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Phosphate Solubilizing of Carob (*Ceratonia siliqua* L) Associative Bacteria Analyzed by Molecular Technique ARDRA

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Abstract: ***We aim use in this study, endophytic bacteria isolated from inside the roots (IRC) and epicotyls (IEC) of carob (Ceratonia siliqua L.) plants collected from different Moroccan regions and previously screened by Rep-PCR. 30 IRC and 4 IEC strains selected on the basis of Rep-PCR representative groups and 8 carob symbiotic bacteria (RCM) were tested for their solubilizing activities and antibiotic resistance and analyzed by PCR-RLFP technique. 70 % and 50 % of carob associative bacteria and RCM strains respectively were solubilizing phosphate in culture media with P solubilization index (SI) ranged from 1,63 to 2,13 for IRC and IEC and from 1,25 to 1,5 for RCM. Amplified ribosomal DNA analysis (ARDRA) of the isolates based on cleavage with four restriction enzymes yielded 25 genotypes with IRC and 3 and 5 differents profiles respectively with IEC and RCM. Dendrogram based on upon UPGMA analysis of ARDRA patterns showed a wide genetic diversity and divided the strains into six clusters at 50 % of similarity level. Two clusters revealed relatedness with the rhizobial reference strains used and four others clusters which contain mainly associative endophytic bacteria were more heterogeneous and very distinct from all the other strains.***

Keywords: Associative bacteria, carob (*Ceratinia siliqua* L), Phosphate solubilization, ARDRA

1. Introduction

Rhizospheric, endophytic associative and endophytic symbiotic bacteria play an important role in the enhancement and retain of agricultural and forestry soils fertility. Called Promoting Growth Plants Bacteria (PGPB), endophytic associative bacteria may promote plant growth and development in terms of increase germination rates, biomass, leaf area, nitrogen content, chlorophyll content, hydraulic activity, roots and shoot length, yield and tolerance to abiotic stress like drought, flood and salinity [1]. Associative bacteria have directly or indirectly divers positive effect on their host plants. Directly, they contribute significantly in biological fixation and reduction of nitrogen molecular [2, 3], increase and influence phytohormone production such as auxins, gibberellins, ethylene, cytokinins and abscisic acid [4], increase the availability of selected soils minerals *via* solubilization of the nutrients such as phosphprus [5] and siderophores production [6]. Indirectly, they also support plant growth by improving growth-restricting conditions either *via* biocontrol activities by production of antagonistic substances [7] or by inducing resistance against plant pathogens [8, 9] and systemic tolerance such as presence of toxic metals, drought, salt and extremes of temperature [10, 11, 12].

The improvement of soil fertility is one of the most common strategies to increase agricultural yield. In addition to biological nitrogen fixation, phosphate solubilization is equally important [1, 13]. Phosphate is known to be the second most limiting compound for plant growth. Although, many of the soil is rich in phosphate but they are in insoluble form and cannot be utilized by plants or other soil organisms [12]. Use of these PGPB as environmental friendly biofertilizer helps to reduce the much excessive phosphatic fertilizers. A vast number of PGPB with phosphate solubilzing property have been rpported which included member belong to *Rhizobium* [14], *Mesorhizobium*, *sinorhizobium* [15], *Bacillus* [16], *Pseudomonas* [17], *Burkhoderia* [18, 19]. However, characterizing bacterial isolates on the base of biochemical and biological tests like phosphate solubilizing trait is not very enough to distinguish well between the different populations. DNA marker un-affected by environmental and physiological factors, present a good tool for identification and speed analysis of the genetic diversity among bacterial population or collection.

The use of molecular methods, such us restriction endonulease analysis of 16S rDNA amplified (ARDRA) has provided more information about genomic characterizations of the microorganisms. 16S rDNA genus conserved through the generation and formed by a stable regions and a variable regions [20, 21], is frequently used by several authors for genotypic characterization and diversity analysis of bacteria including associative bacteria [22, 23, 24]. Since, the molecular study using ARDRA marker for carob (*Ceratonia siliqua* L.) symbiotic or associative bacteria is un-available.

