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Carbon metabolism and bacteroid respiration in nodules of chick-pea (*Cicer arietinum* L.) plants grown under saline conditions

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ABSTRACT - The present work investigates the relationships between nitrogen fixation, carbon metabolism and oxygen consumption by bacteroids of *Mesorhizobium ciceri* in root nodules of chick-pea plants. Its aim was to establish whether some of the compounds which accumulate under salt stress may be used as respiratory substrates by bacteroids to fuel their own metabolism and nitrogenase activity. Plants were grown in a growth chamber, and salt stress was induced by adding 50 mM NaCl to the nutrient solution at sowing. The data presented here show a rise in fermentative metabolism in nodules of chick-pea plants exposed to high salinity, and suggest that proline, lactate or ethanol, may play an important role as energy-yielding substrates for bacteroids in this plant species. The bacteroids could utilize glucose as a respiratory substrate both under control and saline conditions, while malate did not appear to be the preferred substrate in the presence of salt.

KEY WORDS - *Cicer arietinum,* bacteroid respiration, carbon metabolism, N_2 fixation, salinity

ABBREVIATIONS - ARA = acetylene reduction; PEPC = phosphoenolpyruvate carboxylase (EC 4.1.1.31); MDH = malate dehydrogenase (EC 1.1.1.37); $NADP^+$-ICDH = $NADP^+$-dependent isocitrate dehydrogenase (EC 1.1.1.42); ADH = alcohol dehydrogenase (EC 1.1.1.1); 6PGDH = 6-phosphogluconate dehydrogenase (EC 1.1.1.44).

Salinity reduces the yield of legume crops by its negative effects on growth and nitrogen fixation. The effect of salinity on nitrogen fixation has been well studied, and different mechanisms are known to affect root nodule formation. However, the hypothesis which relates nitrogenase inhibition directly to the lack of photosynthate supply to nodules (HUNT & LAYZELL, 1993) must be discarded, given that photosynthesis is less sensitive to water and salt stress than is nitrogen fixation (SOUSSI *et al.*, 1998), and that these conditions induce the accumulation of total soluble sugars in nodules (FOUGÈRE *et al.*, 1991; GONZALEZ *et al.*, 1995).

Sucrose is supplied to the nodule via phloem, and its metabolism leads to the synthesis of dicarboxylic acids, which are the main products of sucrose degradation supplied to bacteroids to support nitrogen fixation in

legume nodules (DAY & COPELAND, 1991; GORDON *et al.*, 1995). However, sucrose metabolism is inhibited under stress (RAMOS *et al.*, 1999), resulting in a lower supply of dicarboxylic acids to bacteroids. In fact, the decrease of organic acid contents, such as malate or oxaloacetate, in nodules of stressed plants has been well documented in soybean (FOUGÈRE *et al.*, 1991), faba bean (TRINCHANT *et al.*, 1998) and chick-pea (SOUSSI *et al.*, 1999).

On the other hand, the respiratory capacity of bacteroids is severely depressed by salinity, as reported by DELGADO *et al.* (1993) after measuring the oxygen consumption of bacteroids isolated from nodules of pea plants grown under salt stress, and this effect could be partially reverted by the addition of respiratory substrates such as succinate or malate. Salinity promoted the accumulation of glucose and soluble amino acids, such as proline, glycine-betaine, and glutamate in root nodules. These compounds, in addition to their controversial role in osmoregulation (PEREZ-ALFOCEA *et al.*, 1994; PETRUSA & WINICOV, 1997) are suspected of providing energy to bacteroids (PEDERSEN *et al.*, 1996).

