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Carbohydrates as carbon sources in rhizobia under salt stress

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Abstract

Eight strains of rhizobia isolated from Mediterranean legumes were tested for their ability to use various carbohydrates in the presence of different NaCl concentrations. This study used API 50CH galleries which permitted rapid screening of the effect of salt on the utilization of 49 different carbohydrates. In absence of salt, the strains used a large variety of carbohydrates as energy and carbon sources. Salt tolerant strains nodulating *Trigonella foenum-graecum*, *Cytisus arboreus* and *Adenocarpus decorticans*, grew on a wide range of carbohydrates in hyper-osmotic media containing 500 µM or 1 M NaCl. Esculin was the preferred carbon source in highly salt stressed media. In milder saline conditions, 175 or 350 µM NaCl, two salt-stress sensitive strains, isolated from *Genista erioclada* used xylose, ribose, mannose, gluconic acid, galactose, sorbitol, fructose, and the disaccharides, maltose, sucrose, cellobiose and trehalose, but could not grow on mannitol. An unexpected result was that some carbon sources are utilized in presence of salt but not in its absence. The uptake of these carbohydrates is probably activated by salt stress.

Keywords: Carbohydrates, salt stress, rhizobia, Mediterranean legumes, osmoprotection, salt stress

1. Introduction

Soil salinisation is an important cause of land degradation in the world. Saline soils are unsuitable for cultivation of many crop plants because of lack, or unavailability of nitrogen (Zahran, 1999). Cultivation of plants that fix atmospheric nitrogen via symbiotic systems may reduce the severity of this problem (Anthraper and Dubois, 2003). Salinity affects the survival and growth of rhizobia in soil (Zahran, 1999), alters the protein and lipopolysaccharide content of cells, reduces the number of rhizobia in legume inoculants, inhibits the infection process, affects root nodule function, and reduces plant growth and photosynthesis (Soussi et al., 1999; 2001; Cordovilla et al., 1999a,b; Mezni et al., 2002). Rhizobia that colonize saline environments require strategies that protect them against the harmful effects of salt stress such as the accumulation osmoprotectants to counteract cell dehydration (Csonka and Hanson, 1991).

Osmoprotectant substances include glutamate, betaines, ectoine, pipecolic acid and some sugars. These are reported to be of great importance in the salt tolerance of rhizobia (Boncompagni et al., 1999; Gouffi et al., 1998, 1999, 2000; Jebbar et al., 1992; Le Rudulier et al., 1984; Le Rudulier and Bernard, 1986; Le Rudulier; 1993; Talibart et al., 1994). The availability of betaines is limited in arid environments but many carbohydrates and sugars are naturally present in the soil and the rhizosphere. The effect of salinity on the utilization of carbon sources, or the ability of carbon sources to counteract the effect of salinity in rhizobia, has not been much studied. However, ElSheikh and Wood (1989) reported that carbohydrate utilization in *Rhizobium* was affected by the salinity of the growth medium.

Trehalose has been shown to play a role in maintaining a positive turgor of bacterial cells when exposed to high salt osmolarity (Boos et al., 1990; ElSheikh and Wood, 1990; Klein et al., 1991). External trehalose is utilized as a carbon source but it does not contribute to osmoprotection in *E. coli* (Larsen et al., 1987; Strom et al., 1986). However, in *Sinorhizobium meliloti*, trehalose accumulates as a result of biosynthesis or transport and participates in osmoregulation (Smith et al., 1990, 1994). Other disaccharides such as sucrose, maltose, cellobiose, gentibiose, turanose, and palatinose are reported to be nonaccumulated osmoprotectants for rhizobia (Gouffi et al., 1999).

The aims of the present work were to assess salt tolerance in eight strains of rhizobia isolated from Mediterranean legumes, to analyze the effect of different

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salt concentrations on the ability of rhizobia to utilize carbon sources and to test the osmoprotective effect of carbohydrates in rhizobia.

2. Material and Methods

Origin of the strains

Rhizobium strains were isolated from legumes in the North Eastern region of Morocco which is known for its semi-arid to arid climate (Table 1). The strains were isolated from nodules using the method of Vincent (1970) and maintained at 4°C on YEM agar slants. Eight strains were selected to test the effect of salt concentration on carbohydrate utilization in rhizobia. The strains were selected on the basis of their level of tolerance to salt and comprised four salt stress sensitive strains from *Genista erioclada* (broom) (2) and *Leucaena leucocephala* (lead tree) (2), one moderately salt-tolerant strain from *Cytisus arboreus* (Moroccan broom) and three highly salt-tolerant from *Adenocarpus decorticans* (silver broom) (1) and *Trigonella foenum-graecum* (fenugreek) (2).

Table 1. Strains of *Rhizobium* used to study tolerance to NaCl.

