. Indeed, bacterial strains of ArgS and Pisl showed viscosity when cultivated in liquid medium. It is known that microorganisms contribute in water and nutrients uptake by plant roots [13] being a key biotic aggregating agents in soil [14]. Also Yang *et al.* [15] reported some rhizospheric bacteria to help plants to resist abiotic stress especially draught by hormone secretion involved in roots growth.

Table 3. Total rhizosphere amylolytic bacteria and specific activities of the best isolates.

Isolate source	Number of colonies	Colony diameter (mm) (Y)	Starch degradation zone diameter (mm) (X)	Amylase specific activity (X/Y)
Phoenix dactylifera	18	1.8	3.9	2.16
		2.9	4.8	1.65
Ficus carica	5	1	2.8	2.8
		1	5.8	5.8
Opuntia Ficus-indica	35	0.9	3.9	4.33
		0.5	2.8	5.6
Globulus Labill	24	1	6.8	6.8
		0.6	3.9	6.5
Ceratonia siliqua young	5	0.9	3.9	4.33
		0.8	5.8	7.25
Pistacia lentiscus L	20	1	6.4	6.4
		0.8	5.8	7.25
Argania Spinosa L	5	0.9	4.9	5.44
		1	3.9	3.9
Ceratonia siliqua L aged	25	0.8	3.9	4.87
		0.7	3.8	5.42

Table 4. Enzyme activity using crude amylase of rhizospheric isolates

Isolate source	Enzyme activity (Lysis zone, mm)
Phoenix dactylifera	6
Ficus carica	4
Eucalyptus globulus labill	5
Pistacia lentiscus	8
Argania Spinosa L	12
Ceratonia siliqua L young	3
Ceratonia siliqua L aged	2
Opuntia ficus-indica	0

4. Conclusion

The obtained results are encouraging and provided precious data about the diversity of rhizospheric bacterial flora and the specificity of each studied plant flora. Also, high amylase production by some of these bacteria was detected. These outcomes concerning bacterial flora should be valued and followed by molecular and metagenomics study. The ecological aspect also should be deepened and soil analysis and climate study should be realized.

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