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Influence of carbon source on the production of exopolysacharides by *Rhizobium sullae* and on the nodulation of *Hedysarum coronarium* L. legume

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The exopolysaccharides (EPS) produced by species of the *Rhizobium* genus are essential for establishing symbiotic nitrogen fixation with legumes. *Rhizobium* strains were isolated from the wild plant *Hedysarum coronarium* L. collected in Constantine, Algeria. The effect of carbon source on the production of EPS and the infectivity of the bacteria were studied for the different *Rhizobium* isolates. Strains were cultivated on YMA where mannitol was replaced by different sugars. Good bacterial growth was found for sorbitol, sucrose, glucose and maltose. High production of EPS is obtained in the presence of mannitol, sucrose and maltose. Infectivity of the isolates was influenced by the carbon source; high infectivity was recorded in the presence of mannitol and sucrose. A relationship between carbon source, EPS production and nodulation capacity was observed.

Key words: *Rhizobium sullae*, *Hedysarum coronarium*, exopolysaccharides (EPS), symbiosis.

INTRODUCTION

The establishment of symbiotic nitrogen fixation is an important phenomenon for certain plants. This process occurs in the root of leguminous plants within specialized structures called nodules. The development of nodules is induced when the leguminous plants enter in symbiotic association with soil bacteria of the Rhizobiaceae family (Lepek and D’Antuono, 2005). Recognition between the symbionts is made possible through the exchange of molecular signals (Chataigné, 2007). Exopolysaccharides (EPS) produced by *Rhizobium* species are macromolecular complexes essential for the establishment of the symbiotic relationship between *Rhizobium* and leguminous plants. EPS are considered as signaling molecules which play an essential role in the formation of the infection thread and in the invasion process, which are key steps for the formation of root nodules (Chuang-Yien, 2000). The production of EPS is influenced by the conditions for bacterial growth. The carbon source is one of the factors which influence the biosynthesis of EPS (Ruas-Madiedo and de Los Reyes, 2005).

The perennial legume *Hedysarum coronarium* L. is a member of the tribe Hedysareae, subtribe Euhedysarinae whose natural range in the Mediterranean basin stretches from Morocco, Algeria, and Tunisia to southern Spain, Balearic Islands, Corsica and central to southern Italy. This plant is known under the vernacular names of sulla, Spanish sainfoin, and Spanish esparcet (Quezel and Santa, 1962; Tola et al., 2009). Bacteria isolated from the nitrogen-fixing root nodules of sulla have been described and studied by different authors. This bacterial species is described and named *Rhizobium sullae* by Squartini et al. (2002). The objective of this work was to highlight the production of EPS by *Rhizobium sullae* strains, specific partner of *Hedysarum coronarium* L., to study the

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influence of carbon source on the production of EPS and to determine the effect of the variations in the biosynthesis of the EPS on the process of nodulation.

MATERIALS AND METHODS

Isolation and purification of bacterial strains

The bacterial strains used in this study were isolated from the root of *Hedysarum coronarium* L. plant collected according to the method described by Vincent (1970) and Somasegarant and Hoben (1994). The nodules were sterilized by 0.1% acidified mercuric chloride and ethanol, and then washed thoroughly in at least 10 changes of sterile distilled water. The isolation of bacteria was achieved by the method of Vincent (1970). The colonies growing on the three media: YMA-Congo red, glucose peptone agar bromocresol purple (GPA-BCP), YMA-bromothymol blue (YMA-BTB) were ascertained to correspond to the phenotypic descriptions recommended by Vincent; based on the shape and color of the colonies. Five isolates were selected (S1, S2, S3, S4, S5) and strain *Rhizobium sullae* A6 (A. Benguedouar, University of Constantine, Algeria) was used as a reference strain.

Utilization of different carbon sources

To study the effect of the carbon source, strains were cultivated on YMB and YMA medium where mannitol is replaced by other sugars. The growth was estimated by measuring optical density at 600 nm, after 48 h of incubation at 28°C. Rate of growth and the visual evaluation of degree of colonies viscosity were determined by observation of colonies obtained on YMA medium after incubation at 28°C for 48 h.

