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Full Length Research Paper

Long-term salt stress responsive growth, carbohydrate metabolism, proline and anti-stress enzymes in *Nicotiana tabaccum*

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We investigated the long-term responses of tobacco tissues to salt stress, with a particular interest for growth parameters, proline (Pro) accumulation, and carbohydrate metabolism. Exposure of 17-day-old tobacco plants to 0.2 M NaCl was followed by a higher decrease in dry matter in roots than shoots with a decrease of both Chlorophyll a and b. Analysis of potassium, sodium and chloral accumulation showed dramatic effect to K^+ accumulation especially in shoots, whereas Na^+ and Cl^- accumulation were strongly increased in shoots with NaCl treatment, but with minor effects in the roots. Salinity stress conditions induced a strong increase in sugar free content in the shoot and root and Pro and 2-oxoglutarate biosynthesis in tobacco tissues. However, salt-stress resulted in significant increase in the activity of NADPH and NADH malate dehydrogenase dependency, particularly in the roots, and this effect was reversed with $NADP^+$ and NAD^+-dependent malate dehydrogenase, but the activity of these enzymes was also maintained significantly higher in the absence of NaCl. Significant decreases in NADPH-depend isocitrate dehydrogenase was also observed after long-term salt treatment in tobacco plants. In addition, a gradual increase was observed in the *in vitro* aminating GDH activity, but the *in vitro* deaminating activity remained unchanged under salinity stress. These results confirmed the hypothesis that the salinity-generated MDH and GDH induction act as anti-stress enzymes in ammonia detoxification and production of Glu for Pro synthesis.

Key words: Tobacco, carbohydrate metabolism, salinity stress.

INTRODUCTION

During salinity stress, plants induce processes that regulate the osmotic adjustment and maintain sufficient cell turgor for growth to proceed (Miflin and Habash, 2002). Such adjustment requires the control of intracellular inorganic ions in the cytoplasm, via vacuolar sequestration, and accumulation of organic compounds compartmented mainly in the cytoplasm (Bohnert et al., 1995). These organic solutes are termed osmolytes, which raise osmotic pressure and protect some macromolecular structures against denaturation (Bourot et al., 2000). Pro is the most diversely nitrogenous osmolytes accumulated under osmotic stress conditions in plants (Hanaoka et al., 2002). In addition, Pro was used as a carbon and nitrogen source or as an osmoprotectant (Bohnert et al., 1995). Malate dehydrogenase (EC 1.1.1.37) (MDH) is an enzyme in the citric acid cycle that catalyzes the conversion of malate into oxoglutarate and vice versa. Pyruvate in the mitochondria is acted upon by pyruvate carboxylase to form oxaloacetate, a citric acid cycle intermediate. In order to get the oxaloacetate out of the mitochondria, malate dehydrogenase reduces it to malate, and it then traverses the inner mitochondrial membrane. Once in the cytosol, the malate is oxidized back to oxaloacetate by cytosolic malate dehydrogenase. The degree of

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stimulation by sodium chloride increases with increasing substrate concentrations. Differences between the properties of the enzymes isolated from plants grown with and without sodium chloride are described. The action of NaCl on MDH activity appeared to be a general ionic effect as judged by the response of the enzyme activity in the presence of iso-ionic concentrations of other salts and isoosmotic mannitol. Thus, the response of the MDH from five of the salt marsh plants to NaCl is similar to that of glycophytes (Galvez and Gadal, 1995). The proposed role of glutamate dehydrogenase (GDH; mitochondrial NADH dependent EC 1.4.1.2) is particularly noteworthy. This has been subject to continued controversy (Schneidreit et al., 2005). GDH is one of the few enzymes capable of releasing amino nitrogen from amino acids to give a keto-acid and NH_3 that can be separately recycled to be used in respiration and amide formation, respectively. GDH may be expected to function in the deaminating direction in tissues that are converting amino acids into transport compounds with a low C/N ratiosuch as germinating seeds and senescing leaves. Aubert et al. (2001), using MR and labelling studies and coupling glutamate oxidation to glutamine synthesis, recently produced strong experimental evidence that GDH does function in this direction in isolated mitochondria (Arnon, 1949). The activity of NADP-ICDH in plants treated at long-term with salinity decreased in leaves and roots. The physiological roles of NADP-ICDH in the adaptation of plants to high salinity are discussed. Inhibition of phosphoenolpyruvate carboxylase by the inorganic NaCl depended on the source of the enzyme. Ribulose-1, 5-diphosphate carboxylase was less salt-sensitive than the phosphoenolpyruvate carboxylases. Under stress conditions, such as salinity, increased proteolytic activity results in increased intracellular hyperammonia and toxicity if not efficiently removed (Lin et al., 2002). Ammonium ions are incorporated into Gln and Glu by glutamine synthetase/glutamate synthase, respectively (Meloni et al., 2004). In addition, glutamate dehydrogenase (GDH) (EC 1.4.1.2) catalyzes the reductive amination of 2-oxoglutarate (2OG) and the oxidative deamination of Glu *in vitro* and is abundant in plant tissues (Meloni et al., 2004). The exact physiological roles of GDH in plant carbon and nitrogen metabolism and remobilization in plants remain largely speculative. Intracellular hyperammonia due to abiotic stress (Hernandez et al., 2000) results in increased aminating GDH activity *in vitro.* In salt-tolerant cultivars, aminating GDH activity increases with increasing salt stress (Hodges, 2002).