Host plants	Number of isolates from nodules	Strains selected for study
Genista erioclada	26	Ge111 and Ge3
Leucaena leucocephala	30	LlAS6 and LlCS6
Cytisus arboreus	16	Cyt8
Adenocarpus decorticans	73	Ad41
Trigonella foenum-graecum	75	S9D and S3G

Seeds sampling and germination

Seeds and root nodules of *Adenocarpus decorticans* were collected from plants naturally growing in an acidic siliceous soil in the mountains of Beni-Znassen (1400 m), a sub-humid region in the North-East of Oujda city. Seeds (and nodules) of *Cytisus arboreus* were collected from fields near Debdou (about 150 km in the South-East of Oujda) a semi-arid zone where the soil is alkaline with a pH of 8.3 ±0.1. Nodules of *Genista erioclada* were collected in Tikermine Mountain around Zaio (about 100 km in the north west of Oujda). Seeds of *Leucaena leucocephala* were kindly provided by colleagues of Laboratoire Commun de Microbiologie (LCM), IRD, Dakar, Senegal.

Seeds of *Adenocarpus decorticans* and *Leucaena leucocephala* were scarified before use via a 30 min exposure to concentrated H_2SO_4, followed by thorough washing in sterile distilled water. To promote germination, seeds were transferred to sterilized water agar medium (0.6% w/v) in Petri plates at 26°C in the dark. After germination, seedlings were aseptically transferred as required.

Seeds of *Cytisus arboreus* and *Genista erioclada* were surface sterilized by 5 min exposure to 3% sodium hypochlorite and steeped in a boiling water bath for 30 seconds and placed in the refrigerator for a night in an icy bath before transfer to the water agar plates. Seeds of *Trigonella foenum-graecum* were surface sterilized by 5 min exposure to 3% (w/v) calcium hypochlorite, then thoroughly rinsed with sterile distilled water and transferred to plates containing water agar. All isolates were tested to confirm their ability to nodulate their original hosts. Seedlings were grown in plastic pots (diameter, 7 cm; height, 14 cm) containing sterilized and nitrogen free sand. Pots were placed in a growth chamber at 23°C, with illumination for 16 h/day, and watered with a dilution of Jensen nitrogen-free solution (Vincent, 1970). The *Rhizobium* strains were grown on YEM broth for 36 hours and inoculated onto seedling roots with 2 ml of a suspension containing approximately 10^8 cells/ml. Nodulation was checked after one to three months.

Effects of salt concentrations on the growth of rhizobia

The effect of NaCl concentrations on the growth of the strains was assessed after 6 days incubation period, by observing the appearance of colonies on solid YEM and by determining the absorbance at 600 nm in a mineral liquid medium containing per litre: NH_4Cl, 0.5 g; K_2HPO_4, 0.35 g; KH_2PO_4, 0.27 g; $MgSO_4$ ($7H_2O$), 0.1 g; $CaCl_2$ ($2H_2O$), 40 mg; $CuSO_4$ ($5H_2O$), 80 mg; H_3BO_3, 2.8 mg; Na_2MoO_4, 90 mg; $MnSO_4$ ($4H_2O$), 2 mg; $ZnSO_4$ ($7H_2O$), 0.2 mg; biotin, 1 mg; Thiamin-HCl, 2 mg; pure agar (Missbah et al., 1996).

On YEM solid media, growth was considered as positive when the diameter of colonies reached at least 1 mm. In the liquid mineral media growth was positive when the OD values at 600 nm were at least 1.5 times higher than the controls. NaCl was added to the media before autoclaving to give the following concentrations: 175; 350; 525; 700; 875; 1050; 1225; 1400; 1575 and 1750 mM. All assays were done in duplicate. The minimal inhibitory concentration of each strain was determined on solid YEM. Strains were streaked on solid YEM, containing the different salt concentrations. Two replicates were used for each assay

API 50 CH galleries or strips (bioMerieux, Marcy-l'Etoile, France, www.biomerieux.com), is a standardized system for the study of carbohydrate metabolism of bacteria. The system consists of 49 microtubes containing dehydrated carbohydrates and one control microtube containing no substrate. These tubes are inoculated with a

bacterial suspension which reconstitutes the media. During incubation, metabolism produces colour changes and the strips are read visually and growth or lack of growth is noted. Even weak growth or change in colour is considered a positive result.

Suspensions of the cells in a mineral medium (Missbah et al., 1996) were transferred into each of the 50 wells of the API 50 CH strips. The mineral medium used to inoculate the galleries contained per liter: NH_4Cl, 0.5 g; K_2HPO_4, 0.35 g; KH_2PO_4, 0.27 g; $MgSO_4$ ($7H_2O$), 0.1 g; $CaCl_2$ ($2H_2O$), 40 mg; $CuSO_4$ ($5H_2O$), 80 mg; H_3BO_3, 2.8 mg; Na_2MoO_4, 90 mg; $MnSO_4$ ($4H_2O$), 2 mg; $ZnSO_4$ ($7H_2O$), 0.2 mg; biotin, 1 mg; Thiamin-HCl, 2 mg, and 3 g of agar. All wells were overlaid with sterile paraffin oil to make anaerobiosis. Strips were moistened and covered as recommended by the manufacturer and incubated at 28°C. Changes in colour and appearance of growth were monitored after 1, 2 and 7 days. Aesculin hydrolysis (revealed by a change to a darker colour or black) was represented by a positive sign (+), while a negative sign (–) represented no change.