Carob (*Ceratonia siliqua* L.) tree, leguminous belong to the *Ceasalpinoideae* subfamily, is natural native from most countries of the Mediterranean basin and from some world regions like Southwestern Asian, Australia, USA, Mexico and South Africa [25]. It was considerate by Martins-Loucâo and Rodriguez-Barrueco [26] to be no nodulate and unable to fix atmospheric nitrogen. But, in 1996 Bryan et al. detected, *in vitro culture*, the enzyme nitrogenase activity internal the carob roots and bourgeon. The symbiotic bacteria of carob plant (RCM) and natural associative bacteria were also reported respectively by Misbah et *al*. [27] and El-Refarey [28]. In this research we aim (i) to study

the phosphate solubilization activity and the resistance to antibiotic substances of carob associative bacteria isolated from roots (IRC) and epicotyls (IEC) and carob symbiotic bacteria described by Misbah et *al*. [27] and (ii) to use molecular marker ARDRA for genetic diversity analysis.

2. Materials and methods

2.1 Plant Material

The seeds and soils sample were collected from eleven Moroccan localities. Scarification, sterilization and germination of seeds were carried out as described by konate et al. [29, 30]. On the base of Rep-PCR data, eight provenances were retained for this study: Aïn Safa, Demnate, Essaouira, Ouad Lou, Ouazzane, Sidi Bou Othmane, Taounate and Tetouan.

2.2 Bacteria Material

30 endophytic bacteria isolate from roots coded IRC (Isolate Root Carob) and 4 endophytic bacteria isolate from epicotyls coded IEC (Isolate Epicotyls Carob) representing the various Rep-PCR groups (data not showed) and also 8 strains nodulating carob (RCM) plant and previously described by Misbah et al. [27] were used. In addition, thirteen reference strains belong to the different genre *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Agrobacterium* were used.

2.3 Biological Characters

42 isolates of carob plants (34 associative and 8 symbiotic bacteria) were tested *in vitro* in the presence of iron of phosphate insoluble and antibiotic substances

2.3.1 Solubilization of Phosphate Mineral

Ability of mineral phosphate solubilizing of the associative (IRC and IEC) and symbiotic (RCM) bacteria was assayed on Pikovskaya agar [31]. The isolates bacteria were spotted onto agar medium containing inorganic phosphate composed by ($(g.l^{-1})$[agar, 15; glucose, 10; phosphate bi-calcium, 5; yeast extract, 0,5; $(NH_2)2SO_4$, 0,5; KCl, 0,2; $MgSO_4$, $7H_2O$, 0,1], bromophenol blue, 4ml/l; $MnSO_4$ and $FeSO_4$ in trace; $pH_{6,7}$) and incubated at 28°C for up to 7 days. The presence of halo zone around the bacterial colony was considered as indictor for positive mineral phosphate solubilization. Solubilization index (SI) was determined using following formula [32]:

SI = (colony diameter + halo zone) / colony diameter

2.3.2. Resistance to antibiotic substances

This test was realized on YEM agar containing the following filter sterilized antibiotic at variable concentrations ranging from 10 to 100 $\mu g.ml^{-1}$ (ampicillin, chloramphenicol, erythromycin, nalidixic acid, rifampicin, spectromycin, streptomycin and tetracycline).

2.4 DNA extraction

DNA extraction was carried by method of alkaline treatment according to De Bruij [33] protocol.

