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Amino acid accumulation in sink and source tissues of *Coleus blumei* Benth. during salinity stress

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Abstract

The purpose of this study was to analyse the accumulation of amino acid in source and sink tissues of variegated *Coleus blumei* Benth. leaves during an extended exposure to salinity stress. The imposed stress resulted in a reduction in shoot height and leaf size, as well as a reduction in total protein and nitrogen content in both the sink and source tissues. At the same time, accumulation of low molecular weight nitrogen-containing compounds in *Coleus* leaves was observed, which peaked within the first 10 d of exposure to salinity, and then declined, but remained slightly elevated for the remainder of the study. A number of amino acids were found to accumulate in both the sink and source tissues, including arginine, asparagine, and serine. A larger proportion of asparagine and less arginine was observed in the sink tissue than the source tissue of the salinity-stressed plants. This difference may reflect the mobility of these compounds in the phloem. No proline was found to accumulate in either the source or sink tissue during the exposure to salinity. From the pulse–chase labelling of stressed *Coleus* leaves it can deduced that some of the observed accumulation of amino acids and amides observed is due to *de novo* synthesis and not simply the result of protein degradation.

Key words: Salinity stress, *Coleus blumei*, carbon partitioning, amino acids, compatible solutes.

Introduction

Salinity stress is a major factor in limiting crop productivity throughout the world and has recently been the focus of much research. The ability of plants to cope with salinity stress is an important determinant of crop distribution and productivity in many areas, so it is important to understand the mechanisms that confer tolerance to saline environments. Productivity in saline environments is affected by both water availability and excess salts. Biochemical studies have shown that plants under salinity stress accumulate a number of metabolites, which are termed compatible solutes because they do not interfere with biochemical reactions (see reviews by Bohnert *et al*., 1995; Rabe, 1990). These metabolites include carbohydrates, such as mannitol, sucrose and raffinose oligosaccharides, and nitrogen-containing compounds, such as amino acids and polyamines.

The function of compatible solute accumulation is often associated with osmotic adjustment, by lowering the water potential to improve the uptake of water against the external gradient, but a number of other roles for these compounds have been hypothesized in recent literature (Vernon *et al*., 1993; Rabe and Lovatt, 1984; Smirnoff and Cumbes, 1989). Possible roles include: serving as a readily available energy source or as a nitrogen source during limited growth and photosynthesis, detoxification of excess ammonia under periods of stress, and stabilization of enzymes and/or membranes. The effects of stress on plant nitrogen metabolism has been frequently studied, with increases in protein degradation, inhibition of protein synthesis and the accumulation and/or depletion of protein and non-protein amino acids reported in a variety of monocots and dicots (Schubert *et al*., 1995; Fougere *et al*., 1991; Good and Zaplachiniski, 1994). Proline accumulation in response to stress is widely reported, and may play a role in stress adaptation within the cell, which is of great interest to those studying stresses in plants. Other

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nitrogenous compounds that accumulate in response to stress may have important roles in tolerance, but have received little attention compared to proline accumulation in recent years. Also, patterns of solute accumulation during an extended exposure to stress has not been widely studied. Detailed understanding of the biochemical pathways leading to the formation of amino acids and compatible solutes will be important in engineering plants to tolerate saline environments in the future.

Most of the studies of compatible solute accumulation have focused on the photosynthetic tissues, or source tissues. Phloem transport in sieve tubes enables the transfer of assimilates from source tissue to sink tissue, and major components of the phloem sap includes carbohydrates and amino acids. All of the protein-forming amino acids can be found in the phloem, but the chief nitrogen transport compounds in plants are asparagine, glutamine and amides (see review by Atkins and Beevers, 1990). The nitrogen economy of plants appears to be biased towards the synthesis, transport and utilization of a particular compound or a small group of compounds rather than a wide range of components, and the specific compounds utilized varies greatly among plants. Although indications for differential uptake of certain amino acids into sieve tubes has been reported (Schorbert and Komor, 1989), there is little information on the selectivity of amino acid uptake from source leaves into sieve tubes and the influence of the environment on the transport of these compounds. Amino acid transfer from source tissue to sinks is an important regulatory step in the overall nitrogen assimilation in plants, but much information about these processes is still unknown.