Effects of salt on carbohydrate utilization

The effects of salt was first studied on the salt stress sensitive strains, Ge111 and Ge3, that nodulated *Genista erioclada*, and LISC6 and LISA6 that nodulated *Leucaena leucocephala*. The test was carried out on a YEM solid medium lacking mannitol, but contained yeast extract at a NaCl concentration of 50 mg/l (instead of 500 mg/l). Filter sterilized carbon sources were added to the salt medium after autoclaving to give a final concentration of 5 g/l. The growth was checked daily at 28°C. Two replicates were used for each assay Controls contained the carbon sources but no salt, or salt but no carbon source.

A second study was undertaken which involved salt-tolerant strains Ad41 (isolated from *Adenocarpus decorticans*); Cyt8 (*Cytisus arboreus*); S3G and S9D *Trigonella foenum-graecum* and employed the API50CH to study growth on 49 carbohydrates. NaCl concentrations were adjusted to values of 250 mM, 500 mM or 1 M. Media were autoclaved and left to cool in a bath at 50°C.

Rhizobia cultures were grown on YEM slants at 28°C and incubated for 36 hours, then 3 ml of sterile bi-distilled water or of saline solutions 250 mM, 500 mM or 1 M made in bi-distilled water were added. The cultures were suspended in the saline solutions and 3 ml of the suspensions were transferred to mineral media with the same osmolarity. These saline bacterial suspensions ($\approx 10^8$ cells/ml) were used to inoculate the API50CH galleries (Biomérieux, France) as recommended by the manufacturer. The galleries were incubated at 28°C and daily checked for growth. Two replicate galleries were used for each assay. Controls containing no salt (in the medium or in the culture suspensions) were conducted and assessed under the same conditions.

3. Results

Effects of salt on the growth of rhizobia

All the strains studied nodulated their original hosts. Strains S3G and S9D isolated from *Trigonella foenum-graecum,* strain Cyt8 isolated from *Cytisus arboreus* and strain Ad41 isolated from *Adenocarpus decorticans* were highly salt tolerant strains; whereas strains Ge3; Ge111 nodulating *Genista erioclada* and strains LIAS6 and LICS6 isolated from *Leucaena leucocephala* were more sensitive (Table 2). There were no changes in the pH of the media after addition of salt.

Carbohydrate utilization

None of the *Rhizobium* strains grew in controls lacking carbon sources, in presence or absence of added salt. Therefore, any growth observed was attributed to the assimilation of the specific carbohydrate. After 6 days of incubation, all monosaccharides as well as a great number of the disaccharides tested on modified solid YEM were assimilated by strains Ge3; Ge111; LISA6 and LISC6 (Table 3). Trehalose, maltose, cellobiose, raffinose, inositol and sucrose were used by all the four strains. Lactose was not assimilated by the two strains nodulating *Genista erioclada*. However, none of the four strains were capable of assimilating starch, dextrin, and salicin, while gluconate was only used by strains Ge111 and Ge3.

We assessed the growth on 49 substrates in API 50CH galleries and 21 carbohydrates on the solid mineral medium. Substrates on which a rapid growth was observed were considered as the most easily utilized or preferred. For example, with strain Ad41, no growth was observed on lactose, amygdaline or D-lyxose until the third day of incubation at 28°C. The same observation was made with growth of strain Cyt8 on mannitol, xylitol and L-arabitol. Amygdaline and melezitose were slowly metabolized by strain S9D. In strain S3G, all the potentially metabolized carbon sources were assimilated after the second day of incubation (Table 4).

Effects of salt on carbohydrate utilization

Low-salt tolerant strains

We determined the maximal concentration of salt tolerated by each strain. Then, we determined the minimal inhibiting concentration (MIC) on the same medium (results not shown). The MICs were used to apply the salt stress on the growth of rhizobia.

Table 2. Effect of salt concentration on growth of rhizobia on solid YEM medium.

Strains used in this experiment	NaCl concentration in mM 0	175	350	525	700	875	1050	1225	1400
Ge111	+	–	–	–	–	–	–	–	–
Ge3	+	+	–	–	–	–	–	–	–
LIAS6	+	+	–	–	–	–	–	–	–
LICS6	+	+	–	–	–	–	–	–	–
Cyt 8	+	+	+	+	+	–	–	–	–
Ad 41	+	+	+	+	+	+	+	–	–
S9D	+	+	+	+	+	+	+	–	–
S3G	+	+	+	+	+	+	+	+	+

Table 3. Carbohydrate utilization in presence or absence of salt (in 175 or 350 mM of NaCl) by *Rhizobium* strains GE3, GE 111, LIS5 and LIS6 which have a low tolerance to salt.

