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Contribution to the Study of the Relationship between Gammaproteobacteria and Rhizobia in Legume Species of the Genus *Hedysarum*

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ABSTRACT

The nodular contents of the leguminous species of the genus *Hedysarum* (*H. carnosum, H. spinosissimum subsp capitatum and H. pallidum*) consist of cultivable bacteria belonging to the class Gammaproteobacteria, while the rhizobia of these leguminous species are not cultivable. *Rhizobium sulla* is the specific micro-symbiont of the *H. coronarium* leguminous species. The aim of this study is to see if the Gammaproteobacteria that inhabit the nodules of the three species of the genus *Hedysarum* (*H. carnosum, H. spinosissimum subsp capitatum and H. pallidum*) could have acquired the symbiotic criteria from rhizobia and cause nodulation in these host legume species by the transformation process. Transformation is carried out by extracting the plasmid DNA from the *R. sulla* strain and incorporate it into the bacterial strains of the Gammaproteobacteria class. Nodulation assay is performed to evaluate the ability of transformed strains to cause nodulation in legume species. The results showed that although there has been a transformation, there is a total failure of nodulation in the three legume species. This is explained by the specificity of molecular signals between bacteria and host plants as well as the genetic information carried by the plasmid is not sufficient to achieve a complete symbiotic relationship.

Key words: Gammaproteobacteria, Nodulation, *Hedysarum, Rhizobium*, Transformation.

INTRODUCTION

Symbiotic nitrogen fixation is the signature feature of legumes in which the microsymbiont collectively called as rhizobia can reduce atmospheric nitrogen (N_2) into ammonia; otherwise, N_2 is metabolically unavailable to higher plants. The fixed nitrogen is generally used for plant growth or the excess of fixed nitrogen is released into the rhizosphere for improving soil fertility (Girija *et al.*, 2018; Senthil *et al.*, 2017). Biological nitrogen fixation can be an important factor in sustainable agriculture and can complement fertilizer inputs in crop production (Benselama *et al.*, 2018; Singh and Singh, 2018). The symbiotic leguminous-bacterial association that gives rise to nitrogen fixation is an essential process for the plant but also for the bacteria to have anaerobic conditions and obtain necessary nutrients for its development (Gage, 2004). The specificity of *Rhizobium* for a legume host plant is determined by the exchange of molecules between both symbiotic partners. Each step of the establishment of symbiosis is tightly controlled through a complex network of signaling cascades (Senthil *et al.*, 2017). Among legumes that come into association with symbiotic bacteria we count the plants of the genus *Hedysarum*, which are fairly widespread in the Mediterranean region. Different species of this genus are found in North Africa and Southern Europe (Abdelguerfi-Berrekia, 1988). The genus *Hedysarum* constitutes a significant range of forage legumes in Algeria with ten species distributed in various climatic zones ranging from semi-desert to humid climate zones (Abdelguerfi-Berrekia, 1991). In effect, by dint of their nitrogen fixing potential, species of the genus *Hedysarum* can be used as green fertilizer or as cover plants. The species of the genus *Hedysarum* have mainly been studied for their agronomic interest and foraging properties, but very few studies have focused on their symbiotic partners.

All bacterium nodulating legumes are classified in the rhizobiales order. This is demonstrated for the genus *Hedysarum* when a new species of *Rhizobium* (*R. sullae*) specific for the species *H. coronarium* is described (Squartini

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et al., 2002). This criterion is abandoned following the results obtained by Benhizia *et al.* (2004) when they demonstrated that the genus *Rhizobium* was not the only micro-symbiont of *Hedysarum* and species of Gammaproteobacteria were the cultivable occupants of the nodules of the three species of the genus *Hedysarum (H. carnosum, H. spinosissimum subsp capitatum and H. pallidum*).

Although no cultivable *Rhizobium* can be found, studies of several species of *Hedysarum* and their nodular content have shown that the rhizobia of these legume species are not cultivable and a vast range of opportunistic endophytes are present (Muresu *et al.*, 2008; Tondello *et al.*, 2011; Torche *et al.*, 2014).

The aim of our study is to see if Gammaproteobacteria that co-inhabit nodules of the three species of the genus *Hedysarum* (*H. carnosum, H. spinosissimum subsp capitatum and H. pallidum*) can acquire the symbiotic criteria from rhizobia and if they can have the ability to cause nodulation in these host legume species by application of transformation.

MATERIALS AND METHODS

In this study we have used *Rhizobium sulla* strains, which are the specific isolates of the legume species *Hedysarum coronarium and* strains of the class Gammaproteobacteria isolated from the nodules of others legume species of the genus *Hedysarum* (*H. carnosum, H. spinosissimum subsp. capitatum and H. pallidum*) and identified by Benhizia *et al.* (2004) (Table 1).