Here, the accumulation of nitrogen-containing compounds in the sink and source tissue of *Coleus blumei* were studied throughout a 4 week exposure to salinity. The variegated leaves serve as an ideal model for studying sink and source patterns, as the non-chlorophyllous regions of the mature leaves behave as true 'sinks' for assimilates produced by the photosynthetic areas (Weisberg *et al.*, 1988; Turgeon and Wimmers, 1988; Madore, 1990). The goal of this study was to characterize the partitioning of nitrogen containing compounds between sink and source tissues during an extended exposure to moderate saline conditions.

Materials and methods

Plant material and growth conditions

Coleus plants (*Coleus blumei* Benth. cv. 'Fairway White') were grown from cuttings obtained from plants purchased at a local nursery. Once rooted, 36 plants were transplanted to six sand tanks in a green house at the US Salinity Laboratory located in Riverside CA (Wilson *et al.*, 1992), with six plants present per tank. The plants were allowed to equilibrate to the conditions for 2 weeks, before the salt treatment began. Each tank contained the following (in mol m^{-3}): $Ca(NO_3)_2$, 2.5; KNO_3, 3.0; $MgSO_4$, 1.5; KH_2PO_4, 0.17; Fe (as sodium ferric diethylenetriamine pentaacetate), 0.05; H_3BO_3, 0.023; $MnSO_4$, 0.005; $ZnSO_4$, 0.0004; $CuSO_4$, 0.0002; and H_2MoO_4, 0.0001. After equilibration for 2 weeks in nutrient solution, three tanks of *Coleus* plants were salinized by the addition of a 5 : 1 ratio of NaCl to $CaCl_2$ in three equal increments on consecutive evenings until a salinity level of 60 mM NaCl, 12 mM $CaCl_2$ was reached. The salinity level was maintained over a 1 month period. The experiments were repeated three times.

Leaf tissue amino acid analysis

Three samples of leaf tissue of 0.2 g fresh weight were cut from green and white areas of mature leaves from each tank of control and salinity-stressed plants approximately every 3 d. The samples were immediately frozen on dry ice and kept at $-20\,^{\circ}C$ until the tissue was analysed. The samples were extracted in 80% ethanol and the extracts partitioned by ion exchange chromatography into neutral, basic, and acidic fractions as previously described (Madore, 1990). The basic fractions were taken to dryness, resuspended in 100 μl of a drying reagent consisting of triethylamine : absolute ethanol: water (1 : 1 : 1, by vol.) and dried again. The amino acids were converted to their PITC derivatives, and analysed as previously described (Mitchell and Madore, 1992).

Nitrogen and carbon content

Total nitrogen and carbon content was determined by flash combustion chromatography using a Carlo-Erba C/N/S analyser. Six tissue samples from different control and stressed *Coleus* plants were taken 7, 10, and 13 d after the salinization process began. The green and white regions of the leaves were immediately separated, frozen on dry ice and kept at $-20\,^{\circ}C$ until analysed. The samples were dried and powdered with a morter and pestle, and approximately 10 mg powdered plant tissue from each sample was weighed into tin capsules and the exact weight was recorded. Determination of N and C content was accomplished by automated integration and calculation using a standard curve developed from BBOT standard. Inclusion of samples with a known C and N content (National Bureau of Standards, apple leaf) and replications of unknowns were utilized to evaluate accuracy and precision respectively, during the analytical run. The data shown represents an average of all the samples collected.

Protein content

Leaf tissue (1 g) from green or white areas of several mature leaves from different control or stressed plants were ground on ice using a mortar and pestle in 3 ml of grinding buffer (50 mM HEPES, 50 mM ascorbic acid, 1 mM DTT, 1 mM $MnCl_2$, and 10% ethylene glycol). The extracts were filtered through cheesecloth, transferred to microfuge tubes and centrifuged for 2 min at 10 000 *g* in a microcentrifuge. Portions (0.5 ml) of the supernatant were desalted on Sephadex G25 columns equilibrated with grinding buffer. Protein contents were then determined by the Bradford method (Bradford, 1976).