Carbon source	Strains Ge111		Ge3		LIAS6		LICS6	
NaCl (mM)	0	175	0	350	0	350	0	350
Mannitol	+	–	+	–	+	–	+	–
Glucose	+	+	+	–	+	–	+	–
Xylose	+	+	+	+	+	–	+	–
Ribose	+	+	+	+	+	–	+	–
Mannose	+	+	+	+	+	–	+	–
Gluconic acid	+	+	+	+	–	–	–	–
Galactose	+	+	+	+	+	–	+	–
Sorbitol	+	+	+	+	+	–	+	–
Arabinose	+	–	+	–	+	–	+	+
Fructose	+	+	+	+	+	–	+	–
Inositol	+	–	+	–	+	+	+	–
Rhamnose	+	+	+	–	+	–	+	–
Salicin	–	–	–	–	–	+	–	+
Lactose	–	+	–	–	+	–	+	–
Maltose	+	+	+	+	+	+	+	–
Sucrose	+	+	+	+	+	+	+	–
Cellobiose	+	+	+	+	+	+	+	–
Trehalose	+	+	+	+	+	+	+	–
Raffinose	+	–	+	–	+	+	+	–
Dextrin	–	–	–	–	–	–	–	–
Starch	–	–	–	–	–	–	–	–

Growth was assessed on solid YEM where mannitol was replaced by the carbohydrate mentioned. +: Appearance of colonies on the solid medium. –: No growth observed.

Strain Ge111 which is inhibited by 175 mM of NaCl on YEM modified medium with mannitol as a sole carbon source (the MIC) was exposed to this salt concentration in presence of other carbohydrates. Strains Ge3, Llas6 and Llcs6 were exposed to 350 mM of NaCl. The utilization of some sugars that normally supported growth, e.g. arabinose in strains Ge111, Ge3 and Llas6, was inhibited by the MIC salt concentration. Other carbohydrates for example maltose, sucrose, cellobiose and trehalose in strains Ge111, Ge3 and Llas6 were used in the presence of the MIC of salt (Table 3).

Mannitol was not the preferred carbon source under salt stress as it was unable to sustain bacterial growth under these conditions whereas galactose, cellobiose, sucrose, fructose etc were utilized in saline media. We noted that some sugar, not used by rhizobia under normal conditions become utilizable under salt stress. Hence, salicin was utilized by the two strains nodulating *Leucaena leucocephala* and lactose by strain Ge111 (Table 3). Strain Ge111, in presence of 175 mM of NaCl, was able to grow with all tested carbohydrates other than mannitol, inositol, raffinose and arabinose.

High-salt tolerant strains

The tolerant strains, Cyt8; Ad41; S3G (data not shown) and S9D were exposed to salt stress at 250, 500 and 1000 mM of NaCl, just before inoculation into the galleries (Tables 5, 6, and 7).

When exposed to 250 mM of NaCl, strains Ad41, S3G and S9D continued to use a wide variety of carbohydrates to the same extend as in the absence of this level of NaCl. There was no variation in either the carbohydrate utilization pattern or in the length of time needed to use each substrate. In strain Cyt8, all the sugars used under normal conditions were used in presence of 250 mM NaCl except α-Methyl-D-glucoside, amygdaline, starch and 5-Keto-gluconate. However, there was a delay of two or three days in the growth on some carbon sources. Thus, after 2 days incubation time, only esculin and 2-Keto-gluconate were used, but by the third day, all but D-raffinose, mellibiose, salicin and D-fructose were used. Growth on those 4 carbohydrates was observed by the sixth day of incubation. The long lag phase was independent of the bacterial concentration of the inoculum.

The carbohydrate utilization pattern of the strains S3G and S9D was the same in the presence or absence of 500 mM NaCl. In strain Ad41, a saline stress of 500 mM completely inhibited the use of amygdaline, lactose, D-lyxose and tagatose. Except for D-raffinose and xylitol, whose utilization was retarded for more than 4 days, growth on the different substrates was similar to controls without salt. Under the same salt-stress condition, strain Cyt8 was unable to use the great majority of carbohydrates. Of the 38 substrates used in presence of 250 mM NaCl, only 4 (glucose, N-acetyl glucose amine, esculin and 2-Ketogluconate) were assimilated at 500 mM NaCl. Growth on esculin was observed by the third day of incubation, glucose by the fourth day and N-acetyl-glucose-amine by the sixth day.

Rhizobium strain Ad41 used eleven carbohydrates in presence of 1 M NaCl. Under these extreme conditions, there was no growth by the second day of incubation but on the third day, growth was observed on esculin, trehalose, D-fructose, galactose, D-arabitol, gluconate and mannitol. By the fourth day, glycerol, rhamnose and N-acetyl glucose amine were used and growth on inositol was observed in the sixth day of incubation. Strain Cyt8 was completely inhibited by 1 M NaCl.

In strains S3G and S9D, esculin seemed to be the preferred carbohydrate because of its rapid utilization by the second day of incubation. Growth on mannitol and the other substrates appeared in the third day or later. Inositol and xylose were not used by strain S3G at 1 M NaCl but seventeen of the nineteen carbohydrates used at 500 mM still supported growth of the bacteria. Strain S9D was able to metabolize seventeen sugars of the thirty-four used at 500 mM NaCl.