Transformation of Gammaproteobacteria strains

Extraction of plasmid DNA from *Rhizobium sulla* strains by alkaline lysis

Strains of *Rhizobium sulla* A6 and F were seeded in 2 ml of YMB medium (Vincent, 1970) and incubated 24-28h at 30°C. The extracting of the plasmid DNA was carried out according to the protocols described by Birnboim (1983), it is a fast technique for the extracting of plasmid DNA in an alkaline medium which makes it possible to eliminate the chromosomal DNA without diminishing the yield of plasmid.

Transformation of Gammaproteobacteria strains by thermal shock

The state of competence of the Gammaproteobacteria strains was provoked by the culture on YMB medium supplemented with 50 mM $CaCl_2$ and incubated at 30°C for 24 hours. 5 µl of each extracted plasmid DNA were added to 150 µl of a competent bacterial strain. The mixture was placed in the ice for 15 to 20 minutes and quickly transferred to the oven at 42°C for 45 seconds to cause a thermal shock. Then, quickly another 1 or 2 minutes in the ice. Finally, 500 µl of YMB+ $CaCl_2$ medium (50 Mm) were added and incubated at 30°C for 40 minutes (Gharzouli, 2013).

Transformed strains selection:

After incubation, 100 to 200 µl of the culture were spreaded on the YMA medium (Vincent, 1970) containing 0,02% calcofluor white and incubated at 30°C. for 24 hours (Gharzouli, 2013). Calcofluor is a dye of cellulose and capsular structures. It is fluorescent under ultraviolet light (UV). The transformed colonies will be fluorescent and viscous, indicating the production of exopolysaccharides, specific components of rhizobia (Strufi *et al.*, 1998).

Nodulation assay

A nodulation test under controlled microbiological conditions was performed to evaluate the ability of transformed strains to nodulate *Hedysarum* host plants. The test was performed according to the Gibson tube method (Vincent, 1970).

After sterilization of the seeds of each leguminous species of *Hedysarum*, they were put to germinate in TYA dishs (Beringer, 1974) , in total darkness at room temperature for 3 to 7 days, until the appearance of the rootlets. Young seedlings were transferred to assay tubes containing 10 ml of 1% solidified Fähreus medium (Fähreus, 1957) at the rate of one seedling per tube. Each tube was inoculated with 1 ml of a transformed strain. After four weeks, the plants were harvested to observe the result of the nodulation.

RESULTS AND DISCUSSION

In this study we tried to apply the bacterial transformation process based essentially on the extraction of plasmid DNA from *Rhizobium sulla* strains nodulating the leguminous species *Hedysarum coronarium* and incorporate it into bacterial strains of Gammaproteobacteria class isolated from the nodules of others leguminous species of the genus *Hedysarum* (*H. carnosum, H. spinosissimum subsp capitatum and H. pallidum*). This is for the purpose to see if

Table 1: Strains used in this study.

	Strains	Host plant	Géographic origine	Source
A6	*Rhizobium sullae*	*Hedysarum coronarium*	Constantine, Algeria	A.Benguedouar-Constantine
F	*Rhizobium sullae*	*Hedysarum coronarium*	Pise, Italy	S. Casella- Pise
HS1	*Pseudomonas* sp. NZ096	*Hedysarum s.ssp.capitatum*	Constantine, Algeria	Y.Benhizia-Constantine
HP7	*Enterobacter kobei*	*Hedysarum.pallidum*	Sétif, Algeria	Y.Benhizia-Constantine
Hca1	*Pseudomonas* sp.KD	*Hedysarum carnosum*	Biskra, Algeria	Y.Benhizia-Constantine

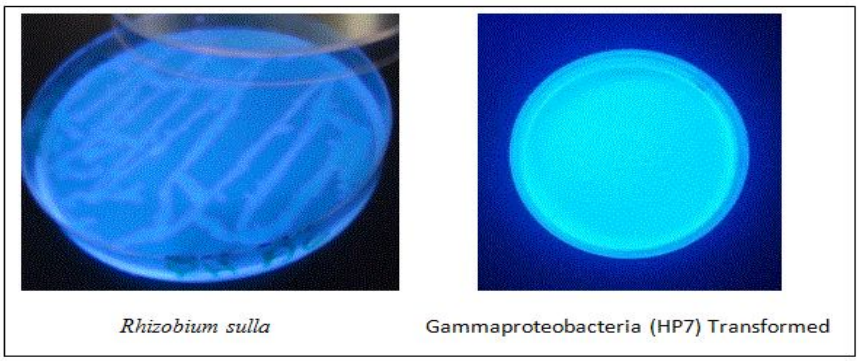

Fig 1: Strains under UV on YMA – Calcofluor.

the bacterial strains of Gammaproteobacteria class that occupy the nodules and co-inhabit rhizobia can acquire the symbiotic criteria of the latter by transformation.