Detection of EPS production

A strain producing EPS forms mucoid and brilliant colonies on YMA medium. By taking the colony with a platinum loop, there will be formation of long viscous filaments (Dupont, 1998). Strains were cultivated on YMA with 0.05% ruthenium red at 28°C for 24 h. Colonies producing large amounts of EPS appear white in a pink background (Dupont, 1998). The EPS production was tested by the ability of the strains to form fluorescent colonies as determined by illumination with UV light, on YMA agar containing 0.02% calcofluor white (Hotter and Scott, 1990).

Extraction and purification of EPS

EPS production was tested on YMA solid medium where mannitol was substituted with other sugars to estimate the influence of different carbon sources on its production. EPS were extracted as described by Kaci et al. (2005) with some modifications. Petri dishes were incubated at 28°C for 5 days. Mucoid colonies were scraped with a sterilized spatula, and resuspended in sterile KCl (0.85%). Bacterial cells were separated from their EPS by centrifugation (12800 g for 30 min, at 4°C). The supernatant containing EPSs were vacuum-filtered through membranes of 45 μm pore size to eliminate cells and large cellular fragments. EPSs were precipitated with three volumes of absolute cold ethanol. Ethanol-precipitated EPSs were collected by centrifugation for 15 min at 4500 g, at 4°C. EPSs were dried, weighed, and lyophilized.

Colorimetric assay

EPSs were quantified by the Dreywood anthrone/sulfuric acid method (1946) (Dreywood, 1946).

Plant assay

Bacterial strains were tested for their ability to infect the host plant (*Hedysarum coronarium* L.) and induce formation of nodules in bacteriologically controlled conditions. This test was performed in modified Leonard jar, a method suggested by Vincent (1970). Seeds of *H. coronarium* L. were surface-sterilized and germinated as described by Vincent (1970). Germinated seeds were aseptically planted in modified Leonard’s bottle-jar containing the sand-vermiculite mixture watered with Fahraeus nutrient solution. Then, plants were inoculated with exponentially-grown rhizobia, cultivated in the presence of different carbon sources. Pots were placed at room temperature where it was possible to modulate the light conditions for 2 to 3 months. Five strains (S1, S2, S3, S4, and A6) were tested with the following seven sugars: mannitol, sucrose, glucose, galactose, maltose, lactose and sorbitol.

RESULTS AND DISCUSSION

Cultivation phenotypes

The isolates showed good growth on medium YMA after 48 h, which became abundant after 3 to 5 days. Isolates and reference strain *R. sullae* A6 have low absorption of Congo red and do not acidify GPA medium with bromocresol purple after 24 h. These traits were specific to bacteria nodulating legumes, especially the genus *Hedysarum* (Benhizia at el., 2004). Strains acidified the YMA medium in the presence of bromothymol blue after 48 h of incubation. According to Beck et al. (1993), fast growing rhizobia cause an acid reaction on YMA medium containing bromothymol blue, with a shift in the medium color from green to yellow.

Growth on different carbon sources

All five *Rhizobium* strains and the reference strain were found to utilize a wide range of carbon sources (Table 1). Good growth was observed in the presence of sorbitol, sucrose, glucose, maltose and mannitol, whilst arabinose and xylose were poorly utilized by the isolates. The results obtained were comparable to those quoted in the review of Struffi et al. (1998). The isolates and the reference strain show abundant growth on YMA medium after 48 h of incubation. For sugars that support good growth (sorbitol, sucrose, glucose, maltose, mannitol), the degree of viscosity of the colonies is important. The less developed colonies show less viscosity (Table 1). In fact, the fast-growing strains of *Rhizobium* have a generation time of less than 4 h, and form circular convex colonies, usually translucent. In contrast, slow-growing strains of *Bradyrhizobium* have a generation time of 6 to 8 h, and form circular convex colonies, and they are rarely translucent (Jordan, 1984). Most rhizobia use mannitol, glucose, arabinose, fructose, and sucrose as carbon sources (Vincent, 1970; Werner, 1992). Kumari et
al. (2009) showed that among the carbohydrates, monosaccharides (glucose, galactose, arabinose, fructose, raffinose and xylose) gives best growth, followed by sugar alcohols (mannitol), disaccharides (lactose, maltose, and saccharose) and polysaccharides (starch and cellulose). Fast growing rhizobia have the capacity to produce the enzyme NADP-dependent phosphogluconate dehydrogenase which allows them to use a wide range of sugars (Martinez - Romero et al., 1991). On the other hand, slow-growing rhizobia do not have a NADP-dependent phosphogluconate dehydrogenase, and assimilate only some carbon substrates (Jordan, 1984).