In the present work, growth, nitrogen fixation, and enzyme activities of carbon metabolism in the host-plant cytosol and in bacteroids were evaluated in plants of chick-pea (*Cicer arietinum* L.) grown under saline conditions. Moreover, bacteroids isolated from root nodules were incubated in the presence or absence of some energy-yielding compounds, and the oxygen consumption was estimated. The aim was to assess the relationships between carbon metabolism, nitrogen fixation and oxygen consumption by bacteroids in nodules of chick-pea plants, in order to establish whether some of these compounds, which accumulate under salt stress, may be used as respiratory substrates by bacteroids to fuel their own metabolism and nitrogenase activity.

MATERIALS AND METHODS

Plants and growth conditions

Chick-pea (*Cicer arietinum* cv. ILC1919) seeds were obtained from the International Center for Agricultural Research in Dry Areas (ICARDA). The seeds were surface-sterilized and inoculated at the time of transplanting with *Mesorhizobium ciceri* strain ch-191. In previous experiments, this strain was selected as salt-tolerant from a collection of strains that were cultured under free-living conditions in the presence of 400 mM NaCl (data not shown). Plants were watered with a nutrient solution (RIGAUD & PUPPO, 1975), and salt stress was induced by adding 50 mM NaCl to the nutrient solution at sowing. Meanwhile, control plants were maintained in a NaCl-free solution. All plants were grown in Leonard jars in a growth chamber under the conditions described previously (SOUSSI *et al.*, 1998). Harvest was at the beginning of the flowering period, 4 weeks after transplanting.

Nitrogen fixation assays

Nitrogenase (EC 1.7.99.2) activity was determined by acetylene reduction on nodulated roots of six plants as described by HERDINA & SILSBURY (1990). The nodulated root sample was incubated at room temperature in vials containing C_2H_2 (10%, v/v) in air and sealed with serum caps. Aliquots of 0.2 ml were taken after 5 and 10 min and analysed for ethylene in a Perkin Elmer 8600 gas chromatograph equipped with a Porapak R column (LIGERO *et al.*, 1986).

Bacteroid and nodule cytosol preparation

Bacteroids were isolated as described earlier by DELGADO *et al.* (1993). Nodules were homogenized with a mortar and pestle in 50 mM Na-phosphate buffer, pH 7.4 and a 1:3 ratio of polyvinylpolypyrrolidone to nodule weight. The homogenate was filtered through four layers of cheesecloth and centrifuged at 250 *g* for 5 min to remove cell debris. The supernatant was recentrifuged at 10,000 *g* for 10 min to sediment the bacteroids. The bacteroid fraction was washed twice with 50 mM Na-phosphate buffer (pH 7.4) containing 0.3 M sucrose and 2 mM $MgSO_4$. The bacteroid suspension was adjusted to a final volume of 2 ml of 25 mM Na-phosphate buffer (pH 7.4) per g of nodules. Nodule cytosol was obtained after centrifugation of the homogenate from crushed nodules at 30,000 *g* for 20 min.

Bacteroid oxygen consumption

After isolation, the bacteroids were incubated at 25°C in the reaction chamber of an oxygen electrode (Hansatech DW, Hansatech ltd.), connected to a microprocessor (Hanna H4818, Hanna Instruments) that registers readings in mv. The incubation medium contained 25 mM oxygen-saturated Na-phosphate buffer (pH 7.4), the bacteroid preparation (1-2 mg protein), and 10 mM succinate, glucose, malate, fumarate, lactate, glutamate, proline, pyruvate, ethanol, glycine-betaine, oxaloacetate, acetate or citrate, while for the control no exogenous substrate were added to the reaction mixture. The disappearance of the dissolved oxygen was registered at intervals of 1 min for 5 min, values remaining linear for least 12 min. Three repetitions were made per trial. Bacteroid respiration was expressed as µmol oxygen (mg protein)$^{-1}$h^{-1}.

