African Journal of Biotechnology Vol. 11(63), pp. 12619-12629, 7 August, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1103
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Effects of NaCl on growth and activity of enzymes involved in carbon metabolism in leaves of tobacco (*Nicotiana rustica*)

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Accepted 8 July, 2011

The adverse effects of salt should not be the same in tobacco plants exposed to a permanent and transient high concentration of NaCl in its environment. Experiments were conducted in order to verify the hypothesis of reversibility of NaCl effects. The study of this reversibility is checked by monitoring a number of parameters in pre-stressed plants and then, replaced in normal conditions. Plants previously grown for 30 days on basic medium were treated for 7 days with 200 mM NaCl and then placed back on the basic culture without NaCl for 10 days. The results show that NaCl suppression leads to a resumption of growth with a decrease in the concentration of sodium (Na^+) and chloride ions (Cl^-). Hence, potassium content (K^+) increases gradually in the leaves to reach the level obtained with unstressed plants. At the same time, there is a stimulation of the activities of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase isoenzymes (NAD, NADP, NADH and NADPH-MDH) and isocitrate dehydrogenase (ICDH) after NaCl had been removed. Along with the boosting of the activity of these enzymes involved in the process of carbon assimilation, there is a gradual decrease in soluble sugars content, suggesting a resumption of the normal activity of photosynthetic assimilation process. All these results verify our hypothesis and can be explained by the ability of the plant to dilute the effects of Na^+ and Cl^- during the recovering period. An important result of this study is that a transient salinity is not necessarily followed by a significant depreciation in product yield or quality.

Key words: Tobacco, NaCl, reversibility, phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), isocitrate dehydrogenase (ICDH).

INTRODUCTION

Salt stress imposes a major environmental threat to agriculture and its adverse impacts are becoming a more serious problem in regions where saline water is used for irrigation. The effects of salinity have undergone little study in certain plants of agricultural interest. Salt accumulated in the plants generates both osmotic and ionic stress and has toxic effects on numerous biochemical processes. The few studies that have examined the effect of salinity on the production of biomass in tobacco plants have shown negative effects on production in this crop (Flowers et al., 1986; Aragon and Alvarez, 1988; Sifola and Postiglione, 2002; Ruiz et al., 2006; Razavizadeh et al., 2009).

Carbon metabolism could play a significant role to discern the behaviours of plants in response to salinity, via enzymatic and/or metabolic processes. Among the key enzymes of carbon metabolism, dehydrogenases are considered important in generating reducing powers which are utilized in various metabolic activities including reductive biosynthesis of amino acids; fatty acids in growing tissues also replenish the mitochondrial compartment with reducing powers (Chen et al., 1988; Hodges, 2002). Isocitrate dehydrogenase (ICDH) catalyzes the reversible conversion of isocitrate to 2-

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Abbreviations: PEPC, Phosphoenolpyruvate carboxylase; **MDH,** malate dehydrogenase; **ICDH,** isocitrate dehydrogenase; **2-OG,** 2-oxoglutarate.

oxoglutarate (2-OG) and links C and N metabolism (Gallardo et al., 1995). ICDH activity is mainly localized in the cytosol, although, minor activity has been found in mitochondria, peroxisomes, and chloroplasts in higher plants (Fieuw et al., 1995). By supplying the cytosol with 2-OG as a primary acceptor for ammonium assimilation, ICDH have a key function in the biosynthesis and export of amino acids and in the supply of NADPH to the cytosol (Hodges, 2002). Malate dehydrogenase (MDH) catalyzes the interconversion of oxaloacetate (OAA) and malate and exists in different isoforms (Gietl, 1992a). The isoforms localized in subcellular organelles like peroxisomes, mitochondria, and cytosols are NAD-dependent, whereas the chloroplastic one is NADP-dependent (Gietl, 1992a). Differential expressions of MDH isoforms and changes in its activity have been reported in many plant species under abiotic stresses (Kingstonsmith et al., 1997; Kumar et al., 2000). Phosphoenolpyruvate carboxylase (PEPC) also play an important role within carbon metabolism, it is a ubiquitous cytoplasmic enzyme in higher plants, catalyzes the irreversible β-carboxylation of phosphoenol- pyruvate (PEP) to yield OAA and inorganic phosphate, a reaction that is involved in several metabolic contexts in plants (Chollet et al., 1996). PEPC has a major anaplerotic function of replenishing the tricarboxylic acid (TCA) cycle with intermediates, which are withdrawn for amino acid synthesis (Miyao and Fukayama, 2003). The photosynthetic carbon assimilation process mainly regulated by these enzymes and sugars was found to be greatly depressed by high levels of NaCl. Changes in carbon metabolism have rarely been studied in relation to salt stress. Recent works have reported the inhibition of PEPC, MDH and ICDH activities by NaCl in chick pea and legumes nodules (Soussi et al., 1998; Lopez et al., 2008; Lopez and Lluch, 2008), in pea leaves (Popova et al., 2001), in rice (Kumar et al., 2000), in maize and wheat leaves (Abdel-Latif, 2008). Considering the key role of ICDH and MDH in generating and shuttling reducing equivalents amongst different subcellular organelles and the important role played by PEPC in replenishing the TCA cycle with intermediates to meet the demand of carbon skeletons for synthesis of organic acids and amino acids (Miyao and Fukayama, 2003).

This study was undertaken to evaluate the behaviours of these enzymes and how carbon metabolism was modified in tobacco seedlings submitted to 7 days period of high salt treatment (200 mM NaCl) then by return to non-saline conditions for 10 days, in order to specify the possible roles of these enzymes in tobacco leaves and to get more insight into the mechanism of carbon assimilation.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of tobacco (*Nicotiana rustica,* Soufi var.) were germinated on moistened filter paper at 25°C in the dark. The seedlings obtained were transferred to continuously aerated nutrient solutions containing 8 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 32.9 µM Fe-K-EDTA, and micronutrients: 30 µM H_3BO_4, 5 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 1 µM $(NH_4)_6Mo_7O_{24}$. The nutrient solution was renewed every 3 days. Plants were grown in a growth chamber: 26°C/ 70% relative humidity during the light period and 20°C/ 90% relative humidity during the dark period; photoperiod: 16 h daily with a light irradiance of 150 $\mu mol.m^{-2}.s^{-1}$ at the plant canopy. Plants grown hydroponically for 30 days are first placed for 7 days on a culture medium with 200 mM NaCl (Khelil et al., 2007) and then placed back on the basic culture without NaCl for 10 days. Control plants were untreated plants (0 mM NaCl). Plants were harvested 3 h after the beginning of the light phase. The dry weight (DW) was measured after 48 h of desiccation in an oven at 60°C.