MATERIALS AND METHODS

Plant material and growth condition

Seeds of tobacco (*Nicotiana tabaccum*) were sterilized in 10% (v/v) hydrogen peroxide and after imbibition, the seeds were germinated on moistened filter paper. After 7 days, uniform seedlings were transferred to plastic beakers filled with continuously aerated, basal nutrient solutions. At the age of 10 days after transplant, sodium chloride was added to the medium as NaCl at 0 and 200 mM. After one week of NaCl treatment, one part of plants was separated into shoots and roots and the samples were stored in liquid nitrogen for subsequent analysis or dried at 70°C for at least three days in order to determine both dry material and ionic contents. The rest tobacco plants continued the growth and were harvested eventually after 2, 9, 11, 13, 15, 17 days.

Carbohydrate analysis

Proline was determined by the ninhydrin assay as described previously (Thakur and Dev Sharma, 2005). Concentrations of L-malate in pineapple leaf samples were determined spectrophotometrically according to the method of Crecelius et al. (2003) (Drong et al. 2000). 2-oxoglutarate was measured in perchloric acid extracts (Srivastava and Singh, 1987). Sugar free content was measured in the soluble and residual fractions of ethanol-water extract (Srivastava and Singh, 1987).

Potassium, sodium and chloride determination

Ions were extracted from fresh tissue in 70% (v/v) ethanol and analyzed by HPLC and conductimetric detection. Cation analysis was done from ethanol extracts eluted with 25 mM H_2SO_4. For anion analysis, the extracts were diluted in acetonitrile, and separation was obtained on a Dionex Ion Pac AS11, eluted with a linear gradient of 12 to 35 mM KOH.

Chlorophyll concentration

Chlorophyll a and b were extracted and estimated following Arnon (1949) (Aubert et al., 2001). Absorbance of the solution was then measured at 645 and 663 nm in order to determine the concentrations of chlorophyll a and b respectively. Chlorophyll concentrations were expressed as mg g^{-1} fresh weight.

Enzyme assays

Malate dehydrogenase, MDH (EC 1.1.1.37) enzyme was extracted with 100 mM tris-HCl buffer (pH: 8.3), 5 mM DTT, 2 mM EDTA, 5 mM $MgCl_2$, 150 mM β-mercaptoethanol and polyclart AT %. MDH activity was measured according to the method of Crecelius et al. (2003) (Drong et al. 2000). The homogenate was centrifuged at 4°C. NAD(P)H-MDH activity was determined in a final volume containing 25 mM NADH or NADPH, 25 mM Tris-HCl at pH 8, 1 mM EDTA and 0.5 mM oxaloacetate. The NADH- or NADPH-MDH activities were measured by following the absorbance changes at 340 nm. Enzyme activities were expressed per min and g FW. The $NAD(P)^+$-MDH activities were measured in the reaction mixture containing 25 mM, at pH 8 to 9 tampon Tricine de sodium, 1mM EDTA, 50 mM malate, 20 mM glutamate, and 0.2 M hydrazine. The reaction was initiated by the addition of 2.5 mM $NAD(P)^+$. The results were expressed at µM $NAD(P)^+$red/g FW/min.

Isocitrate dehydrogenase

ICDH (EC. 1. 1. 1. 42), *in vitro* enzyme activity was determined as described by Crecelius et al. (2003). All operations were carried out at 4°C. Samples were ground with an ice-cold mortar and extracted in the presence of phosphate potassium buffer, containing (14 mM) mercaptoethanol and 1% PVP. The enzymatic reaction was

measured in the presence of potassium phosphate buffer (100 mM, pH 7.5), isocitrate (2.5 mM) and $MgCl_2$ (5 mM). One unit of ICDH was defined as the reduction of $NADP^+$ per min at 30°C at 340 nm.

Glutamate dehydrogenase

In vitro enzyme activity of GDH (EC 1.4.1.2) was determined as described previously (Luts et al., 1999), and values were presented as units ± SE. One unit of GDH was defined as the oxidation of 1 µmol NADH or reduction of NAD^+ per min at 30°C.

RESULTS

Effect of long-term stress treatments on the growth

Salt stress was imposed on the tobacco plants 3 weeks by adding 200 mM NaCl to the growth medium, and two physiological parameters; dry matter and chlorophyll a and b contents acetylene were measured during the stress period merely to establish the degree of stress that affects the plants. By day 17 of the treatment, the individual biomass of shoot and root tissues decreased by more in the roots than shoots (Figure 1A and B). Biomass of the control tobacco tissues strongly increased at day 17. Chlorophyll a and b decreased in the shoots at day 9 of NaCl treatment (Figure 1C and D), but a significantly increase was observed in the control plants.

Nutrient content and the changes of K^+ concentrations in the organs of tobacco seedlings are presented in Figure 2. The application of NaCl caused a decrease in K^+ concentration both in the shoots and roots; the effect was most marked in the shoots in the end of the period of treatment, but at day 13, an increase was observed in the root tissues (Figure 2B). In the control plants, a high increase of K^+ concentration was noticed. After 7 days of NaCl treatment, an increase in Na^+ content was observed both in the shoots and roots (Figure 2C and D). There was no effect in the Na^+ content of the control plants. After 17 days of stress, the increase in intracellular Cl^- was 2.5-fold in the shoots than the Na^+ content (Figure 2C and E) and about 1.34-fold in roots (Figure 2D and F). An endogenous content in Cl^- always exceeded that of Na^+. Thus, the rapid increases in Na^+ and Cl^- contents in shoots can be more likely correlated with strong K^+ lost in the same organ.