Enzyme extracts

Enzyme activities were determined in the nodule cytosol and in the soluble fraction of bacteroids. Nodules (4-5 g) were homogenized with 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM Na_2-EDTA, 10 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 1 mg ml^{-1} bovine serum albumin and 10% glycerol. Bacteroids were resuspended in 25 mM Na-phosphate buffer (pH 7.5) and sonicated for 5 min at 15 s intervals in a circuit refrigerated to 4°C. The soluble fraction of bacteroids, removed after centrifugation at 30,000 *g* for 30 min, was used for enzyme assays and protein quantification.

Enzyme assays

The activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), alcohol dehydrogenase (ADH, EC 1.1.1.1) and malate dehydrogenase (MDH, EC 1.1.1.37) was assayed as described previously (SOUSSI *et al.*, 1998). The PEPC assay in nodule cytosol was coupled to MDH activity, which in this case was so high that it was not necessary to add exogenous MDH to the reaction mixture. The activity of 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) was assayed with a reaction mixture consisting of 0.5 mM $NADP^+$, 10 mM $MgCl_2$, 2.5 mM 6-phosphogluconate in 100 mM bicine-KOH (pH 8.5) and the appropriate amount of extract (COPELAND *et al.*, 1989). In prior experiments (data not shown), it was found that the assay of glucose 6-phosphate dehydrogenase provided no additional information than that gained with the 6PGDH assay, which is simpler and thus 6PGDH activity was chosen.

The assay of $NADP^+$-isocitrate dehydrogenase ($NADP^+$-ICDH, EC 1.1.1.42) activity was optimized from CHEN *et al.* (1988). The reaction mixture included 0.5 mM $NADP^+$, 5 mM $MgCl_2$ and 1 mM isocitrate in 100 mM bicine-KOH (pH 8.5). All these activities were assayed at 30°C by monitoring the change in absorbance at 340 nm due to oxidation of NAD(P)H or reduction of NAD(P).

The soluble proteins in nodule extracts were determined by the Bradford method (BRADFORD, 1976), and bovine serum albumin was used as standard.

Statistical design and analysis

The experimental layout was a randomized complete block design. Values of growth and nitrogen fixation were means of 6 replicates per treatment, and the values of enzyme activities, oxygen uptake and protein content are the mean of four replicates. All results were subjected to analysis of variance, and the least significant difference (LSD) test between means was performed.

RESULTS

Plant dry weight and root-to-shoot ratios reflect reductions due to salinity that were not statistically significant (Table 1). Salt stress inhibited acetylene reduction activity of chick-pea nodules by about 13% compared with controls, but significantly stimulated nodule dry weight (43%; Table 1).

The activity of PEPC and 6PGDH in the nodule cytosol increased under saline conditions by roughly 15% and 10%, respectively (Table 2). Meanwhile, MDH and ICDH activities decreased significantly with salt by 16% and 28%, respectively. The reduction by about 3% in ADH activity was not statistically significant.

Enzyme activities followed an opposite trend in the bacteroids, MDH, ICDH and ADH being significantly increased with salt (Table 3). In fact, ICDH activity rose by about 10%, while MDH and ADH activities increased more than 40%. However, 6PGDH activity was reduced by approximately 10% by salt.

Oxygen consumption by the bacteroids was measured in the absence (control) or presence of exogenous substrates (Figure 1). In the absence of exogenous sub-

TABLE 1

Effect of salt stress on plant dry weight (PDW, g $plant^{-1}$), root-to-shoot ratio (RSR), nodule dry weight (NDW, g $plant^{-1}$), and acetylene reduction activity (ARA, µmol C_2H_4 g^{-1}NDW h^{-1}) in plants of *Cicer arietinum* inoculated with *Mesorhizobium ciceri* strain ch-191, and grown under saline conditions (50 mM NaCl) or without salt for 4 weeks after transplanting.