Transformation of Gammaproteobacteria strains

We had started by plasmid DNA extraction from *Rhizobium sulla* strains. After inducing the competence state in Gammaproteobacteria strains, they were transformed by the heat shock method. Benhizia, (2006) proved that comparatively to *Rhizobium sulla* strains, Gammaproteo--bacteria strains are devoid of a symbiotic plasmid. The character used to select transformed strains is the ability of *Rhizobium* strains to produce exopolysaccharides (EPS). The selection of transformed strains is facilitated by the use of calcofluor. Calcofluor is a dye of cellulose and capsular structures. It is fluorescent under ultraviolet light. Colonies of strains producing exopolysaccharides are fluorescent on an agar medium containing calcofluor, while colonies that do not secrete them are non-fluorescent (Leigh and Lee, 1988). In our study, the detection of strains producing exopolysaccharides and thus transformed is evidenced by the culture on the YMA medium containing 0.02% Calcufluor (Gharzouli, 2006). After observation the Petri dishes in a dark room and using a UV lamp, we were able to select the transformed strains that give under UV, fluorescent, smooth and viscous colonies, comparable aspect to that of rhizobia strains (Fig 1).

Realization of the *Rhizobium*-legume symbiosis that permits the plant to benefit from atmospheric nitrogen fixation is one of the most important consequences of plasmid information (Benguedouar, 2000). The study of plasmid transmission constitutes an effective way for the control of their functional and evolutionary role (Tejerizo *et al.*, 2010). The plasmid pSym has an important diversity of genes including the synthesis genes of exopolysaccharides which intervene in the establishment of symbiosis. The extracellular polysaccharides produced by rhizobia are essential for the establishment of symbiotic nitrogen fixation with legumes (Chataigné, 2007).

Our results are in agreement with the results of Strufi *et al.* (1998). Gharzouli (2006) reported that the low fluorescence on YMA medium containing 0.02% calcufluor indicates a mutation at level of the involved genes in the production of succinoglycan (EPS1). Among the roles that have been proposed for rhizobial exopolysaccharides, the identification of specific active signaling during colonization and elongation of the infection thread, organization of the root cytoskeleton and redirection of tip growth of root hairs (Morgante, 2005), also the inhibition of plant defense responses structural requirement for this process and the electrophoretic mobility of rhizobial cells (Ghosh and Maiti, 2016).

Nodulation assay

After one month of culture, the nodulation is evaluated. The results obtained show a total failure of nodulation for the three leguminous species of *Hedysarum* tested (Fig 2). This failure may be due to the specificity of the signal molecules responsible for nodulation and therefore the specificity of the genetic information carried by the transferred plasmid. The nodulation assay carried out by Benhizia (2006) by inoculating Gammaproteobacteria strains which lacked a symbiotic plasmid showed a deficiency in nodules, which explains the absence of the genetic information essential for nodulation. Moreover, for the nodulation assay carried out by Torche (2006) on the same leguminous plants, when there was a doubled inoculation of a strain Gamma proteobaria and a strain of *Rhizobium sulla* A6, the results show that there is a lack of nodules and that the presence of the rhizobial strain has no influence on nodulation which also explains the specificity of the strains for nodulation. Specificity in the association *Hedysarum coronarium*-*Rhizobium sulla* has been verified in several times. In a study carried out in Australia where no species of the genus *Hedysarum* is present, Casella *et al.* (1984) were able to introduce the plant and the symbiotic bacterium, knowing that there are no rhizobia capable of nodulating *Hedysarum coronarium.* During this study, cross-inoculation was attempted with other species of the genus *Hedysarum* and *Onobrichis viciifolia* belonging to the same tribe. It results that the symbiont bacterium *Hedysarum coronarium* nodulates almost all other species of the genus *Hedysarum,* but the azotofixator function is not detectable, while the symbiont of *Onobrichis viciifolia* is unable to nodulate *Hedysarum coronarium*. *Rhizobium sulla* is specific for the species *Hedysarum coronarium,* the other species of