The important result emerging from this work is that some sugars which are not normally used by rhizobia in absence of salt are used in its presence, except in the case of strain Ad41. Thus, in strain Cyt8 growth became possible on rhamnose, sorbose, gluconate and fucose in salt stressed conditions at 250 mM. Strain S9D assimilated erythritol, D-arabinose, D-fucose and L-fucose in presence of 500 mM NaCl but not in its absence. However, this phenomenon was not generally observed in presence of 1 M NaCl. Salicin was the sole carbohydrate not used by strain S3G in control conditions but used in presence of 1 M NaCl.

4. Discussion

The eight strains of rhizobia isolated from Mediterranean legumes use a large variety of carbohydrates as sole carbon and energy sources. All the strains showed a pattern of carbon sources utilization typical to fast growers (Graham, 1964; Chakrabarti et al., 1981, 1987). Fast growing rhizobia are nutritionally diverse in their utilization of carbon sources, whereas slow growing rhizobia appear to be nutritionally fastidious (Stowers, 1985). The utilisation of a wide range of carbohydrates may be the key to surviving and competing for nodule occupancy (Jensen et al., 2002). In arid and semi arid regions, the irregular rainfall as well as the succession of drought and rain periods leads to large changes in the osmolalities of soil and the rhizospheric microorganism environment.

Many carbohydrates such as raffinose, sucrose, rhamnose and trehalose are present in the rhizosphere around legume plant roots and germinating seeds (Gage and Long, 1998; Bringhurst and Gage, 2000; Bringhurst et al., 2001). Transport and utilization of soil carbohydrates is critical for competitive root colonization by *Rhizobium* (Jensen et al., 2002). When submitted to a salt stress, the rhizobial strains used in the present study showed a variation in the utilization of carbohydrates in accord with their salt tolerance pattern. Strains which tolerate high salt concentrations continue to use (or are able to recover their capability to grow on) a wide variety of carbon sources in salt stressed media. However, the growth rates were slow compared with the control in unstressed conditions.

The use of API galleries or the screening test on solid modified YEM does not establish whether it is the ability to transport the carbon sources into the cells or the rate of uptake that is affected by the presence of salt. Nevertheless, we can anticipate that both processes may be affected. This is because, in many cases, there was a delay of 2 to 5 days in the appearance of growth on carbohydrates in presence of salt. We presume that in high-salt media, the catabolic processes and/or the enzyme activities as well as the transport systems may be affected by intracellular ionic forces.

Table 4. Utilization of carbohydrates over a six day period, in the absence of added NaCl, by salt-tolerant *Rhizobium* strains that nodulate some Mediterranean legumes in Morocco.

Carbohydrates	Strains Ad41				Cyt8				S3G				S9D			
	2[a]	3[a]	4[a]	6[a]	2	3	4	6	2	3	4	6	2	3	4	6
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Erythritol	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
D-Arabinose	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Xylose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Adonitol	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+
α Methyl-xyloside	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+
Dulcitol	–	–	–	–	+	+	+	+	–	–	–	–	–	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Mannitol	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+
α Methyl-D-mannoside	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α Methyl-D-glucoside	–	–	–	–	+	+	+	+	–	–	–	–	–	+	+	+
N acetyl glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdaline	–	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+
Arbutine	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Maltose	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+
Lactose	–	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+
Melibiose	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Inulin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
D-Raffinose	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Starch	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–
Glycogen	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xylitol	+	+	+	+	–	+	+	+	–	–	–	–	+	+	+	+
Gentibiose	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
D-Turanose	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+
D-Lyxose	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–
D-Tagatose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fucose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-Fucose	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–
D-Arabitol	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+
L-Arabitol	+	+	+	+	–	+	+	+	–	–	–	–	+	+	+	+
Gluconate	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+
2 Keto-gluconate	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+
5 Keto-gluconate	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–

This experiment was tested in API CH strips. The growth was observed after 2, 3, 4 and 6 days of incubation at 28°C. a): Days of growth. –): No growth observed at the time specified. +): Visible growth.

Table 5. Carbohydrate utilization by strain Ad41 nodulating *Adenocarpus decorticans*, in the presence of 500 mM NaCl (S1), 1 M NaCl (S2), or absence of salt in relation to incubation time.