Detection of EPS

The colonies of all strains were large with a circular form, translucent and mucoid on YMA. The mucoid colonies formed a long viscous filament when taken with a platinum loop (Figure 1A). When the bacterial cell wall is accessible (no blocking by EPS), their staining with ruthenium red is possible. Therefore, colonies which do not produce EPS appear pink, and strains producing EPS appear white (Figure 1B). When grown on YEM agar containing the fluorescent dye calcofluor white, colonies of the isolated strains showed bright fluorescence under ultraviolet light. These results indicate that all isolates and the reference strain are producing EPS. In R. sullae, the production of two types of EPSs: EPS-A and EPS-B have been reported. Their production is responsible for the viscosity of the bacterial colonies when cultivated on solid medium, which is specific to rhizobia (Orgambide et al., 1996). Mutants defective in production of EPS give small, opaque, and non-mucoid colonies on YMA, and show only dim UV fluorescence (Kaufisi et al., 2004; Hotter and Scott, 1990).

Extraction and quantification of EPS

Abundant production of EPS was found for growth on mannitol, sucrose, sorbitol and maltose, with the majority of strains examined. The other sugars used did not stimulate significantly the production of EPS (Table 1). The strains were not affected similarly by the carbon source used. For example, growth on lactose gave a maximum production of EPS by strain S4, a large quantity of EPS was also produced by strain S3, whereas with the other strains (S1, S2, S5, and A6), the production was null. Growth on xylose did not result in any production of EPS with all the strains tested. Growth on lactose for strains S1, S2 and S5 resulted in good growth and high viscosity, whereas the production of EPS was low (Table 1).

Rhizobia synthesize different classes of polysaccharides: Exopolysaccharides (EPS), capsular polysaccharides (KPS), lipopolysaccharides (LPS) and the cyclic glucans. Some of them are secreted to the media, others are exposed on the surface or present in periplasmic space (Lepek and D’Antuono, 2005).