2.5 Amplification of region 16S ribosomal DNA

34 endophytic bacteria, 8 RCM and 13 reference strains, were used in this analysis. Purified template DNA of 60 ng was used by reaction. Amplification of 16S rDNA was performed in a total volume of 70 µl containing 7 µl of 10X reaction buffer (Promega), 11.8 mM $MgCl_2$, 5ppm of each of the following primers: 41f 5'-GCTCAGATTGAACGCTGGGG-3' and 1488r 5'-CGGTTACCTTGTTACGACTTCACC-3' [21], 1.75 mM of dNTP, 4U of *Taq* DNA Polymerase (Promega). The reaction mixtures, after incubation at 95°C for 2 min, were cycled through the following temperature profile: 2 different cycles of the denaturation at 94°C for 40 s, annealing for 1 min, extension at 72°C for 2 min; the annealing temperature was 60°C for the ten first cycles and 50°C for the last 25 cycles. The mixtures were then incubated at 72°C for 5 min. 10 µl of each PCR product was analyzed by agarose gel (1 % w/v) electrophoresis and the visualisation of band has been done with thidium bromide (10 mg/ml) under ultra-violet light.

2.6 Amplified Ribosomal DNA Restriction analysis (ARDRA)

Endonuclease restriction was carried out in 10 µl of PCR product with *MspI*, *HinfI*, *HhaI* and *TaqI* (Promega) as recommended by the manufactures. Digested products were resolved by electrophoresis in 2.4 % agarose gels in TBE at 80 volts for 4 h.

Clustering analysis based on restriction profiles was performed using the Gel Compar II. The Dice similarity coefficient and UPGMA clustering were also used.

3. Results

3.1 Solubilization of Phosphate Mineral

A total of 34 associative isolated form roots (30 IRC) and epicotyls (4 IEC) and 8 symbiotic (RCM) isolates of carob (*Ceratonia siliqua* L) were analyzed for their ability to solubilize phosphate *in vitro*. The results showed that 70 % of carob associative bacteria and 50 % of RCM strains were positively solubilizing phosphate in culture media. However, the high frequency of the isolates able to solubilize phosphate was obtained with IRC strains. Four IRC (42, 53, 61 and 73) isolates exhibited an efficient P solubiliging activity with P solubilization index (*SI*) ranged from 1,63 to 1,70. The low P solubiliging activity was observed with RCM strains with *SI* ranged from 1,25 to 1,5 (Table 1).

Table 1: Test of phosphate solubilization by associative and symbiotic strains of carob

Isolate	*Origin of host plant*	*Colony diameter (cm)*	*Halo zone diameter +colony (cm)*	*SI*
IRC1	Oued Laou	-	-	-
IRC6	Ouazzane	-	-	-
IRC7	Tétouan	-	-	-
IRC9	Taounate	0,9	1,2	1.33
IRC11	Taounate	0,8	1	1.25
IRC15	Tétouan	-	-	-
IRC18	Ouazzane	-	-	-
IRC19	Ouazzane	0,9	1,2	1.33
IRC20	Tétouan	1,7	1,1	1.24
IRC24	Taounate	0,9	1,2	1.33
IRC27	Taounate	0,7	1	1.43
IRC29	Ouazzane	-	-	-
IRC32	Ouazzane	-	-	-
IRC37	Taounate	0,8	1,1	1.38
IRC42	Sidi Bou Othman	1,1	1,8	1.64
IRC43	Tétouan	1,2	1,6	1.33
IRC44	Essaouira	0,9	1,1	1.22
IRC45	Essaouira	1,7	2,3	1.35
IRC47	Tétouan	1,2	1,7	1.42
IRC49	Aïn Safa	1	1,6	1.6
IRC50	Aïn Safa	1,9	1,4	1.36
IRC51	Aïn Safa	-	-	-
IRC53	Taounate	0,8	1,3	1.63
IRC54	Demnate	1,1	1,7	1.55
IRC56	Taounate	0,8	1,2	1.5
IRC57	Taounate	1,2	1,7	1.42
IRC58	Aïn Safa	1,1	1,7	1.55
IRC61	Demnate	1	1,7	1.7
IRC66	Ouazzane	1,6	2,2	1.36
IRC73	Sidi Bou Othman	1,1	1,8	1.64
IEC1	Sidi Bou Othman	1,2	1,4	1.17
IEC4	Sidi Bou Othman	0,8	1,3	1.63
IEC10	Essaouira	0,8	1,7	2.13
IEC11	Sidi Bou Othman	-	-	-
RCM3	Oujda	-	-	-
RCM4	Oujda	-	-	-
RCM5	Oujda	1	1,5	1.5
RCM7	Oujda	-	-	-
RCM9	Oujda	0,7	1	1.43
RCM10	Oujda	1,1	1,4	1.27
RCM11	Oujda	0,8	1	1.25
RCM12	Oujda	-	-	-