Ion analysis

Inorganic ions were extracted from dry matter with 0.5 N H_2SO_4 at room temperature for 48 h (Gouia et al., 1994). K^+ and Na^+ were analyzed by flame emission using a spectrophotometer (Eppendorf, GmbH Hamburg, Germany).

Cl^- was quantified by a colorimetric method (Ben Ahmed et al., 2008) using a digital chloridometer (HaakeBuchler, Buchler instruments Inc., New Jersey, USA). Ammonium (NH_4^+) was extracted from plant material at 4°C with 0.3 mM H_2SO_4 and 0.5% (w/v) polyclar AT. It was quantified according to the reaction of Berthelot modified by Weatherburn (1967).

Enzyme extraction and assays

Enzymes were extracted from frozen leaf stored at -80°C. All extractions were performed at 4°C.

Phosphoenolpyruvate carboxylase

Frozen samples were homogenized using a cold mortar and pestle with 100 mM Tris-HCl buffer pH 8 containing 3.5 mM $MgCl_2$, 1 mM Triton-X, 10% (w/v) glycerol and 1% (w/v) PVP. The homogenate was centrifuged at 40.000 g for 15 min at 4°C and the resulting supernatant was used as enzyme extract. The PEPC activity was measured spectro- photometrically at 340 nm, in a final volume of 1 ml containing 100 mM Tris-HCl buffer pH 8, 12 mM $MgCl_2$, 6 mM $NaHCO_3$, 5 mM NaF, 0.2 mM NADH, 4 mM PEP, 1 mM dithiolthreitol (DTT) and appropriate amount of crude extract (Foyer et al., 1994). Assays were initiated by the addition of the leaf extracts.

Malate dehydrogenase

The extraction buffer contained 100 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT and 50 mM β-mercaptoethanol. The NAD- and NADP- malate dehydrogenase activities were assayed after incubation in the culture medium at 22°C, contained 25 mM tricine sodium buffer pH 8.9, 1 mM EDTA, 50 mM malate, 20 mM glutamate and 200 mM hydrazine. The reaction was initiated by the addition of 2.5 mM NAD(P). The NADH- and NADPH- malate dehydrogenase activities were assayed after incubation in the culture medium at 22°C, contained 25 mM Tris-HCl buffer pH 8, 1 mM EDTA and 0.5 mM oxaloacetate. The reaction was initiated by the addition of 25 mM NAD(P)H (Johnson, 1970).

Isocitrate dehydrogenase

Frozen samples were homogenized using a cold mortar and pestle with 100 mM potassium phosphate buffer pH 7.6 containing 14 mM β-mercaptoethanol and 1% (w/v) polyvinyl- pyrrolidone (PVP). The homogenate was centrifuged at 14.000 g for 15 min at 4°C and the resulting supernatant was used as enzyme extract. Total ICDH activity was measured in 1 ml reaction set containing 100 mM potassium phosphate buffer pH 7.6, 50 mM $MgCl_2$, 50 mM D,L-isocitrate, 5 mM NADP and appropriate amount of crude extract. The reaction was initiated by adding isocitrate and the NADPH formed was followed at 340 nm (Galvez and Gadal, 1998).

Soluble sugars and protein determination

Soluble sugars were extracted from 25 mg of dry matter by homogenisation in 5 ml of 80% (v/v) ethanol with a mortar and pestle. After heating the homogenate in a water bath at 70°C for 30 min, the insoluble fraction was removed by centrifugation at 6.000 g for 15 min. Then, the anthrone was added to the supernatants and the samples were placed in a boiling water bath at 100°C for 10 min. Finally, the samples were placed in ice to stop the reaction. The absorbance of samples was read at 640 nm (Mac Cready et al., 1950; Staub, 1963). Soluble protein content was quantified according to the method of Bradford (1976) with bovine serum albumin as a protein standard.

Chlorophyll determination

Chlorophyll was determined by the method of Mac Kinney (1941). The absorbance of a sample was read at 652 nm after centrifugation.

Statistical analysis

The data are presented in the figures as the average of at least six replicates (plants) per treatment and means ± confidence limits at the P = 0.05 level. Each experiment was conducted in duplicate (twice).

RESULTS

Growth

Figure 1A shows that NaCl suppression results in a resumption of growth activity in the leaves. The foliar biomass was clearly affected by salinity stress. As compared to the control, the reduction was 73% at the end of the salt treatment. At the same time, water content increased after elimination of NaCl to reach the level obtained with control plants. Significant NaCl effects on the leaves stressed plants were recorded starting from 7 days of treatment. At 17 days, the water content decreased by about 21% in salt-stressed leaves (Figure 1B), with respect to the control plants.

Total chlorophyll content was significantly affected by NaCl; it reached 58% at the end of the salt treatment (Figure 1C), relative to the control. In NaCl-treated plants, we can see that since NaCl elimination, the total chlorophyll content increased progressively.

The highest NaCl stress (200 mM) resulted in a higher decrease in soluble protein content in the leaves starting from 7 days of treatment. It reached 90% of reduction (Figure 1D) at the end of the treatment. The elimination of NaCl is accompanied by a gradual removal of the inhibitory effect of salt stress. Moreover, the soluble protein content reached the level obtained with un-stressed plants.

Soluble sugars content

In response to the salt treatment, soluble sugars content were accumulated to higher levels in leaves. At 17 days, this stimulation reached 200% (Figure 2). Since NaCl elimination, soluble sugars content returned progressively to control levels.