Osmolyte synthesis

Proline accumulation was significantly positively affected by salinity at day 17 (Figure 3A and B). Proline does seem to play an important role in the mechanism of salt tolerance in tobacco. The significance of proline accumulation in osmotic adjustment is still debated and varies according to the species. Recently, studies reported that proline was able to counteract salt stress effects in salt-sensitive tomato plant (Hernandez et al, 2000). It is possible therefore that many of the prospective changes in the photosynthetic apparatus that occurred in tissues of tobacco plants under long-term NaCl treatment were effected through changes elicited by increased shoot and root sugar concentrations (Figure 3C and D). Sugar free levels remained constant in the control shoots, but an increase was observed in the roots of the control plants (Figure 3C and D). As shown in Figures 3E and F, malate levels for shoot and root tissues of tobacco plants were cultured under long-term NaCl treatment. In terms of 17 days, malate content was slightly increased in both shoots and roots of tobacco cultured under 200 mM of NaCl 6 and 12-fold respectively. The increase in malate content was lower in the control plants. Plant cell 2-oxoglutarate (2OG) was present at relatively low levels when compared to the other organic acids. Along with tobacco plants cultured under long-term NaCl stress, 2-oxoglutarate was also induced (Figure 3G and H). This organic acid increased by 2-fold in the shoot and 5-fold in the roots at day 17 in salinity stress plants. However, 2-oxoglutarate levels in the control plants showed a smaller but no significant accumulation. In addition, 2-oxoglutarate is a direct regulator of enzymes such as cytosolic pyruvate kinase, mitochondrial citrate synthase, and alternative oxidase activities involve in sugar/organic acid flux between cytosol and mitochondria (Hoai et al., 2003).

Changes in enzyme activities

NADPH-malate dehydrogenase (NADP-MDH; EC 1.1.1.82) is responsible for the reduction of oxaloacetate to malate in the chloroplasts of higher plants. Salt stress resulted in a net decrease in the activity of NADPH-malate dehydrogenase of tobacco tissues (Figure 4A and B). At day 17 it increased to more than 200% compared to the control roots (Figure 4B). The $NADP^+$-malate dehydrogenase in shoots and roots was rapidly activated in the presence of 200 mM of NaCl (Figure 4C and D). In addition, the activity of enzyme in both tissues was highly increased in the control plants grown in the absence of chloride sodium. The difference in activity of these enzymes in different tissues was investigated with their possible role in photosynthesis. The NAD^+-malate dehydrogenase (l-malate; NAD oxidoreductase, EC 1.1.1.37) showed peaks of activity in the root and shoot tissues at day 17 under NaCl stress (Figure 4E and F). After 17-day exposure to salt stress conditions, the increase of NAD^+-MDH activity was 55.5 and 57.3% respectively in the shoots and roots. Furthermore, NAD^+-MDH of the control tobacco was markedly decreased with age. A close relation between the two reduced isoenzyme of MDH, NADPH-MDH and NADH-MDH was observed. This was positively correlated with the result obtained in Figure 3E and F; the findings of malate synthesis. However, in contrast to the NAD^+-MDH, the