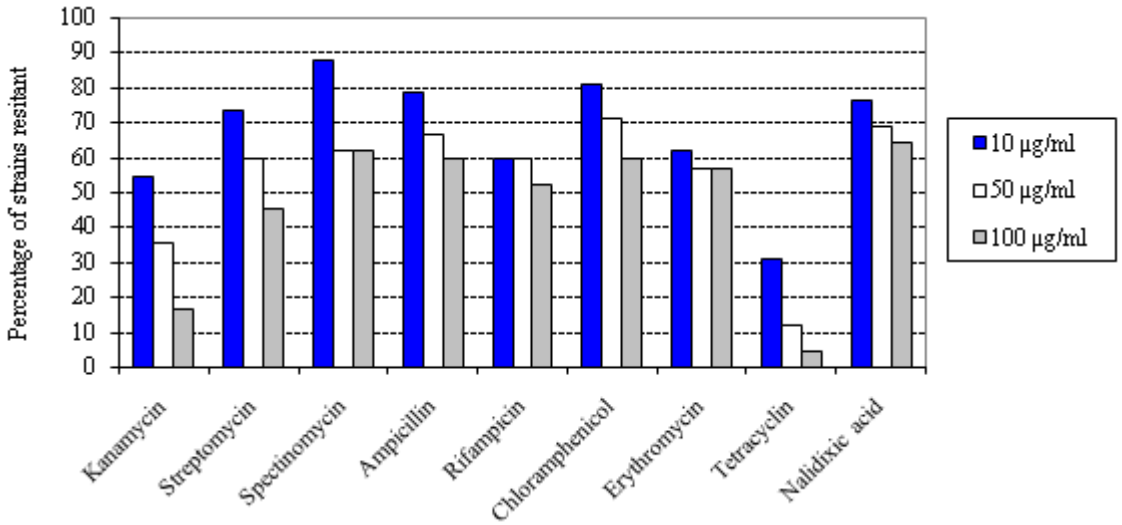

Figure 1: Effect of antibiotic substances on the growth of carob strains

3.2 Resistance to antibiotic substances

Strains were resistant to the concentration of 10 µl/ml of ampicillin, chloramphenicol, erythromycin, nalidixic acid, rifampicin, spectromycin and very sensitive to streptomycin and tetracycline. The same result was also observed with above 60 % of strains that exhibited a high resistance to concentration tested (50 µl/ml). The last two antibiotics (kanamycin and tetracycline) affected significantly the growth of the isolates. However, nalidixic acid has a slight inhibitory effect on the growth of the carob associative and symbiotic bacteria (Fig. 1).