Na^+, Cl^- and K^+ concentrations

In tobacco plants subjected to salinity, after one week of treatment, Na^+ and Cl^- were rapidly accumulated in the leaves (Figures 3A and B); Cl^- had higher accumulation than Na^+. By 10 days of return to non-saline conditions, both Na^+ and Cl^- concentrations had decreased progressively to control levels in leaves. As shown by the data in Table 1, the amount of Na^+ and Cl^- incorporated in the plant after the removal of NaCl from the culture medium decreased gradually during the rest of the experimental period, while the tissue mass increases substantially.

The salt stress resulted in a decrease of K^+ in the leaves (Figure 3C) throughout the period of treatment. At 17 days, K^+ ion concentration decreased by 64%. In stressed plants, we can see that since NaCl elimination, K^+ concentration was partially restored.

NH_4^+ concentrations

Beyond 7 days of treatment, a significant increase in NH_4^+ concentrations by salt stress was recorded at the end of the experimental period (Figure 3D). Excessive amount of NaCl activated the accumulation of NH_4^+; it reached 240% (compared to the control plants) which indicated that the ability of tobacco leaves to assimilate ammonium was reduced under high salinity. This stimulation was partially reversed when the salt was removed from the medium.

Enzyme activities of carbon metabolism

Phosphoenolpyruvate carboxylase activity

The presence of NaCl in the culture medium resulted in a

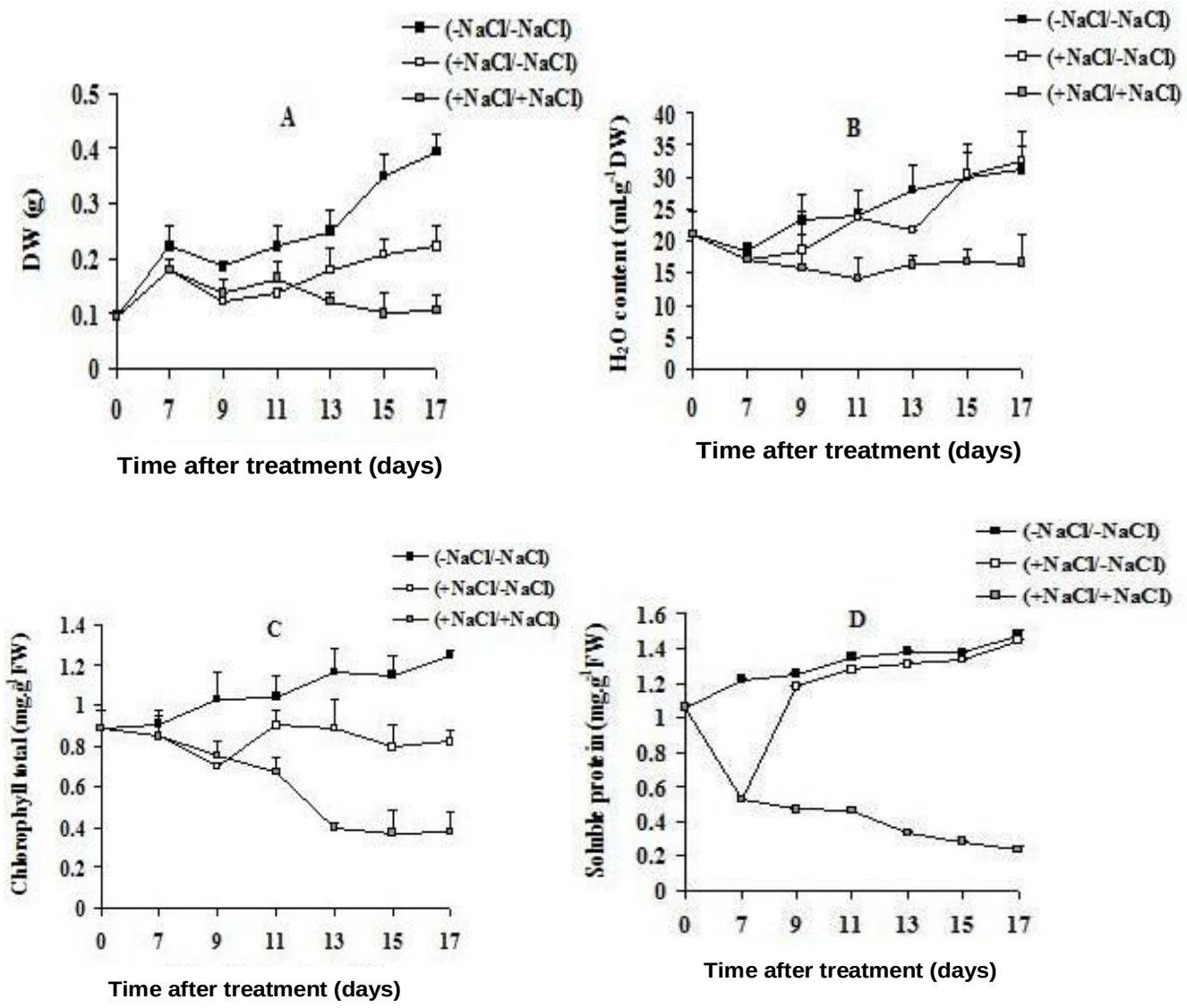

Figure 1. Changes in (A) dry weight (DW) production, (B) water content, (C) total chlorophyll content and (D) soluble protein content in the leaves of plants grown with (+NaCl/+NaCl) or without (-NaCl/-NaCl) 200 mM NaCl during 17 days and plants grown for 7 days with 200 mM NaCl and then placed on a basic culture without NaCl for 10 days (+NaCl/-NaCl). Data are means of six replicates ± CL at 0.05 levels, CL are not shown when they are smaller than the symbol.

decrease in the leaf PEPC activity. It was lowered by 37% (Figure 4A) with respect to control plants. Transferring the plants to non-saline conditions previously subjected to NaCl improved PEPC activity.

Isocitrate dehydrogenase activity

In stressed plants, NADP-ICDH activity decreased gradually after 7 days. However, this reduction reached only 30% in the leaves (Figure 4B) at the end of the treatment. The elimination of NaCl from the culture medium over 7 days of treatment is accompanied by a gradual removal of the inhibitory effect of salt stress.