NaCl (mM)	PDW	RSR	NDW	ARA
0	1.02	0.65	0.021	52.4
50	0.93	0.59	0.030	45.6
LSD (0.05)	0.18	0.09	0.005	5.7

TABLE 2

Effect of salt stress on the enzymatic activities of 6-phosphogluconate dehydrogenase (6PGDH), phosphoenolpyruvate carboxylase (PEPC), isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH) and alcohol dehydrogenase (ADH), expressed in nmol (mg protein)$^{-1}$ min^{-1}, in nodule cytosol of *Cicer arietinum* plants inoculated with *Mesorhizobium ciceri* strain ch-191, and grown under saline conditions (50 mM NaCl) or without salt for 4 weeks after transplanting.

NaCl (mM)	6PGDH	PEPC	MDH	ICDH	ADH
0	292	462	19390	411	829
50	341	508	16353	296	807
LSD (0.05)	14	25	953	28	68

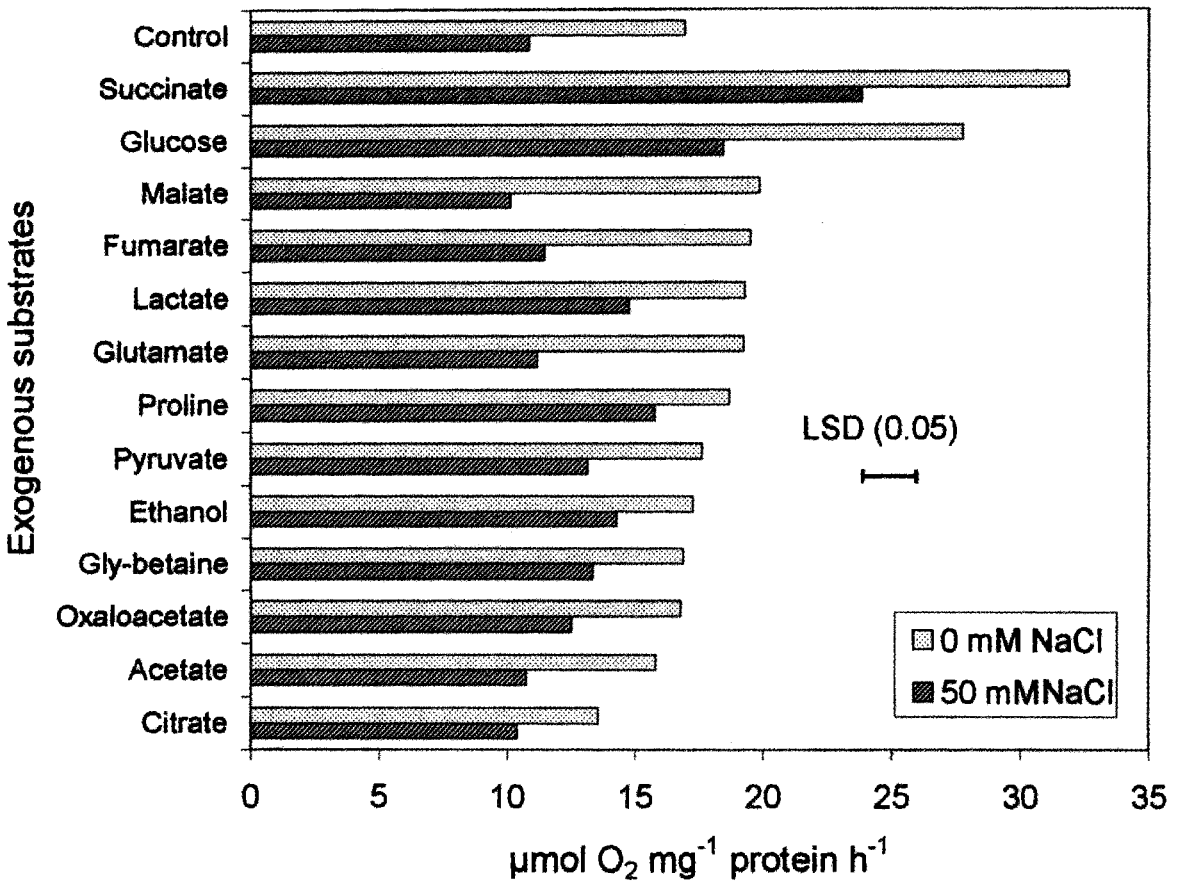

FIGURE 1 - Oxygen uptake, in the absence (control) or presence of exogenous respiratory substrates, by bacteroids isolated from root nodules of *Cicer arietinum* plants inoculated with *Mesorhizobium ciceri* strain ch-191, and grown under saline conditions (50 mM NaCl) or without salt for 4 weeks after transplanting.