$^{14}CO_2$ pulse chase

These experiments were completed on both control and stressed *Coleus* plants exposed to 8 d of salinization. Branches (containing 10–20 leaves) were excised from the plant using a razor blade. The cut ends were immediately immersed in water, and the stems were recut at the base under water to eliminate any trapped air in the xylem. After a 1 h incubation period under a water-filtered 150 W light source, the branch was enclosed in a

plastic bag. The branches were then pulsed with 3.7 MBq $^{14}CO_2$ for 1 min as previously described (Flora and Madore, 1993). At specific time points throughout the 20 min chase in ambient air, three leaves were randomly removed from the branches at each time interval, separated into green and white regions, and immediately frozen in aluminium-foil envelopes in dry ice. The leaves were stored at $-20\,^{\circ}C$ until extracted and processed as described above. The radioactivity in each ion exchange fraction and in the insoluble residue was determined by scintillation counting. The experiment was repeated three times and the data shown is an average of all samples collected.

Phloem exudate

The experimental protocols for these procedures were essentially as previously described (Flora and Madore, 1993). Prior to labelling, branches containing at least four mature leaves were excised from each plant and immediately immersed in 20 mM NaEDTA (pH 7.0) to inhibit callose formation. The plant was enclosed in a plastic bag and exposed to $^{14}CO_2$ (produced by the acidification of 1.0–1.5 MBq of $NaH^{14}CO_3$) as previously described (Flora and Madore, 1996). After a 30 min labelling period, the plastic bag was removed, and the plant was left under the light for a 30 min chase period in ambient air. Branches were then immersed in water, and individual leaves were removed with a razor blade at the base of the petiole. The petiole was then re-cut under water and placed in a microfuge tube containing 1.5 ml of 20 mM EDTA (pH 7.0). The amount of ^{14}C exuded by each labelled leaf was monitored at 30 min intervals by scintillation counting until the total label exuded from each leaf had reached approximately 50 Bq. The exudate was separate into neutral, acidic, and basic fractions as described above, and the amount of radioactivity in each fraction was determined by scintillation counting. For each experiment the exudate was collected from eight leaves (from four different plants) for both stressed (10 d after salinization) and control plants. The experiment was repeated twice and the data shown represents an average of all data collected.

Results

Growth rates

The growth of *Coleus* shoots were affected by the presence of NaCl in the growing medium. During the course of the experiment the control plants grew an average of 1.8 cm d^{-1}, whereas the plants subjected to the saline conditions grew at a rate of approximately 0.7 cm d^{-1} (Fig. 1). Under the stress conditions, there was also a reduction in the size of each leaf by approximately 50%, the size of the control leaves averaged 55.6 cm^2 and the stressed leaves averaged 26.5 cm^2 at day 30. The non-photosynthetic tiuss accounted for approximately 33% of mature leaves in both the control and stressed plants. In all cases, the white non-photosynthetic sink tissue remained healthy (no necrosis or excision of the region was observed) throughout the duration of the experiments.

C and N content

In the source and sink tissue of control plants, carbon accounted for approximately 45% and 40%, respectively,

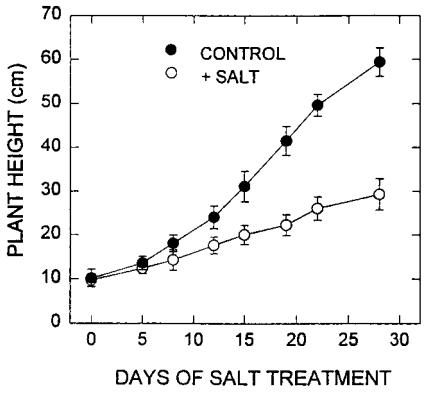

Fig. 1. Changes in shoot height in *Coleus* plants during salinity stress. Day 0 represents the morning before the salinization process began. Plant height of nine different plants was measured (in cm) approximately every 3 d during the salinization treatment (±se).

of the total dry weight. The carbon content did not change significantly when salinity stress was imposed. In the control plants, the nitrogen content accounted for 4.4% of the dry weight in the source tissues, and 4.7% of the sink tissues. Under salinity stress, there was a reduction in total nitrogen content to 3.2% in source tissues in 3.3% in the sink tissue.