3.3 RFLP analysis of PCR-amplified 16S rDNA (ARDRA)

We performed ARDRA technique to compare the 34 new endophytic carob bacteria to the 8 RCM strains previously described by Misbah et al. [27] and to the thirteen reference strains belonging to different genus of rhizobia and *Agrobacterium*. Nearly the full-length 16S rDNA region was amplified with universal primers 41f and 1488r (Fig. 2)

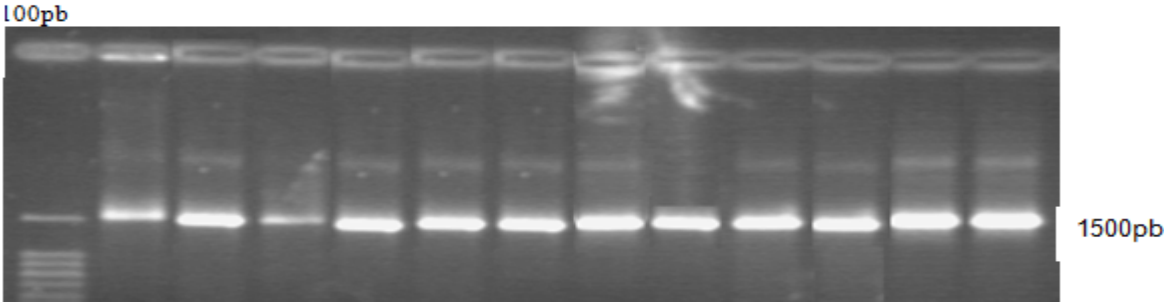

Figure 2: Genomic profiles obtained par by amplified of 16S rDNA region of carob endophytic bacteria 100pb *A. rh* IRC1 IRC29 IRC37 IRC47 *R. ga* IEC1 IEC4 IEC10 *R. so* RCM9 RCM10 *B. ja* 100pb

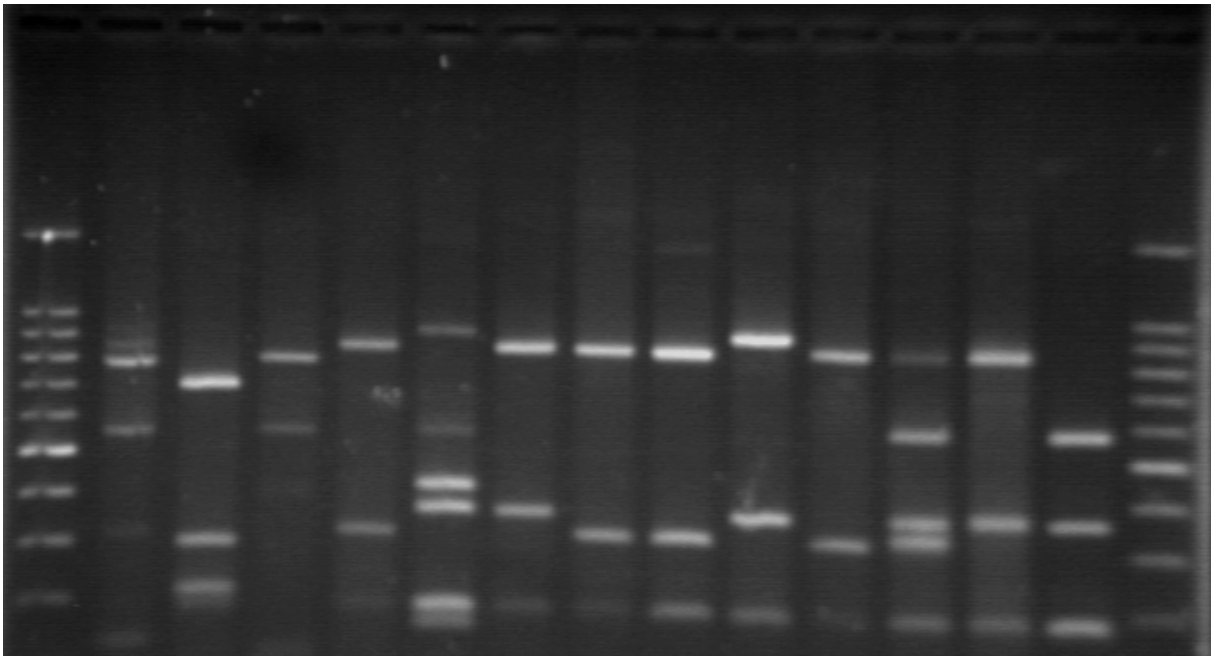

Figure 3: Type of restriction patterns of PCR-amplified 16S rDNA restricted with *Taq* I obtained with IRC, RCM and reference strains (A.rh = *Agro. rhizogenes*; R. ga = *R. gallicum*; *S. kostiens*; B. ja = *B. japonicum*) used in this study