Malate dehydrogenase *activity*

For MDH activities, dependent on oxidized cofactors (NAD or NADP), which catalyzes the reaction towards the synthesis of OAA, we found that in the leaves (Figures 5A and B), MDH activity was increased by NaCl treatment, with a peak occurring at 9 and 7 days for NAD- and NADP-MDH, respectively and then it decreased throughout the treatment; it reached 20% at the end of the experimental period, for both activities.

For MDH activities, dependent on reduced cofactors (NADH or NADPH), which catalyzes the reaction towards the synthesis of malate, we found that exogenously applied NaCl caused a gradual inhibition of both MDH

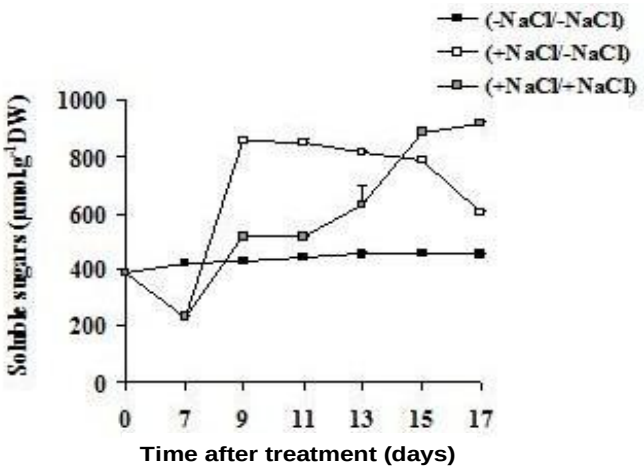

Figure 2. Changes in soluble sugars content in the leaves of plants grown with (+NaCl/+NaCl) or without (-NaCl/-NaCl) 200 mM NaCl during 17 days and plants grown for 7 days with 200 mM NaCl and then placed on a basic culture without NaCl for 10 days (+NaCl/-NaCl). Data are means of six replicates ± CL at 0.05 levels, CL are not shown when they are smaller than the symbol.

activities (Figures 5C and D). Whereas, the foliar NADPH-MDH activity (Figure 5D) became significantly higher than in the control plants at 13 days; it was increased about 180%. After this peak, the NADPH-MDH activity in leaves dropped progressively throughout the treatment.

By 10 days of return to non-saline conditions, NAD(H)- and NADP(H)-MDH activities were partially restored and, in some cases, the levels obtained were similar to those obtained with control plants (Figure 5).

DISCUSSION

Cultivation of glycophytic plants in saline soils causes reduced plant growth and can thus have a severe effect on crop yield (Tester and Davenport, 2003). This study evaluated the physiological response of *Nicotiana rustica* grown under high level of salinity (200 mM) during 17 days. In our experiments on tobacco, symptoms of salt stress were reflected in a lower foliar DW (Figure 1A). The negative effect of salt on tobacco growth has previously been described by Flowers et al. (1986), they found a marked decrease of DW at 200 $mol.m^{-3}$ NaCl (200 mM; 1 M = 10^3 $mol.m^{-3}$). Also, Aragon and Alvarez (1988) found that both yield and quality of flue-cured tobacco leaves decreased in salt-affected soil when saline waters were used for irrigation. Sifola and Postiglione (2002) confirmed previous experimental data and defined tobacco as a crop of intermediate tolerance to salinity. More recently, Razavizadeh et al. (2009) found that the growth of tobacco plants was decreased by NaCl levels of 150 mM and above; this decrease was severe at 300 mM, whereas a level of 400 mM was lethal to the plants.

Effects of NaCl on growth were associated with a significant reduction in total chlorophyll content (Figure 1C). This may be caused by the depressive effect of salinity on the absorption of some ions, such as Mg and Fe, which are involved in the chloroplast formation (Hanafy et al., 2002); or by the increase of chlorophyllase activity (Sivstev et al., 1973). When glycophytes are exposed to salt, growth is reduced, but it is unclear if this reduction is caused by inhibition of photosynthesis or by nutrient deficiency in growing tissues (Munns, 1993; Huang and Redmann, 1995; Rejili et al., 2007).

Salinity affects plants by direct toxicity of Na^+ and Cl^- absorbed and interfering with the uptake of essential nutrients (Greenway and Munns, 1980; Flowers and Flowers, 2005). The decrease in DW production, observed during 17 days in the presence of NaCl 200 mM, was associated with a high accumulation of Na^+ (Figure 3A) and Cl^- (Figure 3B) in foliar tissues, whereas K^+ concentration in the leaves decreased significantly (Figure 3C). Our results agree with those reported in tomato by Alian et al. (2002) and in wheat by Zheng et al. (2008). Our study also showed that the concentration of Cl^- is higher than that of Na^+ in tissues (Figures 3A and B). One important strategy of several species to tolerate salinity was to use NaCl as an osmoticum and to compartmentalize the Na^+ and Cl^- ions primarily in the leaf vacuoles (Ben et al., 2009). Tolerance to salt is also related to the capacity of the plant to control the absorption and the export of Cl^- to aerial parts (M'Charek et al., 2005). Levitt (1980) considered the accumulation of Cl^- in the leaves as the main reason of toxicity of the NaCl, corroborating that in the presence of salt, the resistant cultivars accumulate less Cl^- than the sensitive ones. This points out that the salt injury effect, as observed in this study, could be attributed to Cl^- toxicity (Wahid and Ghazanfar, 2006; Ben et al., 2008; Perez-Tornero et al., 2009). Chloride accumulation is probably the main factor for reduced growth and yield in avocado (Wiesman, 1995) and, citrus (Bar et al., 1998) and the results obtained in this study support these findings. It is likely that leaves employed vacuolar compartmentation to avoid elevated levels of cytoplasmic Cl^- (Llyod et al., 2002) and consequent damage to cytoplasmic damage. If Cl^- and Na^+ are sequestered in the cell vacuole, organic solutes like sugars should accumulate in the cytoplasm to balance the osmotic pressure in the vacuole and to permit the maintenance of turgor (Gouia et al., 1994; Munns, 2002). The high levels of Na^+ generate a kind of competition on the level of the sites of K^+ absorption, as the two ions could be transported by a common protein (Nui et al., 1995) and thus, limited the absorption of this essential element for plant growth and development (Rejili et al., 2007).