Protein content

The loss in total tissue N was reflected in the decline in total soluble protein in the sink and source tissues of *Coleus* which occurred during salinity stress (Fig. 2). In the control tissues, the average soluble protein values were 4.25 $\mu g\ \mu l^{-1}$ in the source tissues, and 2.23 $\mu g\ \mu l^{-1}$ in the sink tissues. By day 25 of the salt treatment the source tissues exhibited a 25% reduction in soluble protein content, and in sink tissues a 20% reduction was observed.

Total soluble amino acid content

In contrast to the effects observed for total N, the imposed salinity treatment raised the free amino acid levels significantly in the source (Fig. 3A) and sink (Fig. 3B) tissues during the first 10 d of the salinity exposure, by more than a 2-fold increase in both. The amino acid levels in the stressed tissues slowly declined for the remainder of the study, but remained slightly elevated compared to the control tissues. Total soluble amino acid content was similar in sink and source tissues of the control plants (Fig. 3A,B).

Specific amino acid accumulation

The addition of salt to the growing media had a significant effect on the amino acids composition of both the source (Fig. 4) and sink (Fig. 5) tissues of *Coleus*. In source tissues, increased levels of asparagine (Fig. 4A), arginine (Fig. 4B), alanine (Fig. 4C), serine (Fig. 4D), valine (Fig. 4F), glutamine (Fig. 4G), isoleucine (Fig. 4H), and

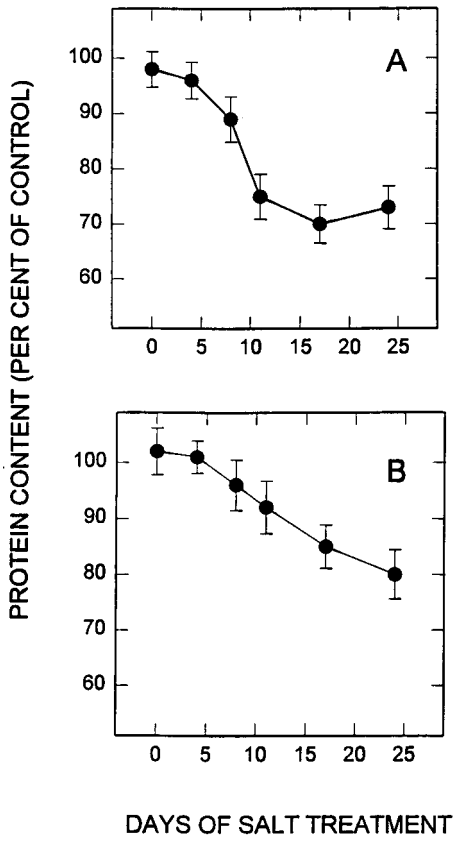

Fig. 2. Effects of salinity on soluble protein content in source (A) and sink (B) tissues of *Coleus* during a 1 month exposure to salinity ($n=3$, $\pm$se).

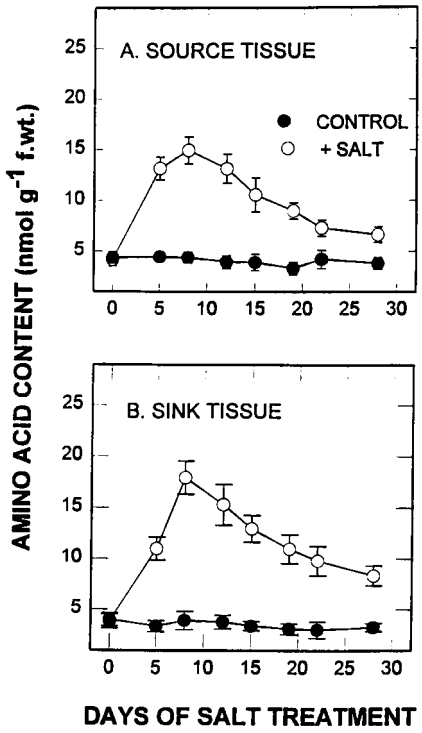

Fig. 3. Total soluble amino acid content in source (A) and sink (B) tissues of *Coleus* in control and salinity-stressed plants determined by HPLC ($n=9$, $\pm$se).