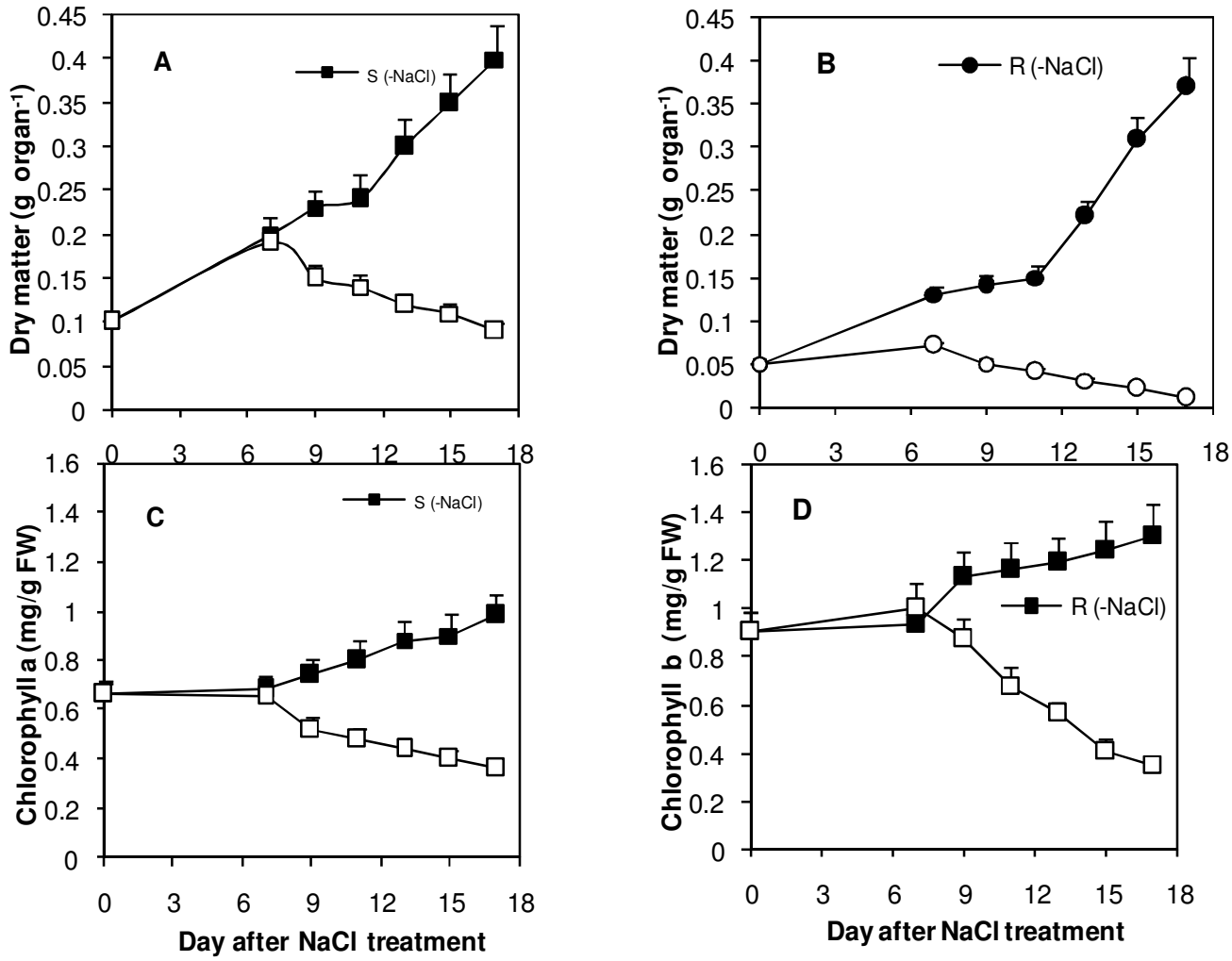

Figure 1. Effect of salt stress on shoot (A) and root dry weight (B), chlorophyll a content (C) and chlorophyll b (D) content of *N. tabaccum* plants. The plants were grown in the absence (●, ■) or presence (○, □) of 200 mM NaCl medium for 17 days. Values are the means ± SE of triplicates from five independent experiments.

reduced form of MDH, that is NADH-MDH activity decreased with NaCl long-term treatment both in the shoot and root tissues (Figure 4G and H). After 17-day exposure to salt stress conditions, the decrease of NAD^+-MDH activity was 5.5 and 57.3% respectively in the shoots and roots. Furthermore, NAD^+-MDH of the control tobacco was markedly increased with age. A close relation between the two reduced isoenzyme of MDH, NADPH-MDH and NADH-MDH could be observed. This was positively correlated with the result obtained in Figure 4C and D; the findings of malate synthesis. Glutamate dehydrogenase (GDH) may be a stress-responsive enzyme, as GDH exhibits considerable thermal stability, and *de novo* synthesis of the α-GDH subunit is induced by senescence. At day 17, NaCl treatment induced intracellular ammonia, and *in vitro* GDH aminating activity in the tissues from tobacco plant organs (Figure 5A and B). After 17 days, the control plants showed an increase in NADH-GDH activities but remained lower than that in the stressed plants. Along with upregulation of α-GDH by NaCl, 2-oxoglutarate was also induced. Treatment with NaCl also elicited several increase in reactive oxygen species (ROS) and immunoreactive α-polypeptide and GDH activity (data not shown). Ammonium ions also mimic the effects of salinity in the induction of NADH-GDH in both shoots and roots. These results, confirmed in tobacco tissues, support the hypothesis that the salinity-generated ROS signal induces α-GDH subunit expression, and the aminating GDH assimilate ammonia, acting as antistress enzymes in ammonia detoxification and production of Glu for Pro synthesis. The results of deaminating activity of GDH (NAD^+-GDH) showed a remarkable decrease especially in the shoots, but no effect was observed in the roots (Figure 5C and D). NaCl stressed plants were able to maintain the Glu content in significant level necessary to