Carbohydrates	2 days			3 days			4 days			6 days		
	C[a]	S1[b]	S2[c]	C	S1	S2	C	S1	S2	C	S1	S2
Control	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	+	+	–	+	+	–	+	+	+	+	+	+
Erythritol	+	+	–	+	+	–	+	+	–	+	+	–
D-Arabinose	–	–	–	–	–	–	–	–	–	–	–	–
L-Arabinose	+	+	–	+	+	–	+	+	–	+	+	–
Ribose	+	+	–	+	+	–	+	+	–	+	+	–
D-Xylose	+	+	–	+	+	–	+	+	–	+	+	–
L-Xylose	–	–	–	–	–	–	–	–	–	–	–	–
Adonitol	+	+	–	+	+	–	+	+	–	+	+	–
α Methyl-xyloside	–	–	–	–	–	–	–	–	–	–	–	–
Galactose	+	+	–	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	–	+	+	–	+	+	–	+	+	–
D-Fructose	+	+	–	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	–	+	+	–	+	+	–	+	+	–
L-Sorbose	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	+	+	–	+	+	–	+	+	+	+	+	+
Dulcitol	–	–	–	–	–	–	–	–	–	–	–	–
Inositol	+	+	–	+	+	–	+	+	–	+	+	+
Mannitol	+	+	–	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	–	+	+	–	+	+	–	+	+	–
α Methyl-D-mannoside	–	–	–	–	–	–	–	–	–	–	–	–
α Methyl-D-glucoside	–	–	–	–	–	–	–	–	–	–	–	–
N acetyl glucosamine	+	+	–	+	+	–	+	+	+	+	+	+
Amygdaline	–	–	–	+	–	–	+	–	–	+	–	–
Arbutin	+	+	–	+	+	–	+	+	–	+	+	–
Esculin	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	–	+	+	–	+	+	–	+	+	–
Cellobiose	+	+	–	+	+	–	+	+	–	+	+	–
Maltose	+	+	–	+	+	–	+	+	–	+	+	–
Lactose	–	–	–	+	–	–	+	–	–	+	–	–
Melibiose	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	+	+	–	+	+	–	+	+	–	+	+	–
Trehalose	+	+	–	+	+	+	+	+	+	+	+	+
Inulin	–	–	–	–	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	–	–	–	–	–	–
D-Raffinose	+	–	–	+	–	–	+	–	–	+	+	–
Starch	–	–	–	–	–	–	–	–	–	–	–	–
Glycogen	–	–	–	–	–	–	–	–	–	–	–	–
Xylitol	+	–	–	+	–	–	+	–	–	+	+	–
α Gentibiose	–	–	–	–	–	–	–	–	–	–	–	–
D-Turanose	–	–	–	–	–	–	–	–	–	–	–	–
D-Lyxose	–	–	–	+	–	–	+	–	–	+	–	–
D-Tagatose	+	–	–	+	–	–	+	–	–	+	–	–
D-Fucose	–	–	–	–	–	–	–	–	–	–	–	–
L-Fucose	–	–	–	–	–	–	–	–	–	–	–	–
D-Arabitol	+	+	–	+	+	+	+	+	+	+	+	+
L-Arabitol	+	+	–	+	+	–	+	+	–	+	+	–
Gluconic acid	+	+	–	+	+	+	+	+	+	+	+	+
2 Keto-gluconic acid	+	+	–	+	+	–	+	+	–	+	+	–
5 Keto-gluconic acid	–	–	–	–	–	–	–	–	–	–	–	–

This experiment was tested in API CH strips. The growth was observed after 2, 3, 4 and 6 days of incubation at 28°C. a): Controls containing no NaCl. b): Utilization of carbohydrates at 0.5 M of NaCl. c): Utilization of carbohydrates at 1 M of NaCl. +): Growth. –): no growth observed.

Table 6. Carbohydrate utilization by strain Cyt8 isolated from *Cytisus arboreus*, in the presence of 250 mM NaCl (S1), 500 mM NaCl (S2), or absence of salt (C) in relation to incubation time.