The PCR products were individually restricted with the four endonucleases *MspI*, *HinfI*, *HhaI* and *TaqI*. Although; each digestion enzyme produced divers profiles and polymorphic banding patterns (Fig. 3). All enzymes yielded together 25 genotypes with IRC, 3 and 5 different profiles respectively with IEC and RCM.

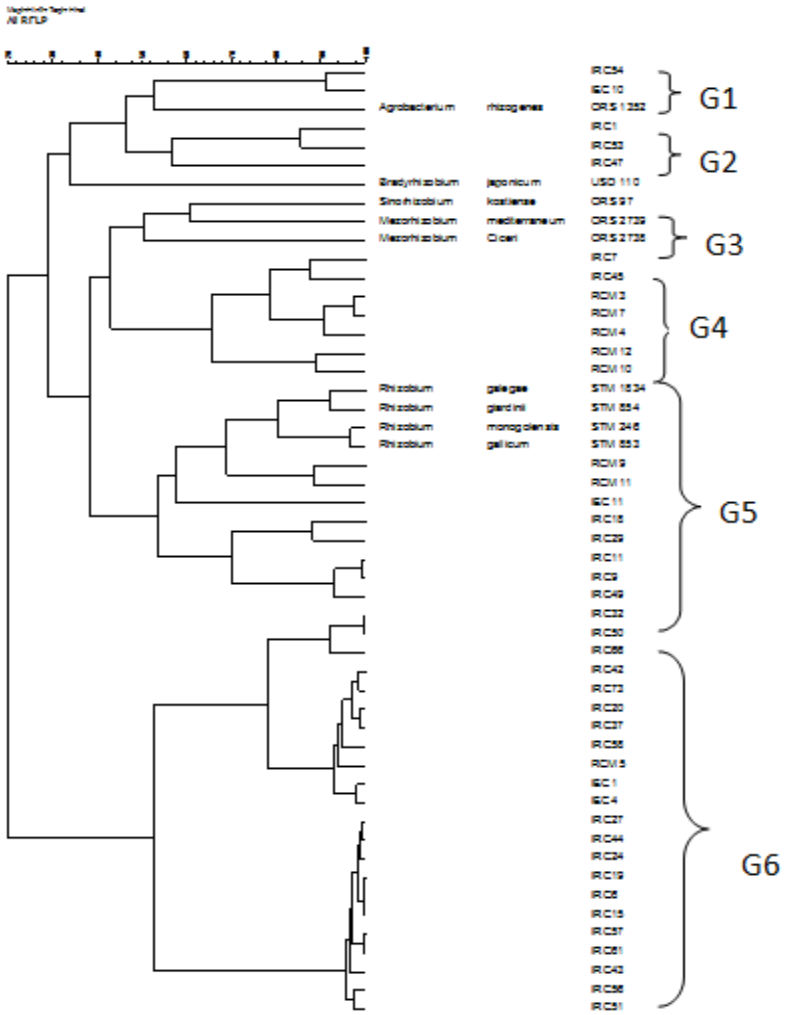

Figure 4: Dendrogram constructed with Dice similarity coefficient of GelCompar II on the basis of ARDRA patterns of selected carob endophytic bacteria (IEC and IRC), symbiotic bacteria

RCM (Missbah et al. 1996) and reference strains.
The clustering analysis of combined *MspI*, *HinfI*, *HhaI* and *TaqI* restriction patterns showed a wide diversity among endophytic strains isolated from roots and epicotyls (Fig. 4). The result showed six main clusters at a mean Dice similarity coefficient of 50%.