glutamic acid (Fig. 4I) were observed. The accumulation of these compounds peaked between 5 and 15 d after the saline conditions were imposed, and returned to levels observed in the control plants by day 30 in most cases. The exception to this is arginine (Fig. 4B), alanine (Fig. 4C) and valine (Fig. 4F), which remained elevated throughout the course of the study. In the source tissue of stressed plants the largest increase occurred in the level of arginine (Fig. 4B), but in the sink tissue the largest increase was observed in the level of asparagine in the tissue (Fig. 5A). In general, similar patterns of accumulation were observed in the sink tissues (Fig. 5A–I) and the source tissue, as previously discussed. Notably, no change in proline concentration was observed in either the source (Fig. 4E) or sink (Fig. 5E) tissues. Other amino acids exhibited either slight declines or showed no significant changes (Table 1).

Pulse chase and phloem exudate

By 8 d after the imposed salinity stress, the distribution of ^{14}C-label between amino acids, organic acids, starch or sugars in the *Coleus* leaves exposed to $^{14}CO_2$ was altered (Fig. 6). Following the label through the time intervals of the chase with ambient air provides information on carbon fixation and the biochemical pathways utilized in the leaves. In the stressed plants, there was an increase in the amount of label incorporated into the amino acids (Fig. 6A), and a decrease in the amount of label incorporated into sugars (Fig. 6B) and starch (Fig. 6D). No significant difference was observed in the amount of label incorporated into organic acids and sugar phosphates between the control and stressed plants (Fig. 6C). There was also an increase in the amount of ^{14}C-labelled amino acids found in the phloem exudates of stressed *Coleus* plants labelled with $^{14}CO_2$, compared to the levels observed in the control plants. At 10 d after exposure to salinity, $27.2\pm2.1\%$ of total ^{14}C in the phloem was incorporated into amino acids, as compared to $16.6\pm1.9\%$ for control plants.

Discussion

This study documents the accumulation of N-containing compounds at eight time points over a 1 month exposure to salinity. The addition of NaCl to the growing medium of *Coleus* resulted in a number of stress responses including reduced growth, decreased protein content and the accumulation of a number of low molecular weight nitrogen-containing compounds. The imposed stress did not result in the excision of the sink areas in the leaves, as would be expected if the plants were severely limited for carbon. The accumulation of amino acids peaked between

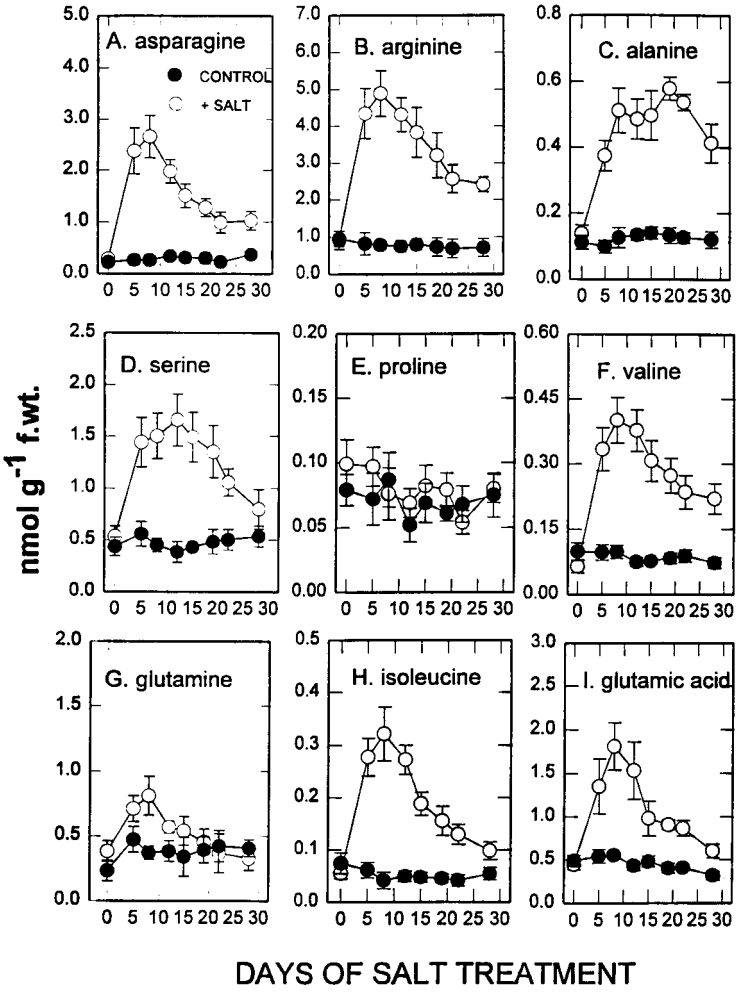

Fig. 4. Levels of specific amino acids in the source tissue of control and salinity-stressed *Coleus* plants ($n=9$, $\pm$se).