Carbohydrates	2 days			3 days			4 days			6 days		
	C[a]	S1[b]	S2[c]	C	S1	S2	C	S1	S2	C	S1	S2
Control	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	+	–	–	+	+	–	+	+	–	+	+	–
Erythritol	–	–	–	–	–	–	–	–	–	–	–	–
D-Arabinose	+	–	–	+	+	–	+	+	–	+	+	–
L-Arabinose	+	–	–	+	+	–	+	+	–	+	+	–
Ribose	+	–	–	+	+	–	+	+	–	+	+	–
D-Xylose	+	–	–	+	+	–	+	+	–	+	+	–
L-Xylose	–	–	–	–	–	–	–	–	–	–	–	–
Adonitol	+	–	–	+	+	–	+	+	–	+	+	–
α Methyl-xyloside	+	–	–	+	+	–	+	+	–	+	+	–
Galactose	+	–	–	+	+	–	+	+	–	+	+	–
D-Glucose	+	–	–	+	+	–	+	+	+	+	+	+
D-Fructose	+	–	–	+	–	–	+	–	–	+	+	–
D-Mannose	+	–	–	+	+	–	+	+	–	+	+	–
L-Sorbose	–	–	–	–	–	–	–	–	–	–	+	–
Rhamnose	–	–	–	–	+	–	–	+	–	–	+	–
Dulcitol	+	–	–	+	+	–	+	+	–	+	+	–
Inositol	+	–	–	+	+	–	+	+	–	+	+	–
Mannitol	–	–	–	+	+	–	+	+	–	+	+	–
Sorbitol	+	–	–	+	+	–	+	+	–	+	+	–
α Methyl-D-mannoside	–	–	–	–	–	–	–	–	–	–	–	–
α Methyl-D-glucoside	+	–	–	+	–	–	+	–	–	+	–	–
N acetyl glucosamine	+	–	–	+	+	–	+	+	–	+	+	+
Amygdaline	+	–	–	+	–	–	+	–	–	+	–	–
Arbutin	+	–	–	+	+	–	+	+	–	+	+	–
Esculin	+	+	–	+	+	+	+	+	+	+	+	+
Salicin	+	–	–	+	–	–	+	–	–	+	+	–
Cellobiose	+	–	–	+	+	–	+	+	–	+	+	–
Maltose	+	–	–	+	+	–	+	+	–	+	+	–
Lactose	+	–	–	+	+	–	+	+	–	+	+	–
Melibiose	+	–	–	+	–	–	+	–	–	+	+	–
Sucrose	+	–	–	+	+	–	+	+	–	+	+	–
Trehalose	+	–	–	+	+	–	+	+	–	+	+	–
Inulin	–	–	–	–	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	–	–	–	–	–	–
D-Raffinose	+	–	–	+	–	–	+	–	–	+	+	–
Starch	+	–	–	+	–	–	+	–	–	+	–	–
Glycogen	–	–	–	–	–	–	–	–	–	–	–	–
Xylitol	–	–	–	+	+	–	+	+	–	+	+	–
Gentibiose	+	–	–	+	+	–	+	+	–	+	+	–
D-Turanose	+	–	–	+	+	–	+	+	–	+	+	–
D-Lyxose	+	–	–	+	+	–	+	+	–	+	+	–
D-Tagatose	+		–	+	+	–	+	+	–	+	+	–
D-Fucose	–	–	–	–	–	–	–	+	–	–	+	–
L-Fucose	+	–	–	+	+	–	+	+	–	+	+	–
D-Arabitol	+	–	–	+	+	–	+	+	–	+	+	–
L-Arabitol	–	–	–	+	+	–	+	+	–	+	+	–
Gluconic acid	–	–	–	–	+	–	–	+	–	–	+	–
2 Keto-gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+
5 Keto-gluconic acid	+	–	–	+	–	–	+	–	–	+	–	–

This experiment was tested in API CH strips. The growth was observed after 2, 3, 4 and 6 days of incubation at 28°C. a): Controls containing no NaCl. b): Utilization of carbohydrates at 250 mM of NaCl. c): Utilization of carbohydrates at 500 mM of NaCl. +): Growth. –): no growth observed.

Table 7. Carbohydrate utilization by strain S9D nodulating *Trigonella foenum-graecum* in the presence of 500 mM NaCl (S1), 1 M NaCl (S2) or absence of salt (C) in relation to incubation time.

Carbohydrates	2 days			3 days			4 days			6 days		
	C[a]	S1[b]	S2[c]	C	S1	S2	C	S1	S2	C	S1	S2
Control	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	–	–	–	+	–	–	+	–	–	+	+	+
Erythritol	–	–	–	–	–	–	–	+	–	–	+	–
D-Arabinose	–	–	–	–	+	–	–	+	–	–	+	–
L-Arabinose	+	–	–	+	+	–	+	+	–	+	+	+
Ribose	+	+	–	+	+	–	+	+	–	+	+	–
D-Xylose	+	–	–	+	+	–	+	+	–	+	+	–
L-Xylose	–	–	–	–	–	–	–	–	–	–	–	–
Adonitol	+	–	–	+	+	–	+	+	–	+	+	–
α Methyl-xyloside	+	–	–	+	+	–	+	+	–	+	+	–
Galactose	+	–	–	+	+	–	+	+	–	+	+	–
D-Glucose	+	–	–	+	–	+	+	+	+	+	+	+
D-Fructose	+	–	–	+	–	+	+	+	+	+	+	+
D-Mannose	+	–	–	+	–	+	+	+	+	+	+	+
L-Sorbose	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	+	–	–	+	+	–	+	+	–	+	+	–
Dulcitol	–	–	–	+	–	–	+	–	–	+	+	–
Inositol	–	–	–	+	+	–	+	+	–	+	+	–
Mannitol	+	–	–	+	+	+	+	+	+	+	+	+
Sorbitol	–	–	–	+	+	–	+	+	–	+	+	–
α Methyl-D-mannoside	–	–	–	–	–	–	–	–	–	–	–	–
α Methyl-D-glucoside	–	–	–	+	+	+	+	+	+	+	+	+
N acetyl glucosamine	+	–	–	+	+	+	+	+	+	+	+	+
Amygdaline	–	–	–	–	–	–	+	–	–	+	–	–
Arbutin	–	–	–	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	–	–	+	–	+	+	+	+	+	+	+
Cellobiose	–	–	–	+	+	+	+	+	+	+	+	+
Maltose	+	–	–	+	–	–	+	–	–	+	–	–
Lactose	+	–	–	+	+	–	+	+	–	+	+	–
Melibiose	+	–	–	+	–	–	+	–	–	+	–	–
Sucrose	–	–	–	+	–	–	+	–	–	+	–	–
Trehalose	–	–	–	+	–	–	+	–	–	+	–	+
Inulin	–	–	–	–	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	+	–	–	+	–	–
D-Raffinose	–	–	–	+	–	–	+	–	–	+	–	–
Starch	–	–	–	–	–	–	–	–	–	–	–	–
Glycogen	–	–	–	–	–	–	–	–	–	–	–	–
Xylitol	+	–	–	+	+	–	+	+	–	+	+	–
Gentibiose	+	–	–	+	+	+	+	+	+	+	+	+
D-Turanose	+	–	–	+	–	+	+	+	+	+	+	+
D-Lyxose	–	–	–	–	–	–	–	–	–	–	–	–
D-Tagatose	+	–	–	+	–	+	+	+	+	+	+	+
D-Fucose	–	–	–	–	+	–	–	+	–	–	+	–
L-Fucose	–	–	–	–	–	–	–	+	–	–	+	–
D-Arabitol	+	–	–	+	–	–	+	+	–	+	+	–
L-Arabitol	+	–	–	+	+	–	+	+	–	+	+	–
Gluconate	+	–	–	+	+	+	+	+	+	+	+	+
2 Keto-gluconate	+	+	–	+	+	–	+	+	–	+	+	–
5 Keto-gluconate	–	–	–	–	–	–	–	–	–	–	–	–