strates, a highly significant difference was found between the bacteroids of control plants and those of plants grown with saline treatment. These results confirm that the respiratory capacity of the bacteroids is severely depressed by salinity. The addition of exogenous respiratory substrates significantly boosts oxygen consumption in bacteroids isolated from control nodules (Figure 1). The greatest increase was caused by succinate (88%) and glucose (63%), followed by malate (17%), fumarate (15%), lactate (14%) and glutamate (13%). On the other hand, proline, pyruvate, ethanol, glycine-betaine, oxaloacetate, acetate and citrate brought about no significant effect ($P<0.05$).

The bacteroids isolated from nodules treated with 50 mM NaCl showed different responses: the increase caused by succinate, glucose and lactate was proportionally greater than in the bacteroids from control nodules (Figure 1). However, malate, fumarate, and glutamate did not cause a significant response. On the other hand, other compounds that did not have significant effects on control bacteroids, such as proline, glycine-betaine, ethanol, and pyruvate, increased oxygen consumption in the bacteroids from plants grown with 50 mM NaCl.

TABLE 3

Effect of salt stress on the enzymatic activities of 6-phosphogluconate dehydrogenase (6PGDH), isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH) and alcohol dehydrogenase (ADH), expressed in nmol (mg protein)$^{-1}$ min^{-1}, in bacteroids isolated form root nodules of *Cicer arietinum* plants inoculated with *Mesorhizobium ciceri* strain ch-191, and grown under saline conditions (50 mM NaCl) or without salt for 4 weeks after transplanting.

NaCl (mM)	6PGDH	MDH	ICDH	ADH
0	250	35273	2509	21.7
50	226	60026	2745	52.8
LSD (0.05)	10	2466	47	5.0

DISCUSSION

The effect of salinity on growth was not significant, confirming that the *Cicer arietinum* cultivar ILC1919 is moderately salt-tolerant (SOUSSI *et al.*, 1999). However, the specific nitrogenase activity was significantly inhibited by 50 mM NaCl, a fact possibly related to the decrease in bacteroid respiration and to a lower supply of energy-yielding compounds to bacteroids (DELGADO *et al.*, 1993).

Salinity and other stress conditions limit oxygen diffusion through nodule tissues (WALSH, 1995), thereby stimulating fermentative pathways in root nodules (FERNANDEZ-PASCUAL *et al.*, 1996). In fact, the nodule cytosol activity of PEPC and ADH were markedly stimulated in chick-pea plants under salinity (SOUSSI *et al.*, 1999). The enhancement of PEPC, ADH and lactate dehydrogenase activities may induce the accumulation of malate, ethanol (BEGERSEN & TURNER, 1993) and lactate (TRINCHANT & RIGAUD, 1987). With respect to the bacteroids, the increased activities of MDH and ICDH could be related to the increased supply of substrates for the fermentative pathways, which are augmented under saline conditions, apparently due to the limitation of oxygen diffusion through the nodule caused by salt stress (SERRAJ *et al.* 1995).