5–10 d after the salt treatment began, which corresponds to the lowest photosynthetic rates of the tissues, which decreased 4-fold during the first 10 d of exposure to salinity (data not shown). In this study, it was observed that the stress responses of the sink and the source tissues of *Coleus* leaves were quite similar, as both demonstrated accumulation of several similar nitrogen-containing compounds. This suggests that the imposed stress did not negatively impact the transport of amino acids to the sink tissue. Analysis of the phloem exudate of *Coleus* demonstrated that there was an increase in the amount of nitrogen containing compounds translocated through the phloem during salinity stress, but the mechanisms underlying the translocation of specific amino acids was not affected.

Many environmental stresses, including salinity, affect nitrogen absorption by the roots and its assimilation in the plant. In *Coleus*, a reduction in the total nitrogen content was observed in both the photosynthetic tissue and the white non-photosynthetic tissue. Previous research on the effects of stress on the total nitrogen content in tissues report variable results, which may be due to differences in tissue type and salinity levels studied. However, a reduction in nitrogen content is a common observation (Dubay and Pessarakli, 1995), which is usually explained by a reduction in nitrogen availability, as most glycophytes exhibit reduced nitrogen uptake and reduced nitrate reductase activity in the presence of salinity (Aslam *et al.*, 1984; Rao and Gnanam, 1990).

Although a reduction in the total nitrogen content was observed in *Coleus* during the exposure to salinity, a number of nitrogen-containing compounds were found to accumulate in both the sink and the source tissue, in particular the amino acids arginine, asparagine and serine. The rapid accumulation of amino acids during salinity stress suggest that these compounds may be acting as sinks for excess N in relation to the decreased growth occurring during the imposed stress. Also, researchers have suggested several roles for the accumulation of nitrogen containing compounds during stress, including osmotic adjustment, and serving as available sources of carbon and nitrogen (Dubay and Pessarakli, 1995;