The decrease in tissue water content in salt-stressed leaves of tobacco (Figure 1B) has been interpreted as a mechanism that concentrates solutes in the cell sap,

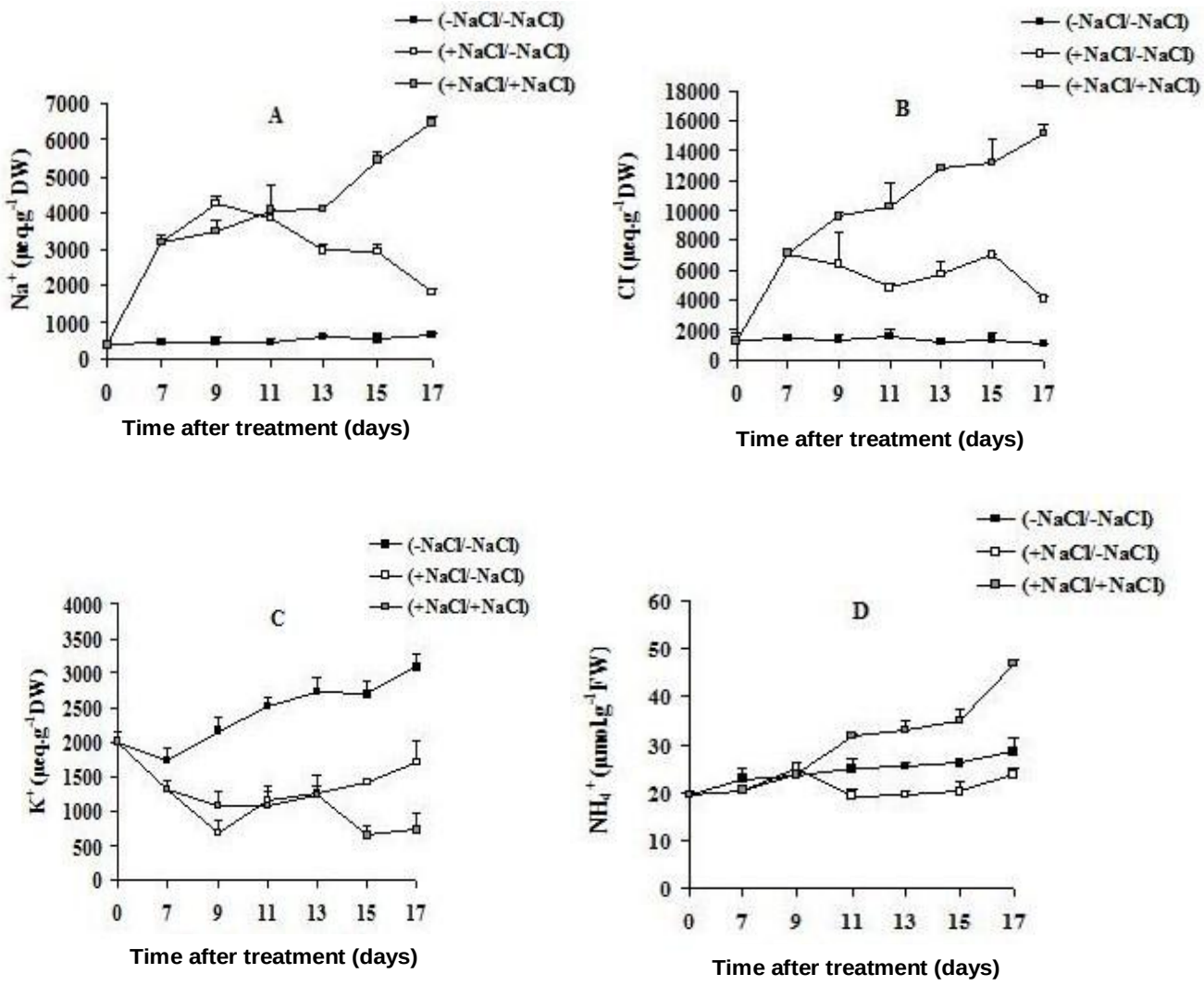

Figure 3. Changes in (A) Na^+, (B) Cl^-, (C) K^+ and (D) NH_4^+ concentrations in the leaves of plants grown with (+NaCl/+NaCl) or without (-NaCl/-NaCl) 200 mM NaCl during 17 days and plants grown for 7 days with 200 mM NaCl and then placed on a basic culture without NaCl for 10 days (+NaCl/-NaCl). Data are means of six replicates ± CL at 0.05 levels, CL are not shown when they are smaller than the symbol.

thereby lowering the osmotic potential and contributing to osmotic adjustment (Lissner et al., 1999). In this study, the soluble sugars content in leaves was increased by salinity (Figure 2). Likewise, this coincided with findings of Zheng et al. (2008) and Ruiz et al. (2006). It has been shown that accumulation of sugars is a common response to drought, salinity, and low-temperature stress (Gupta and Kaur, 2005, Wingler and Roitsch, 2008). Sugars act not only as osmoprotectants in helping to maintain osmotic balance under stress conditions, but can also provide an immediate energy source to plants restarting growth after a period of stress-induced dormancy (Yancey, 2005). Carbohydrates can function in osmotic adjustment and osmoprotection, but their major function is to fuel metabolism (Khelil et al., 2007). Rosa et al. (2009) found that the high sugars accumulation observed in salt-treated plants could imply that in these plants the metabolic carbon flux was mainly used for sugars accumulation, necessary to counteract the osmotic imbalance imposed by salt. Thus, this finding can be correlated with decreased activity of carbon assimilation enzymes. Whereas, PEPC, MDH and NADP-ICDH diminished, the content of sugars increased. This supports the idea that the accumulation of carbohydrates is more of a consequence of damage produced by salt stress than of a protective strategy.

The elimination of NaCl from the culture medium after 7 days of treatment was accompanied by a gradual resumption of growth activity and this was followed by a progressive decrease of Na^+ and Cl^- concentrations (Table1) and also a reduction of sugars content to the normal levels.