According to Navarini et al. (1997), high yield of

### Table 1. Effect of carbon source on the growth (OD), degree of viscosity (DV) and yield of production of EPS (mg/g).

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>S1 OD</th>
<th>S1 DV</th>
<th>Yield</th>
<th>S2 OD</th>
<th>S2 DV</th>
<th>Yield</th>
<th>S3 OD</th>
<th>S3 DV</th>
<th>Yield</th>
<th>S4 OD</th>
<th>S4 DV</th>
<th>Yield</th>
<th>S5 OD</th>
<th>S5 DV</th>
<th>Yield</th>
<th>R. sullae A6 OD</th>
<th>R. sullae A6 DV</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>0.327</td>
<td>+</td>
<td>0.382</td>
<td>+</td>
<td>397.5</td>
<td>0.266</td>
<td>+</td>
<td>152.95</td>
<td>0.27</td>
<td>+</td>
<td>1068</td>
<td>0.291</td>
<td>+</td>
<td>18.18</td>
<td>0.37</td>
<td>+</td>
<td>245.9</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.35</td>
<td>+</td>
<td>0.562</td>
<td>+</td>
<td>347.7</td>
<td>0.316</td>
<td>+</td>
<td>433.07</td>
<td>0.24</td>
<td>+</td>
<td>792.8</td>
<td>0.267</td>
<td>+</td>
<td>220.6</td>
<td>0.436</td>
<td>+</td>
<td>93.45</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.246</td>
<td>+</td>
<td>0.237</td>
<td>+</td>
<td>1.56</td>
<td>0.239</td>
<td>+</td>
<td>30</td>
<td>0.28</td>
<td>+</td>
<td>1.9</td>
<td>0.414</td>
<td>+/-</td>
<td>1.25</td>
<td>0.673</td>
<td>+</td>
<td>2921.05</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>0.208</td>
<td>+</td>
<td>0.407</td>
<td>+</td>
<td>224.8</td>
<td>0.169</td>
<td>+</td>
<td>1.2</td>
<td>0.254</td>
<td>+</td>
<td>132.6</td>
<td>0.211</td>
<td>+</td>
<td>208.3</td>
<td>0.423</td>
<td>+</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>0.367</td>
<td>+/-</td>
<td>0.076</td>
<td>-</td>
<td>1</td>
<td>0.13</td>
<td>-</td>
<td>1.09</td>
<td>0.037</td>
<td>-</td>
<td>1.1</td>
<td>0.126</td>
<td>+</td>
<td>1.45</td>
<td>0.341</td>
<td>+</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>0.511</td>
<td>+</td>
<td>1.111</td>
<td>+</td>
<td>0.468</td>
<td>1.8</td>
<td>0.246</td>
<td>+</td>
<td>379.3</td>
<td>0.26</td>
<td>+</td>
<td>117.6</td>
<td>0.32</td>
<td>+</td>
<td>100</td>
<td>0.557</td>
<td>+</td>
<td>1888.88</td>
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<tr>
<td>Lactose</td>
<td>0.262</td>
<td>+</td>
<td>0.605</td>
<td>+</td>
<td>2</td>
<td>0.275</td>
<td>+</td>
<td>285.7</td>
<td>0.13</td>
<td>+</td>
<td>333.3</td>
<td>0.148</td>
<td>+</td>
<td>1.5</td>
<td>0.289</td>
<td>+</td>
<td>1.35</td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.217</td>
<td>+/-</td>
<td>1.30</td>
<td>0.146</td>
<td>1.03</td>
<td>0.116</td>
<td>-</td>
<td>1.25</td>
<td>0.05</td>
<td>-</td>
<td>1.04</td>
<td>0.238</td>
<td>+</td>
<td>2</td>
<td>0.37</td>
<td>+</td>
<td>41.66</td>
<td></td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.291</td>
<td>+</td>
<td>1.09</td>
<td>0.538</td>
<td>228.6</td>
<td>0.304</td>
<td>+</td>
<td>112.8</td>
<td>0.271</td>
<td>+</td>
<td>875</td>
<td>0.118</td>
<td>+</td>
<td>116.6</td>
<td>0.445</td>
<td>+</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>0.113</td>
<td>-</td>
<td>1.2</td>
<td>0.147</td>
<td>1.1</td>
<td>0.122</td>
<td>-</td>
<td>1</td>
<td>0.016</td>
<td>-</td>
<td>1</td>
<td>0.011</td>
<td>-</td>
<td>1.06</td>
<td>0.233</td>
<td>+</td>
<td>1.32</td>
<td></td>
</tr>
</tbody>
</table>

*: High viscosity, +/-: low viscosity, -: no viscosity.
secretion of EPS was found in *Rhizobium hedysari* HCNT1 strain when it was grown on glucose and sucrose. Kumari et al. (2009) reported that when mannitol was used as carbon source in YEM broth, four out of the five isolates isolated from *Indigofera* species studied, showed maximum EPS production, followed by monosaccharides. Mannitol was the most suitable sugar, followed by glucose and galactose for the strain of *Rhizobium* D110 isolated from *Dalbergia lacedolaria* to produce large amounts of EPS (Ghosh et al., 2000). Production of exopolysaccharides is influenced by several growth factors, not only the carbon source, such as nitrogen source and vitamins. These factors can also influence the structural and rheological properties of EPS (Bergmaier, 2002).

**Colorimetric assay**

The anthrone test makes it possible to investigate the sugar constituting EPS. This method allows estimating the proportion of neutral sugars within EPS. The neutral sugar (glucose, galactose) contents vary from 1/4 to 3/4 of total sugar in the polymer. The color of the products obtained depends on the type of sugar constituting the EPS. According to Dreywood (1946), the green color is due to the presence of hexose. Our results showed the presence of large amount of neutral sugars as glucose and galactose.