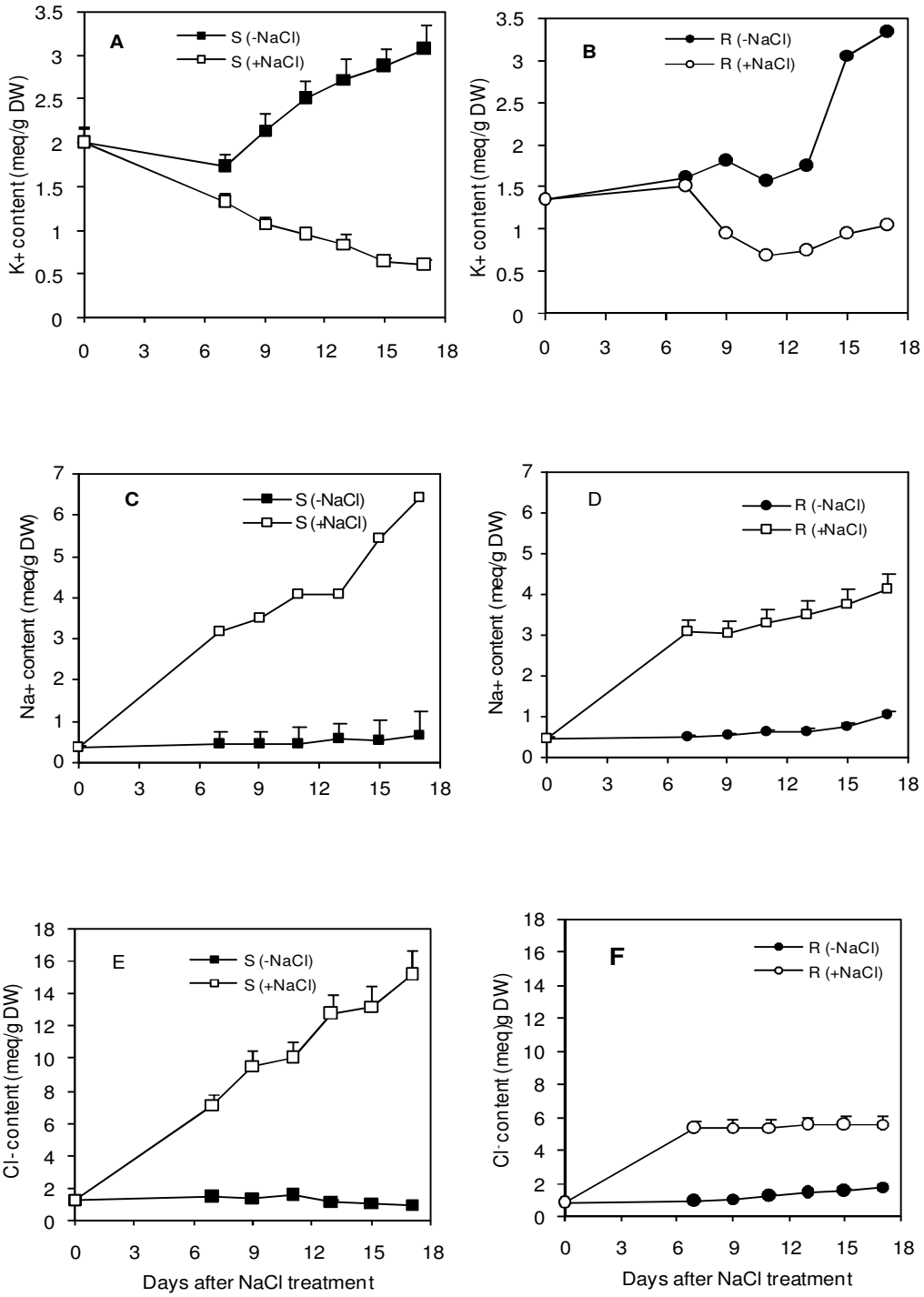

Figure 2. Effect of salt stress on shoot (A) androot K^+content(B), on shoot (C) and root Na^+ content(D), on shoot (E) and (F) root Cl^- content of *N. tabaccum* plants. The plants were grown in the absence (●, ■) or presence (○, □) of 200 mM NaCl medium for 17 days. Values are the means ± SE of triplicates from five independent experiments.

proline synthesis in the stress conditions. Among the amino acids tested as signal molecules, proline (Pro) and glutamate (Glu) were major inducers of GDH and GS expression, respectively (Miflin and Habash, 2000).The contrary effect of NaCl NADH-GDH and NAD^+-GDH was also emphasized. NADP- isocitrate dehydrogenase (ICDH) is a key cytosolic enzyme that links C and N metabolism by supplying C skeletons for primary N