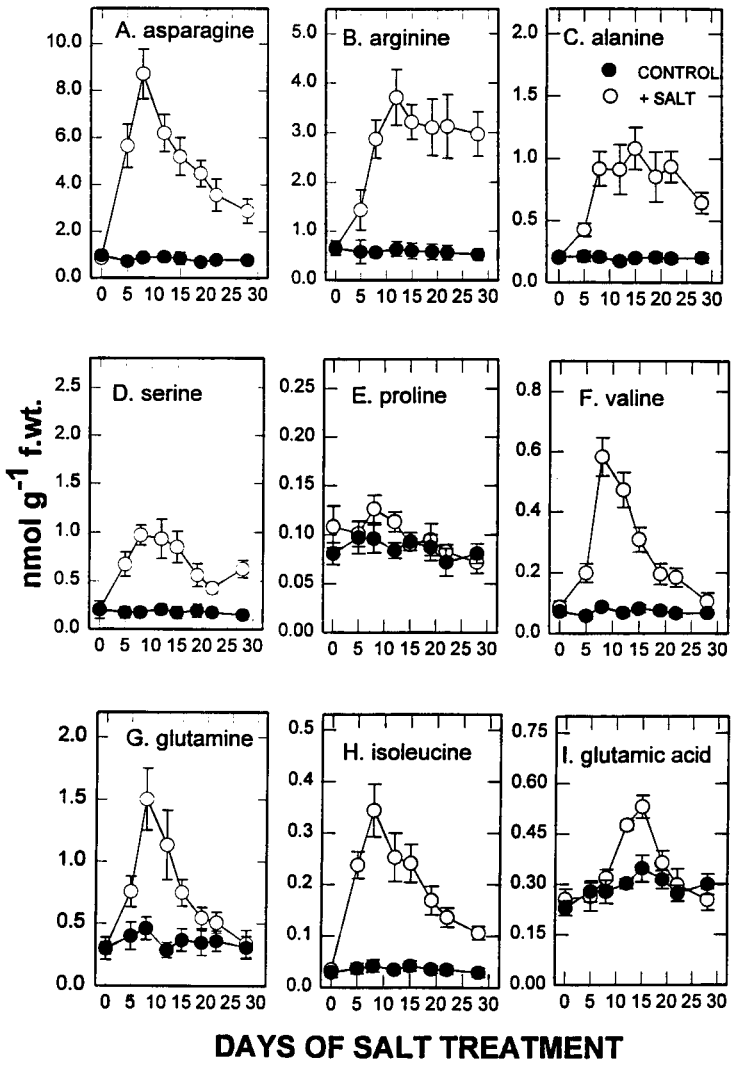

Fig. 5. Levels of specific amino acids in the sink tissue of control and salinity-stressed *Coleus* plants ($n=9$, $\pm$se).

Table 1. *Concentration of amino acids other than those presented in Figs 4 and 5 (nmol g^{-1} FW) 12 d after the salinization process began* (n = *9*, $\pm$*s.e.*)

Amino acid	Source tissue		Sink tissue	
	+Salt	Control	+Salt	Control
Aspartic acid	0.21	0.23	0.31	0.23
Hydroxyproline	0	0	0	0
Glycine	0.23	0.19	0.14	0.09
Homoserine	0	0	0	0
Citrulline	0.01	0.02	0.01	0.02
Histidine	0.10	0.05	0.04	0.02
Tyrosine	0.42	0.21	0.23	0.25
Methionine	0	0	0	0
Cystenine	0	0	0	0
Leucine	0.29	0.15	0.15	0.10
Phenylalanine	0.15	0.08	0.15	0.06
Lysine	0.18	0.07	0.17	0.09
Tryptophan/ Ornithine	0.11	0.06	0.07	0.04

Hanson and Hitz, 1982). The complexity of the roles of these compounds in plant metabolism suggests a diversity of functions in stress responses, dependent on the species, tissue, and environmental stress.

Arginine exhibited the largest proportional increase in the source tissues of *Coleus* leaves during exposure to salinity, followed by asparagine. A possible explanation for arginine accumulation during phosphorus deficiency has arisen from the work by Rabe and Lovatt (1984), that may be applicable to other stresses. They reported that arginine accumulation during stress was the result of de novo synthesis which serves to detoxify ammonia accumulation under periods of reduced growth (Rabe and Lovatt, 1984). Subsequent studies demonstrated increased ammonia levels during water stress (Hake and Lovatt, 1987) and low temperatures (Zheng and Lovatt, 1987), and it was hypothesized that reduced growth results in ammonia accumulation early in exposure to stress (Rabe and Lovatt, 1986). The arginine accumula-