**Plant assay**

Results of the nodulation test differed by carbon source and strain inoculated. The size and number of nodules obtained varied, depending on the strain inoculated and on the sugar used for its growth (Figure 2). Size and color of nodules obtained varied from small (1 to 1.5 mm of length) and/or white and large (2.5 to 3 mm of length) and pink. The five strains showed good infectivity (large number of nodules) when cultivated in presence of mannitol and of sucrose. Strains A6 and S1 gave a maximum number of nodules, using glucose as carbon source. Sorbitol and maltose stimulated nodulation
Figure 3. Average number of nodules per plant of *H. coronarium* for growth of strains S1, S2, S3, S4 and *R. sullae* A6 on different sugars.
moderately. Galactose and lactose are carbon sources with less stimulating effect on nodulation, with the exception of strain S4 which gave a maximum of nodules in the presence of lactose (Figure 3). We noted that in the presence of sugars which stimulated bacterial growth, a large quantity of EPS was produced, and good infectivity was observed by the formation of nodules of significant size and pink color, confirming correlation between amount of EPS and infectivity. Capacity of the rhizobia to produce an infection on the roots of legumes and to induce the formation of the nodules is called infectivity. This property is limited to a specific group of *Rhizobium* and host, where the infection is induced. It is important to test the ability of strains to produce nodules with the original plant from which they were isolated (Beck et al., 1993).

Our results suggest that the carbon source can influence bacterial growth; the rate of EPS produced and therefore, can modulate the nodulation process and the infectivity of strains. *Hedysarum coronarium* L. is one of the leguminous plants characterized by the formation of indeterminate nodules (Tola et al., 2009). The species of *Rhizobium sullae* is the specific partner of *H. coronarium* L. (Squartini et al., 2002). Molecular analyses have revealed that early steps in the establishment of the symbiosis process, including attraction of the bacteria to the plant and formation of the plant nodule, depend upon an exchange of small signaling molecules between the two partners. Bacterial invasion of root nodules requires exopolysaccharide production by *Rhizobium* strains (Mendrygal and Gonzalez, 2000). Bacterial polysaccharides are necessary for a functional *Rhizobium*-legume symbiosis. Exopolysaccharide (EPS), lipopolysaccharide (LPS), capsular polysaccharide, and cyclic beta-(1,2)-glucan play essential roles in the formation of the infection thread and in nodule development (Lloret et al., 1998). *Rhizobium* mutants defective in synthesis of EPS were able to induce formation of nodules; however, they were empty and not infective, containing a reduced number of intracellular bacteria. This structure is referred to as pseudonodule, characterized by a thread of infection not fully extended (Caetano-Anolles et al., 1990).

**Conclusion**

The production of EPS is a principal feature of strains of the *Rhizobium* genus, and also in the species of *R. sullae*, nodulating the leguminous plant *H. coronarium* L. Most studies showed that glucose, sucrose and mannitol stimulate the production of EPS, in our study, in addition of these sugars, in some cases we found an abundant production of EPS in the presence of lactose and maltose. The results of the Anthrone assay showed that the carbohydrate composition of EPS extract varies with strains, and depends on the carbon source. The EPS consists of a high concentration of glucose compared to galactose. Orgambide et al. (1996) showed that the strain of *R. sullae* produce a type of EPS whose glucose content is higher than that of galactose and mannose. Various factors, except for the carbon source, have been shown to regulate both the quantity and structure of EPS, these factors include: osmolarity, nitrogen starvation, phosphate limitation and growth conditions (pH, temperature) (Spank, 2000).

Most strains display their infectivity by the intensity of nodule formation on roots of the host plant. According to the comparison of results of our nodulation tests, the degree of infectivity varies not only depending on the strain inoculated but also according to the carbon source used. The sugar which stimulates the production of EPS stimulates the capacity of nodulation. Many studies of rhizobial mutants showed that EPS also play a major role in the infection of the leguminous plant (Spank, 2000). EPS are involved in early steps of plant infection such as attachment of bacteria to the roots, structuring of the infection threads, bacteroid development, suppression of plant defense responses and protection against plant antimicrobial compounds (Skorupska et al., 2006).

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