Clusters 2, 4 and 6 contain only endophytes and RCM strains. Cluster 6 was formed by a large number of endophytic strains. In fact, this cluster was divided at 54% of similarity to two groups: the first group, formed mainly by IRC stains, one RCM strain and two IEC strains which were revealed identical by REP analysis, and the second group formed only by IRC strains which show more than 96% of similarity. Cluster 1 contains two strains IEC10 and IRC54 which has 53% of similarity with *Ag. Rhizogenes*. Cluster 3 is formed by rhizobial reference strains of *Mesorhizobium* and *Sinorhizobium*. Cluster 5 is divided at 54% of similarity in two groups: the first group which contain an epicotyls isolated strain: IEC11 ant two strains nodulating carob: RCM9 and RCM11 that were tightly related to reference strains of the genus *Rhizobium*. The second group contain only endophytic strains originating from roots.

4. Discussion

Biological test conducted in the presence of insoluble phosphate showed that associative bacteria isolated from roots (IRC) and epicotyls (IEC) of the carob tree (*Ceratonia siliqua* L.) exhibited a similar manner the ability to solubilize phosphate di-calcium *in vitro*. These endophytic bacteria are Gram negative and according Rashith et al. [34], Gram-negative strains are the most efficient in the solubilization of phosphate across the production of organics acids and the phosphatases enzymes. Solubilization index (*SI*) obtained in our study is consistent with the results reported for various rhizobacteria. It is 2.48 for *Rhizobium leguminosarum* bv *Vicia*, 1.41 *Mesorhizobium ceceri*, *M. mediterraneun* and *Sinorhizobium meliloti* [14, 15, 35], 2.15 for *Burkholderia* [18] and 4,1 for *Pseudomonas* sp. [14].

For antibiotic test, the strains of carob tree exhibited a multiple antibiotic resistance. They were highly sensitive to tetracyclin and kanamicin and mostly resistant to nalidixic acid and moderately to the different concentrations tested of ampicillin, chloramphenicol, erythromycin, Nalidixic acid, spectinomycin and streptomycin (Fig. 2). The same result was described for many rhizobacteria. Various strains belonging to the genus *Rhizobium* and *Burkholderia* exhibited a multiple antibiotic resistance [35, 36].

The technology based on DNA digestion by endonucleases results in pattern of bands, is called ARDRA and it offers the opportunity to review polymorphism in the analyzed DNA [37]. Selected endophytic bacteria and RCM strains compared by ARDRA analysis to each other and to thirteen reference strains were present in the all clusters, except cluster 3 (Fig. 4). These endophytes of *Ceratonia siliqua* plant were very heterogeneous and some of them were related to the rhizobial reference strains such as *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Ag. Rhizogenes*. The rhizobia strains that usually known to form nodule organ with legumes plants are recently reported to be as well as natural associative bacteria colonized wide range of host plants non-legumes. Here, we cited some natural associative rhizobia: *Rhizobium leguminosarum* bv. *phaseoli*, *trifolii* and *viceae* [3, 35, 38], *Rhizobium* sp. [14, 39], *Bradyrhizobium japonicum* [40], photosynthetic bradyrhizobia [41], *Sinorhizobium meliloti* [40], *Azorhizobium caulinodans* [42, 43], *Burkholderia brasilensis* [44] nd *Burkholderia cepacia* ([35].

5. Conclusion

The similarity of physiological, cultural, biological and molecular data between endophytes associations (IRC and IEC) and symbiotic (RCM) isolates of the carob (*Ceratonia siliqua* L.) tree confirms that these soil bacteria have the same host plant. On the other hand, these rhizobacteria (PGPB) promote the growth and development of their host, contribute significantly to the favorable installation of carob on poor soils and it resist face various abiotic stresses such as salinity, toxic metals, antibiotic substances, strong drought, ...

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