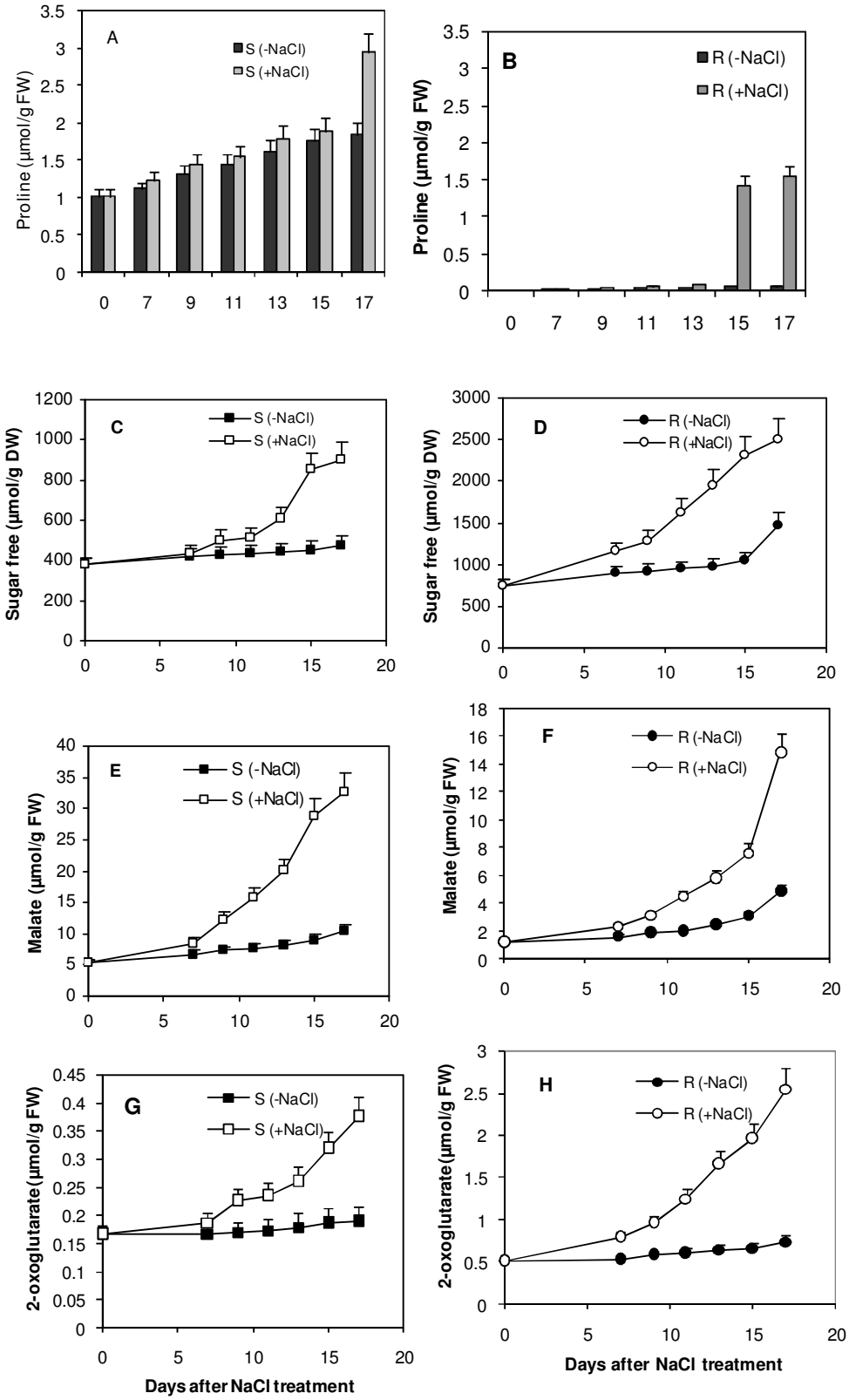

Figure 3. Effect of salt stress on shoot (A) and root proline content (B), on shoot (C) and root sugar free content (D), on shoot (E) and root malate content (F), and on shoot (G) and(H) root 2-oxoglutarate content of *N. tabaccum* plants. The plants were grown in absence (●, ■) or presence (○, □) of 200 mM NaCl medium for 17 days. Values are the means ± SE of triplicates from five independent experiments.

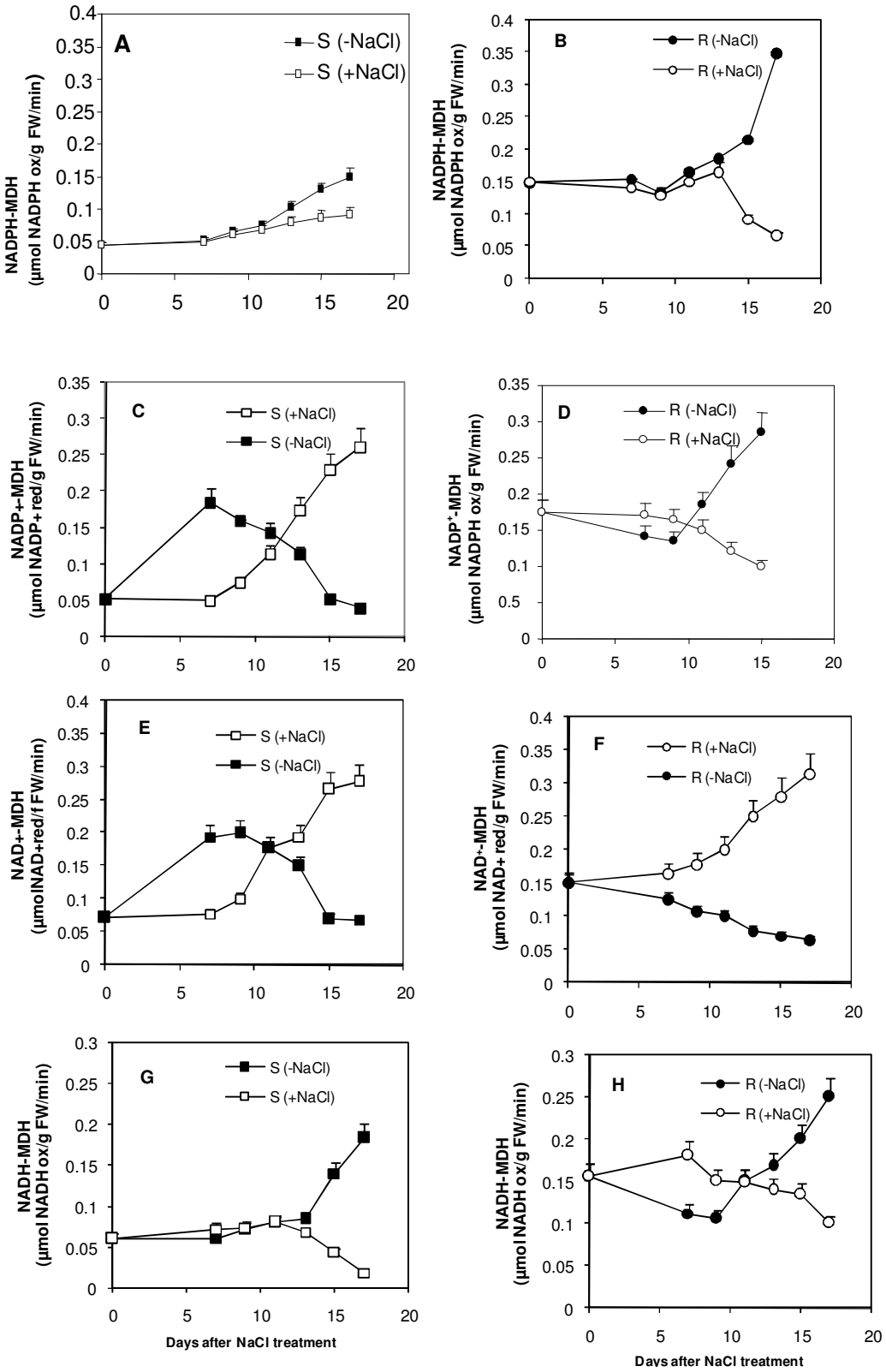

Figure 4. Effect of salt stress on (A) shoot and (B) root NADPH-MDH activity, on (C) shoot and (D) root $NADP^+$-MDH activity, on (E) shoot and (F)root NAD^+-MDH activity and on (G) shoot and (H) root NADH-MDH activity of *N. tabaccum* plants. The plants were grown in absence (●, ■) or presence (○, □) of 200 mM NaCl medium during 17 days. Values are the means ± SE of triplicates from five independent experiments.