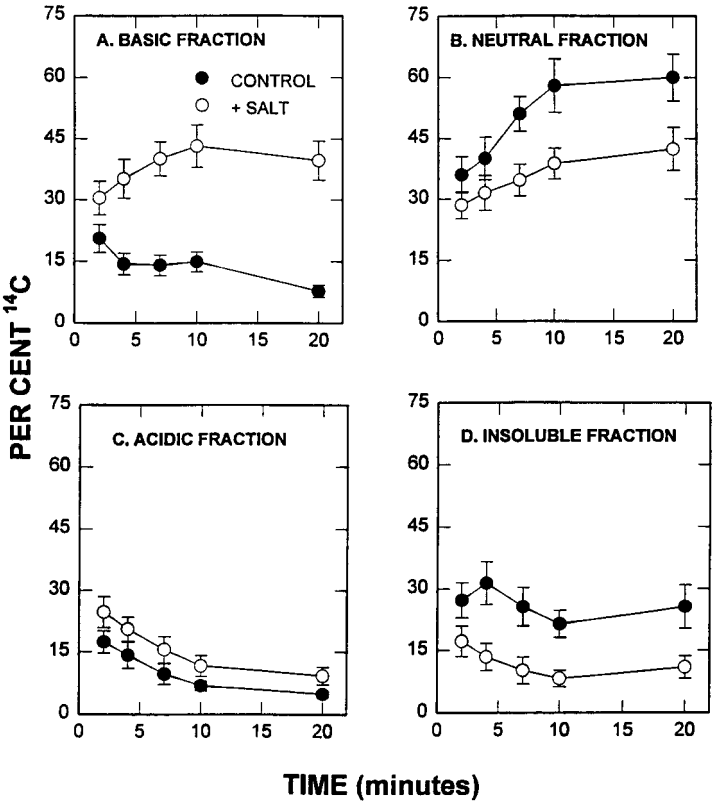

Fig. 6. Partitioning of photosynthetically fixed ^{14}C in control and salinity-stressed leaves, expressed as a percentage of the total label recovered (±se). Time represents the minutes after the plant was first exposed to ambient air. BASIC (amino acid fraction), NEUTRAL (sugars and sugar alcohols), ACIDIC (sugar phosphates and organic acids), and INSOLUBLE (starch).

tion observed in *Coleus* leaves may also be involved in ammonia detoxification as shoot growth was found to be reduced by the imposed stress.

In source tissues, the increase in asparagine that was observed was lower than that of arginine during salinity stress. Asparagine has been shown to accumulate in response to stress in a number of species (see the review by Rabe, 1990). The most commonly accumulating nitrogen containing compounds (including arginine and asparagine) contain at least two amino groups, suggesting that these compounds may be preferentially synthesized in response to stress and serve as important nitrogen sources for metabolic pathways.

In the sink tissues of *Coleus*, the major accumulating amino acids under salinity stress were asparagine, arginine and glutamine. Although arginine accumulates in considerable amounts in *Coleus* sink tissues and other storage tissues, it does not appear to be extensively translocated (see review by Atkins and Beevers, 1990). The increased concentration of asparagine and glutamine in the sink tissues may reflect the mobility of these important protein amino acids in the phloem. Asparagine has long been recognized as an important compound in nitrogen transport, but information concerning the mechanism of transport is lacking. Changes in amino acid composition of phloem sap has been demonstrated in alfalfa in response to water deficit, including increased transport of proline, valine, isoleucine, leucine, glutamic acid, aspartic acid and threonine (Girousse *et al.*, 1996).

No significant change was observed in proline levels in either the sink or the source tissues. Although this is the most commonly reported nitrogen-containing compound to accumulate in response to salinity, other studies have demonstrated no significant change in proline concentrations in response to stress (Ranieri *et al.*, 1989). This study, as well as others (see review by Rabe, 1990) demonstrates the variation in compatible solute accumulation among different species of plants.

Several explanations for the accumulation of free amino acids and amides under stress have been suggested. These include stimulated synthesis, inhibited degradation of amino acids, impaired protein synthesis, and/or enhanced protein degradation (Ranieri *et al.*, 1989). From ^{14}C labelling experiments on *Coleus* during salinity stress, it can be deduced that at least some of the observed accumulation of amino acids and amides is due to *de novo* synthesis, as higher proportions of the label were rapidly incorporated into the basic fraction of the stressed plants as compared to the control plants. Further studies utilizing ^{15}N as a label will prove useful in elucidating

the role of amino acid synthesis in response to salinity stress.

The accumulation of nitrogen containing compounds in *Coleus* leaves peaked within the first 10 d of exposure to salinity, and then declined, but remained slightly elevated for the remainder of the study. This may be an adaptive response to allow the initial stabilization during stress, while long-term responses are occurring. The exception to this is the accumulation of arginine in the sink and source tissues of *Coleus*, which remained elevated during the 2 month exposure to salinity. This may reflect the role of arginine in ammonia detoxification as the growth rates of these plants remain reduced as long as the salinization treatment is imposed.

A number of transient changes in carbohydrate concentrations were also observed in *Coleus* during salinity stress, such as a decrease in glucose and an increase in raffinose concentrations. This data will be presented in a forthcoming publication. It is clear that the mechanisms of stress tolerance involve a number of metabolic pathways changes, cellular components, gene expression, and protein modification. Understanding the regulation of these processes will be essential to developing plants with higher tolerance to salinity, which necessitates the further understanding of the complex molecular responses and biochemical pathways of plants exposed to environmental stresses.

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