Table 1. Changes in the DW production and the amount of Na^+ and Cl^- in the entire plant (whole: leaves + roots), during the period following the elimination of NaCl from the culture medium.

Period after the removal of NaCl	0 day	2 day	4 day	6 day	8 day	10 day
DW (g)	0.24	0.17	0.22	0.265	0.31	0.33
Na^+ (µeq/plant)	6296.53	6128.17	5937.88	4779.31	4503.34	3061.82
Cl^- (µeq/plant)	12443.91	8447.78	6765.95	7775.42	9027.91	5421.84

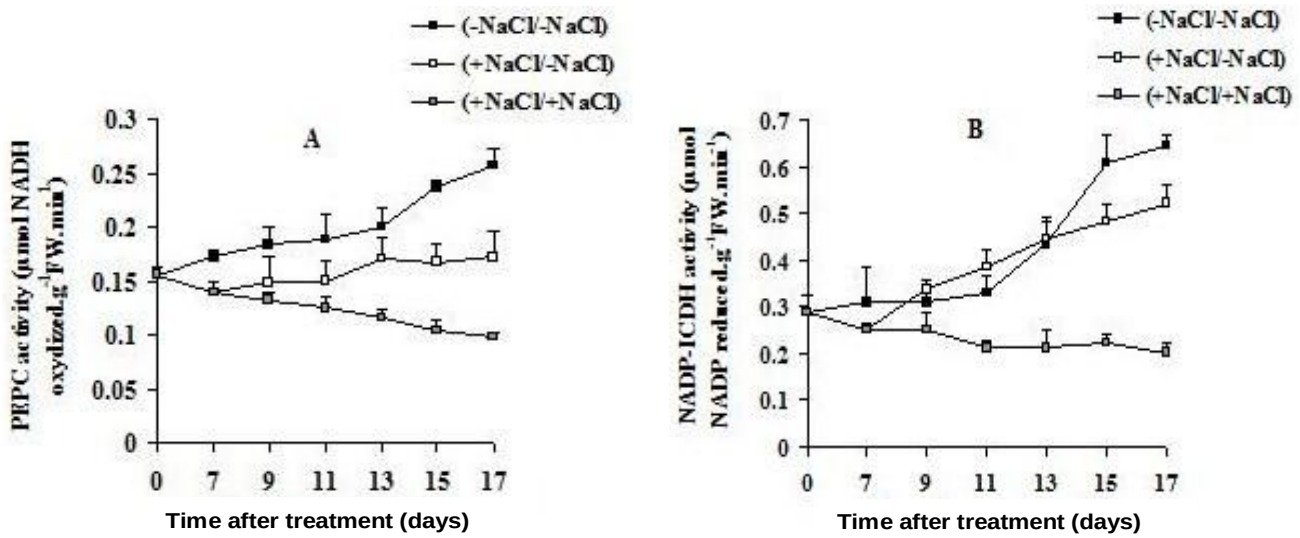

Figure 4. Changes of the (A) PEPC activity and the (B) NADP-ICDH activity in the leaves of plants grown with (+NaCl/+NaCl) or without (-NaCl/-NaCl) 200 mM NaCl during 17 d and plants grown for 7 days with 200 mM NaCl and then placed on a basic culture without NaCl for 10 days (+NaCl/-NaCl). Data are means of six replicates ± CL at 0.05 levels, CL are not shown when they are smaller than the symbol.

Ionic toxicity of Na^+ and Cl^- generally occurs at concentrations in the cytoplasm exceeding 100 mM where inhibition of most enzymes begins (Munns, 2002). The activities of enzymes involved in carbon metabolism decreased during the period of salt treatment in varied proportions depending on the enzyme and the organ (Figures 4 and 5). Abdel-Latif (2008) found that the activity of PEPC in leaves of maize and wheat seedlings subjected to different NaCl concentrations decreased significantly in both species. PEPC contributes to the replenishment of TCA cycle intermediates (Figure 6) when organic acids are directed towards other metabolic pathways such as amino acid or protein synthesis (Stitt, 1999), it provides OAA for the TCA cycle to replace a lower availability of 2-OG. It has been also reported that the activities of PEPC and MDH in leaves of chick-pea (Soussi et al., 1998) and in nodules (Lopez and Lluch, 2008) were decreased by salt. Similar findings have been found by Vu and Allen (2009) in leaves of sugarcane stressed by drought which greatly reduced activities of PEPC and MDH and also Du et al. (1996) reported that drought stress reduces activity of PEPC in mature leaves of sugarcane. Inhibition of the activities of dehydrogenases due to salinity may be one of the possible reasons of decreased growth in tobacco leaves under saline conditions. Kumar et al. (2000) suggest different behaviors of MDH in the two sets of rice cultivars differing in salt tolerance and that salt sensitivity in rice is correlated with decreased MDH activity under salinization. Therefore, NaCl led inhibition of MDH, might limit stomatol opening, TCA cycles, and influence redox state, thus altering both photosynthesis and respiration (Kumar et al., 2000). This could lead to a low supply of OAA and malate. MDH have diversified roles in plant cell metabolism which is evident from their variety of cellular locations and cofactor specificities (Gietl, 1992a). Malate production is essential for growth processes as it replenishes organic acids converted into amino acids and allows the TCA cycle to continue (Figure 6). Cytosolic MDH catalyzes the formation of malate from OAA. This malate enters in mitochondria through the dicarboxylate transporter where mitochondrial MDH catalyzes the conversion of malate to OAA (Gietl, 1992b; Musrati et al., 1998; Minarik et al., 2002). This OAA is channelized in