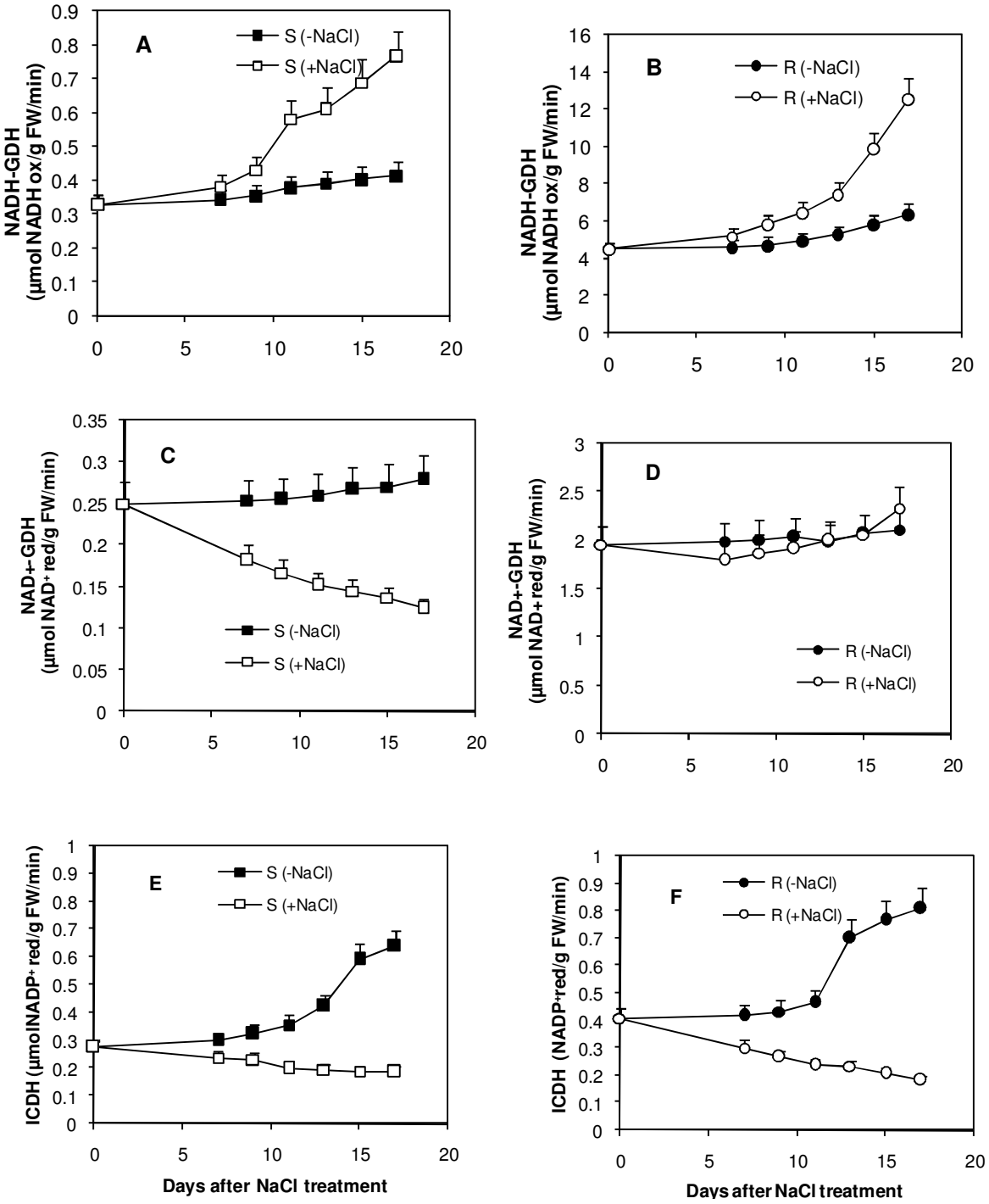

Figure 5. Effect of salt stress on shoot (A) and root NADH-GDH activity (B), on shoot (C) and root NAD^+-GDH (D) and on shoot (E) and root $NADP^+$-ICDH (F) activity of *N. tabaccum* plants. The plants were grown in absence (●, ■) or presence (○, □) of 200 mM NaCl medium for 17 days. Values are the means ± SE of triplicates from five independent experiments.

assimilation in plants. The kinetic properties of the NADP-ICDH activity from shoot and root of control and NaCl treated tobacco plants were analyzed. The activity of NADP-ICDH in plants exposed to long-term of salinity treatment, decreased in shoots and roots (Figure 5E and F). At day 17, this decrease was respectively 30 and 61.8% in shoots and roots of tobacco plants cultured in the presence of 200 mM of NaCl. It appeared, therefore, that a significant increase of ICDH of the control plants was observed from Day 13 both in the shoots and roots.