The respiratory capacity of bacteroids isolated from root nodules of *Cicer arietinum* was reduced by salt stress (Figure 1), as reported for such legumes as alfalfa and pea grown under water or salt stress (BEKKI *et al.*, 1987; DELGADO *et al.*, 1993). In addition, after incubation in the presence of different compounds, oxygen uptake by bacteroids has shown different responses (BERGERSEN, 1997; UDVARDI & DAY, 1997). In the present work the greatest increase of oxygen uptake was induced by succinate, both in control bacteroids (88%) and in those isolated from nodules subjected to salinity (119%). Therefore, succinate appears to be the preferred substrate for bacteroids in the *Cicer arietinum-Mesorhizobium ciceri* symbiosis.

The second greatest increase in bacteroid oxygen consumption from chick-pea control nodules resulted when these were incubated in the presence of glucose (Figure 1). In addition, the acceleration in respiration rate induced by glucose in bacteroids from nodules treated with 50 mM NaCl was proportionally higher than in control nodules. Data in the literature concerning hexose utilization by bacteroids is contradictory. TRINCHANT *et al.* (1981) observed that glucose can maintain nitrogen fixation in bacteroids of common bean and soybean nodules at low oxygen concentrations. The utilization of glucose has also been demonstrated in bacteroids of *Rhizobium lupini* by KRETOVICH *et al.* (1985). On the other hand, according to REIBACH & STREETER (1984), bacteroids of *Bradyrhizobium japonicum* isolated form soybean plants cannot transport glucose. Nevertheless, the results presented here support the utilization of glucose, a compound commonly present in root nodules (FOUGÈRE *et al.*, 1991), by the bacteroids of *Mesorhizobium ciceri* under normal conditions as well as under salt stress.

The respiration of bacteroids was also enhanced by the presence of malate, fumarate, lactate and glutamate in the incubation medium. The malate produced via the PEPC-MDH pathway is used by bacteroids (ROSENDAHL *et al.*, 1990) and is the main substrate to maintain nitrogenase activity (DELGADO *et al.*, 1993). However, our results show that the increase in oxygen uptake induced by malate was lower than that of succinate or glucose in bacteroids of control plants, while it had no significant effect in bacteroids of salt-stressed plants.

The effect of lactate was similar to that of malate in control bacteroids, but in salt-stressed bacteroids it boosted oxygen uptake by 35%. This result supports the findings of TRINCHANT & RIGAUD (1989) in nodules of *Sesbania* subjected to oxygen restriction.

The possible role of proline as an energy source for bacteroids is also controversial in the literature. Our results show that, under saline conditions, proline significantly increased bacteroid respiration (Figure 1), suggesting that proline could be a major energy source for bacteroids, at least under stress conditions. Also the results of STRAUB *et al.* (1997) support the contention that proline plays a significant role in supplying energy for nitrogen fixation. These results disagree with recent observations by TRINCHANT *et al.* (1998) in *Vicia faba*. Thus, this response may depend upon the legume species and on the strain of *Mesorhizobium* used. In particular, the utilisation of glucose and proline by the bacteroid could be a characteristic of the salt-tolerant strain used in this work. Thus, it would be necessary to perform experiments to compare salt-tolerant and –non tolerant strains.

Other compounds, such as pyruvate, ethanol, glycine-betaine, oxaloacetate, acetate and citrate, caused no significant effect under control conditions. However, ethanol and glycine-betaine, which are known to accumulate under saline conditions, significantly increased oxygen uptake by bacteroids in salt-treated plants. PETERSON & LARUE (1981) proposed that ethanol may serve as energy source for soybean bacteroids. Very little information is available in the literature about the accumulation of glycine-betaine in response to osmotic stress

in legume nodules.

In summary, the data presented here show a rise in fermentative metabolism in nodules of chick-pea plants exposed to high salinity, and suggest that some of the compounds which are known to accumulate under salt stress, such as proline, lactate or ethanol, may play an important role as energy-yielding substrates for bacteroids in this plant species. The bacteroids of *Mesorhizobium ciceri* could utilize glucose as a respiratory substrate both under normal and saline conditions, while, in the latter, malate did not appear to be the preferred substrate under saline conditions.

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