This experiment was tested in API CH strips. The growth was observed after 2, 3, 4 and 6 days of incubation at 28°C. a): Controls containing no NaCl. b): Utilization of carbohydrates at 0.5 M of Nal. c): Utilization of carbohydrates at 1 M of NaCl. +): Growth. –): no growth observed.

We noted that salt-stressed strains prefer to use carbohydrates other than mannitol as a carbon source. In the two rhizobial strains nodulating *Genista erioclada*, the most preferred sugars were gluconic acid, sorbitol (polyol), the pentoses xylose and ribose, the hexoses mannose and galactose, and the disaccharides maltose, sucrose, cellobiose and trehalose. In contrast, the strain LIAS6, nodulating *Leucaena leucocephala*, grew better on raffinose (trisaccharide), Inositol (polyol) and the disaccharides, maltose, sucrose, cellobiose and trehalose.

Cellobiose, salicin, and esculin are sugars belonging to a group of saccharides called (beta)-glucosides. Cellobiose and salicin were used by salt stressed cells of strains Ge111, Ge3 and AIAS6. In the strains nodulating *Trigonella-foenum graecum*, *Cytisus arboreus* and *Adenocarpus decorticans*, esculin was the preferred carbohydrate under salt-stress conditions. Esculin [6-(beta-D-glucopyranosyl-oxy)-7-hydroxy-2H-1-Benzopyran-2-one] is a flavonoid known to stimulate significantly nodule formation and nitrogen fixing activity in rhizobia (Novikova, 1994). Esculin should be an osmoprotectant in these rhizobia.

Moënne-Loccoze and Weaver (1996) found that the presence of some plasmids governing the assimilation of many carbohydrates such as glycerol, sorbitol, arabinose and raffinose is necessary for the survival of rhizobia in soil under water and salt stress conditions. Genes involved in the transport and catabolism of rhamnose in *R. leguminosarum* bv. *trifolii* are plasmid encoded and are necessary for the bacterium to be competitive for nodule occupancy (Baldani et al., 1992; Charles et al., 1990; Charles and Finan, 1991; Oresnik et al., 1998). Rhamnose was only used by strain Ge111 in presence of salt. It is a methyl-pentose sugar found as a constituent of pectin in the form of rhamnogalacturonan within the cell walls of dicotyledonous plants (Mc Neil et al., 1984) and in the mucilage of a number of legume plants (Knee et al., 2001).

Among sugars, trehalose is the best known as osmoprotectant in microorganisms (Roberts, 2005). Trehalose is available as a carbon source in the root exudates (Jensen et al., 2002). With the exception of strains LICS6 and Cyt8 which do not use trehalose in presence of 350 and 500 mm of NaCl respectively, all the strains tested used trehalose in absence of salt as well as in its presence. Carbohydrate transport systems may also be induced by intracellular osmotic pressure. Higgins et al. (1987, 1988) have shown that the transport of maltose results from the activation of *malB* genes by the effect of the ionic force of the medium that provokes changes in DNA structure. We suggest that the uptake of fucose and other carbohydrates used only in salt stress conditions are activated by salt stress.

Further research on this topic is warranted because the ability of rhizobia to utilize a variety of carbohydrates is important for successful nodulation and will enable us to increase our understanding of the plant *Rhizobium* symbiosis under conditions of environmental stress. Furthermore, research on the tolerance and performance of *Rhizobium* when exposed to salt may yield results having applications for improving plant growth in saline soils.

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