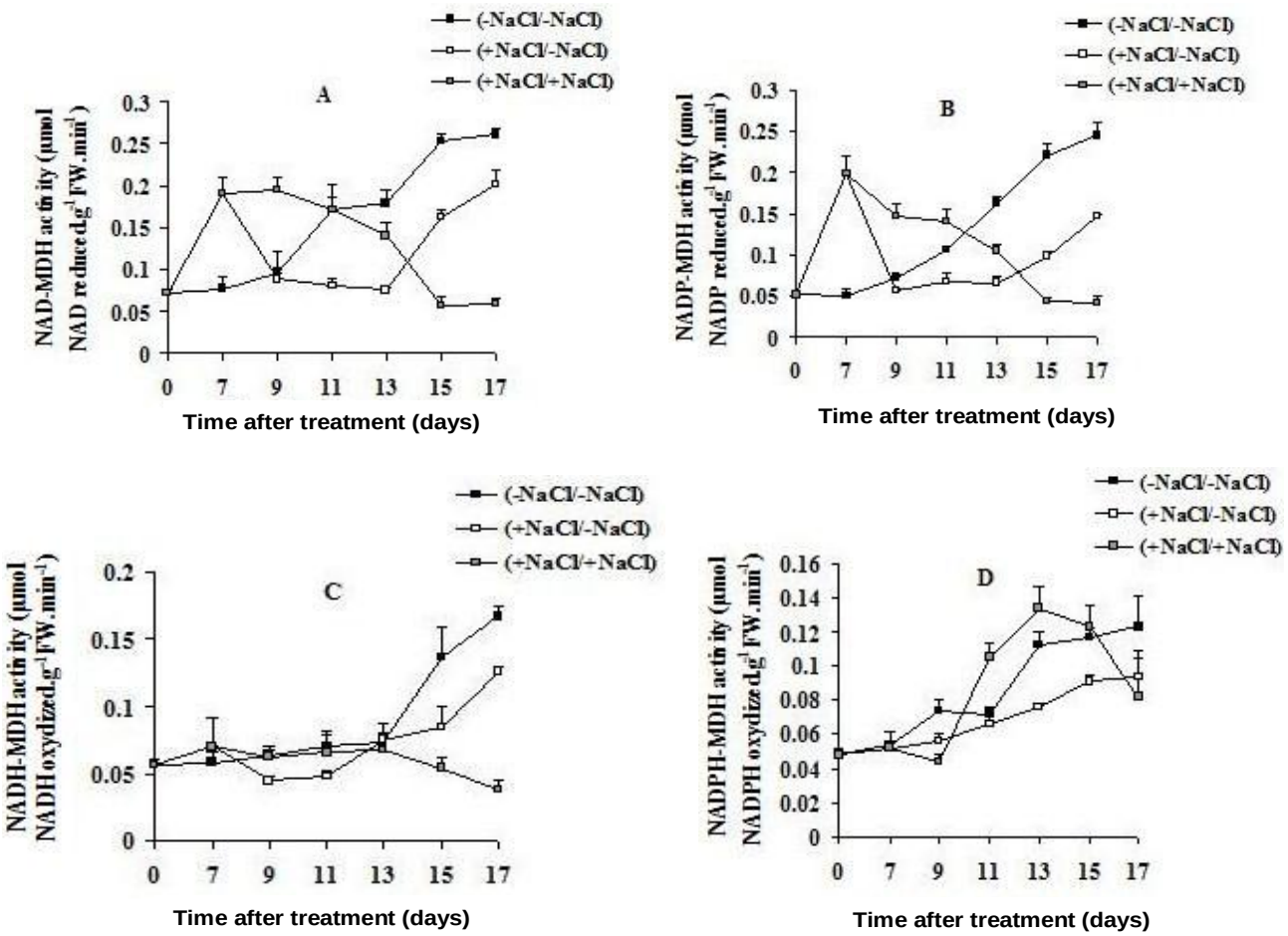

Figure 5. Changes of the (A) NAD-MDH, (B) NADP-MDH, (C) NADH-MDH and (D) NADPH-MDH activity in the leaves of plants grown with (+NaCl/+NaCl) or without (-NaCl/-NaCl) 200 mM NaCl during 17 d and plants grown for 7 days with 200 mM NaCl and then placed on a basic culture without NaCl for 10 days (+NaCl/-NaCl). Data are means of six replicates ± CL at 0.05 levels, CL are not shown when they are smaller than the symbol.

TCA cycle which is able to react with another molecule of acetyl CoA in order to start another turn of TCA cycle (Figure 6). Thus, optimum levels of cytosolic and mitochondrial MDH activities are very important for growth processes as they allow TCA cycle to continue (Minarik et al., 2002). Our results show also that salinity treatment decreased NADP-ICDH activity in leaves (Figure 4B) which was similar to the studies in pea which showed increased activity of the enzyme during the first hour of stress whereas longer exposure resulted in decreased enzyme activity (Popova et al., 2001). The decreased NADP-ICDH activity was unable to meet the requirement for the carbon skeleton for glutamate biosynthesis necessary for ammonium assimilation. It has been reported that this enzyme activity provides carbon skeleton for ammonia assimilation through the GS/GOGAT (Glutamine synthetase/Glutamate synthetase) pathway by the synthesis of 2-OG (Figure 6) (Sheible et al., 2000) and may be also involved in the export and cycling of amino acids (Fieuw et al., 1995).

Levels of almost all organic acids determined in the study of Widodo et al. (2009) were significantly decreased in NaCl-treated barley leaves in the sensitive cultivar of 5 week. This possibly suggested a decrease in the flow of carbon from glycolysis. Lower levels of several metabolites from the TCA cycle may be correlated with reduced metabolic activity (TCA cycle activity) and, therefore, reduced growth. A reduction of photosynthetic activity will limit the availability of energy and photoassimilate for metabolism, resulting in increased catabolism of stored carbohydrates via respiration in order to provide cells with energy. Salinity is known to affect NH_4^+ uptake (Bourgeais-Chaillou et al., 1992). In our study, NaCl treatment resulted in an increase in NH_4^+ concentrations in leaves (Figure 3D). The greater increase in NH_4^+ coincides with the higher decrease in soluble protein content (Figure 1D). It was reported that NaCl stress results in an NH_4^+ accumulation released by increased proteolysis (Debouba et al., 2006a). Moreover, the reduced activity of GS and GOGAT due to NaCl can