DISCUSSION

Salt response is expected to be a complex trait, in accord with the widespread consequences of salt on plants. The water deficit induced by salt results in osmotic stress, while an excess of sodium ions has disastrous effects on numerous key biochemical processes. Plants of *N. tabacum* exposed to 200 mM of NaCl with long-term treatment, underwent extensive osmotic adjustment which accumulated Na^+ and Cl^- as principal solutes for this adjustment. Although the intracellular concentrations of Na^+ and Cl^- correlated well with the level of adaptation, these ions apparently contributed to the osmotic adjustment which occurred during the plant's growth, because the concentrations of Na^+ and Cl^- increased during the period of salinity stress. The average intracellular concentrations of soluble sugars, 2-oxoglutarate, ammonium and malate increased as a function of the period of NaCl treatment. As shown in this work, tobacco can be defined as a salt "includer" and preferentially accumulated salt in the shoots. Thus, 200 mM NaCl caused a significant reduction in the root and shoot growth. Roots appear to be the most vulnerable part of the plant as they are directly exposed to salt, but nevertheless they are surprisingly altered. Our data showed that shoot growth of *N. tabaccum* plants, submitted to high NaCl concentrations, was not affected as much as the root growth. In addition, visible senescence and necrosis symptoms were found on the basal, and oldest leaf of the stressed tobacco plants after 17 days of the 200 mM NaCl treatment (data not shown). These symptoms could be as a result of excess Na^+ and Cl^- ions, which are known to induce chlorosis (Gomes et al., 2006). If excessive amounts of salt enter the plant, they will eventually rise to toxic levels in the shoots and roots, causing premature senescence (Hodges, 2002). The chlorophyll a and b contents of shoots decreased under salt stress. In tobacco, chlorophylls were significantly reduced under NaCl stress, but the rate of decline of chlorophyll b was greater than that of chlorophyll a (Figure 1C and D). The improved decline of K^+ contents in both organs revealed non reduced perturbation of their ionic contents. During salt treatment, Na^+ contents were increased by 16.98- and 8.97-fold in the shoots and roots at day 17 with 200 mM NaCl, respectively. These points to the possible regulation of sodium uptake at the shoot tissues. The increased Na^+ contents were related to the improved decrease of K^+ in the shoots compared to the roots. Saline conditions can influence the different steps of N metabolism, such as uptake and reduce ion and protein synthesis that may be responsible, at least in part, for the observed reduction in plant growth rate (Magalahaes and Huber, 1991). The NaCl treatment induced an increase in proline content in the shoots and roots of tobacco plants. The increase in proline content was positively correlated to the level of salt tolerance. Other functions have been postulated for proline accumulation in stressed tissues; it could be a protective agent of enzymes and membranes (Trinchant et al., 1998). Since 2OG can be synthesized in a number of different subcellular compartments, its mobility could be of major importance in metabolic functioning (Hoai et al., 2003). Although plastids contain several 2OG-producing enzymes, their activities are probably too low to sustain the required ammonia assimilatory capacity of this organelle. In plants, 2OG transport activity is coupled to that of a Glu/malate translocator (Shneidreit et al. 2005). Plant cell 2OG is present at relatively low levels when compared to salinity stressed plants (Figure 3G and H). It has been shown that 2OG levels depend upon metabolic fluxes related to C and N metabolism. Indeed, 2OG levels are dependent on inorganic and organic N content and their related metabolism as well as nitrate and sugar levels (Stitt et al, 1989).Taken together, our data indicated that plant 2OG levels was an agreement with this hypothesis. NADP-malate dehydrogenase (NADP-MDH; EC 1.1.1.82) is responsible for the reduction of oxaloacetate to malate in the chloroplasts of higher plants. In *N. tabaccum*, steady state activity levels for chloroplast NADP-MDH increased transiently in the shoots and roots after salt stress and then increased to levels greater than fourfold higher in the unstressed plants at day 17. When *N. tabaccum* plants were treated with NaCl, an increased Na^+ level was observed in the shoots 3 weeks after treatment, and at this stage of the treatment, the growth of plants was not reduced but the malate metabolism was modified. The malate content increased in the leaves and the activities of NAD and NADP-malate dehydrogenase enzymes also increased. Malate dehydrogenase-NADPH or -NADH dependent activity of shoots and roots decreases in many plants under salt stress (Miflin and Habash, 2002). The primary cause of a reduction of NAD(P)-MDH in the tissues was a specific effect associated with the presence of Cl^- salts in the external medium. This effect of Cl^- seems to be due to a reduction in NAD(P)H synthesis and consequently a lower concentration. Previous studies showed that the GS/GOGAT cycle is mainly responsible for glutamate production under proline demand (Skopelitis et al. 2007). The culture of *N. tabacum* plants in the presence of 200 mM NaCl resulted in a gradual increase of NADH-GDH activities during a 17-days period (Figure 5A and B). The *in vitro* GDH deaminating activity (NAD^+-GDH) remained unchanged in the roots, while decrease was observed in the shoots (Figure 5C and D). Therefore a close correlation between NADH-GDH activity and proline content was further confirmed. Glutamate synthesis requires a carbon skeleton in the form of 2-oxoglutarate, which is mainly produced by NADP-ICDH catalysis. Our results showed that salinity treatments increased $NADP^+$-ICDH activity (Figure 5E and F). The enhanced NADP-ICDH activity met the requirement for the carbon skeleton for glutamate biosynthesis. Based on the above results, we suggest

that NADH-GDH might contribute more glutamate for proline biosynthesis under high salinity condition.

Conclusion

A significant accumulation of proline and carbohydrate were found in tobacco seedlings at long-term salinity treatment. It is the NAD(P)-MDH and NADH-GDH pathway that plays the dominant role in supplying malate and glutamate for proline synthesis. The NADH-GDH pathway seemed to play an important complementary role in assimilating the increased ammonium and maintaining glutamate production, while $NADP^+$-ICDH was significantly decreased.

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