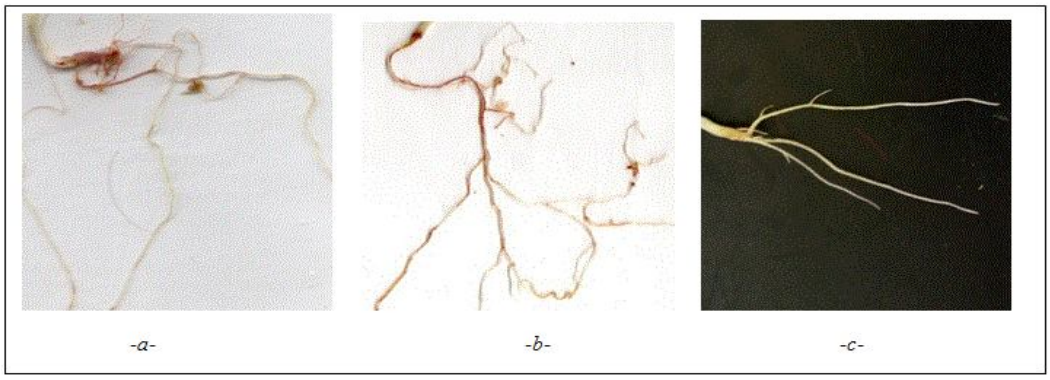

Fig 2: nodulation assay (no root nodule).
-a- H.pallidum inoculated with A6, *-b- H.pallidum* inoculated with (HP7+A6), *-c- H.pallidum* inoculated with HP7 Transformed.

Hedysarum can host other rhizobial species. This has been demonstrated by Torche *et al*., (2014) for the non-cultivable viable rhizobia that reside in the nodules of the two legume species of *Hedysarum* (*H. naudinianum* and *H. perrauderianum*). These bacteria are mesorhizobia that are also different in their sequences.

The establishment of symbiosis is a very complicated process involving a coordinated exchange of signal between legume plants and the symbiots (Ghosh and Maiti, 2016). The involvement of exopolysaccharides in this association has been demonstrated (Gharzouli, 2006). Studies show that succinoglycan (EPS1) is necessary for initiation and elongation of the infection thread during the invasion process (Chuang -Yien Lee, 2000). Exopolysaccharides are produced by a diverse of rhizobia species (Castellane *et al*., 2017). It has been shown that *Rhizobium hedysary* (currently *R. sulla*) produces significant quantities of exopolysaccharides which play a role in nodule induction and nitrogen fixation (Navarini *et al*., 1997). The exopolysaccharides are species- specific complex polymers of different carbohydrate unit secreted by bacterial cell and differ from rhizobial strain to another (Ghosh and Maiti, 2016). The absence of these specific molecules can affect nodulation. Rhizobia mutants deficient in exopolysaccharides synthesis are able to induce nodule formation. These nodules are empty, unable to fix nitrogen and contain a reduced number of intracellular cells (Caetano-Anolles *et al*., 1990). Similarly, the exopolysaccharides deficient mutations of *R.leguminosarum* bv.*trifolii* and *R.leguminosarum* bv *viciae* cause the formation of empty or partially infected nodules and inefficient in clover and vetch (Janczarek and Skorupska, 2003). Further, there is interactions between rhizobial exopolysaccharides and carbohydrate binding proteins, the lectins, on legume root hair surfaces for host recognition (Dudeja *et al*., 2012).

The negative result of nodulation can also be explained by the absence of the other nodulation genes that located on the chromosome of the *Rhizobium* bacteria. Therefore these genes are not transferred during the transformation. The genes involved in nodulation and nitrogen fixation are usually located on one several large plasmids (Psym) or on chromosomal regions (Ding and Hynes, 2009). The genetics of *Rhizobium* is not a simple thing because of the large number of genes involved in the symbiosis and many particularities from strain to another (Pelmont, 1995). Moreover, because of their genetic characteristics, *Rhizobium* strains that nodulate legumes are considered particularly specific to symbiosis (Raposeiras *et al*., 2002). The location of symbiotic genes on the plasmid reinforces the idea that these regions have the potential to be transferred horizontally (Hirsch *et al*., 2001). These replicons contribute to the plasticity of genomes intervening in the process of adaptation and bacterial divergence (Tejerizo *et al*., 2010). The horizontal transfer of genes is one of the strategies that contribute to the generation of genetic variants of the environment that have changed the perception of general aspects of biology (Ibanez *et al*., 2010). Several studies have shown a horizontal transfer of the plasmids carried out by species of the genus *Rhizobium*, this transfer plays an important role in the evolution of rhizobia particularly in their ability to establish a symbiotic nitrogen fixation. Thus, the transfer of plasmids between different bacterial species can play a role in the generation of new strains in soil (Ding and Hynes, 2009).

CONCLUSION

In conclusion, we can say that even if the bacteria of Gammaproteobacteria class are transformed, they cannot cause nodulation because of the specificity of the molecular signals. In addition, the genetic information carried by the transferred plasmid is not sufficient to establish a complete symbiotic relationship.

To complete this study, the aim of the next research is to characterize the non-cultivable viable rhizobia of the leguminous species of the genus *Hedysarum,* look for factors favoring the presence of Gammaproteobacteria in nodules and finally study the causes that lead to the non-cultivable state in rhizobia and seek how to resuscitate them.

Conflict of interest

The authors declare that they have no conflict of interest.

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