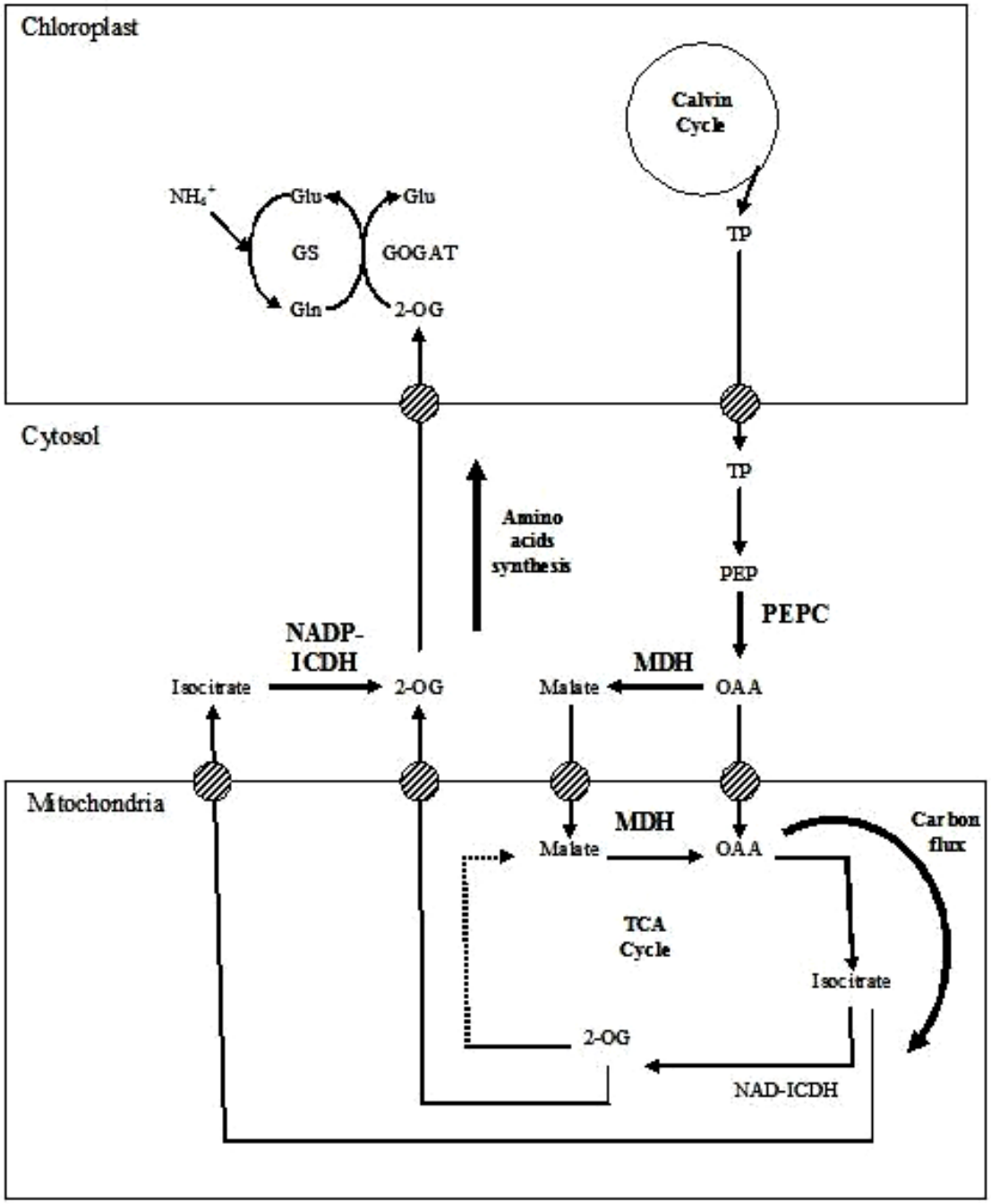

Figure 6. A simplified scheme of carbon metabolism enzymes (PEPC, MDH, NADP-ICDH) pathway in plant cells. TP: triosephosphate, PEPC: phosphoenolpyruvate carboxylase, OAA: oxaloacetate, MDH: malate dehydrogenase, NAD-ICDH: NAD-dependent isocitrate dehydrogenase, NADP-ICDH: NADP-dependent isocitrate dehydrogenase, 2-OG: 2-oxoglutarate, Glu: glutamate, Gln: glutamine, GOGAT: glutamate synthetase, GS: glutamine synthetase. Hatched circles on chloroplast and mitochondria denote membrane transporters.

contribute to the observed increase in NH_4^+ concentrations (Debouba et al., 2006a, b).

Increased PEPC, MDH and NADP-ICDH activities after NaCl suppression may reflect the need of the plant for increased production of carbon skeletons in order to regulate osmotic pressure and to maintain cell ionic neutrality by synthesis of organic acids. This is in support to other works done on different species that showed the reversibility effects of NaCl (Khelil et al., 2007; Slama et al., 2008) and other stresses such as metallic stress (Chaffei et al., 2003) and high temperatures and light intensities (Streb et al., 2003).

Conclusion

In conclusion, our results indicate that the addition of NaCl throughout the experimental period to the culture medium of tobacco induced a decrease in growth, a greater accumulation of sugars, an increase of Na^+ and Cl^-, a nutritional deficiencies shown by the decrease of K^+, an enhanced NH_4^+ production and marked changes in the activity of carbon assimilation enzymes, namely PEPC, MDH and NADP-ICDH. However, despite the clear alterations in growth and physiological parameters that occur in response to salt, the exact nature of the changes in carbon metabolism that are induced by this stress remains unclear and needs further clarification in order to discern whether NaCl effects on plant growth are due to mineral disturbances and/or to metabolic changes. The gradual resumption of normal levels for all parameters studied in tobacco leaves was accompanied by reduced levels of accumulation of Na^+ and Cl^-. This decrease in the concentrations of toxic ions was not due to the rejection of NaCl in the medium. This allows us to conclude that there is reversibility of the deleterious effects of salt once removed from the medium, probably by a dilution of Na^+ and Cl^- by growth. Recovery was substantial on the release of stress, in spite of the duration and magnitude of the stress applied. Hence, it could be inferred that salinity did not cause permanent alterations at some parameters that control production in tobacco plants.

ACKNOWLEDGEMENTS

Tobacco seeds were kindly given by Mr Abdeltif Ben Hsin (Direction Agriculture, Régie Nationale des Tabacs et des Allumettes, Tunisie).

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