Effect of Salinity on Arginine Biosynthesis in Leaves of *Phaseolus vulgaris* L. and *P. acutifolius* A. Gray

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ABSTRACT

The objective of this study was to quantify the effect of salinity on the capacity of leaves of *Phaseolus vulgaris L. and P. acutifolius* A. Gray to synthesize arginine de novo and to determine the relative ability of the two species to tolerate salinity during the vegetative growth phase. Research into the effect of salinity on *P. acutifolius* relative to *P. vulgaris* is limited. Plants were hydroponically cultured until age 5 d and then salinized for 16 d with 40 m*M* NaCl plus 20 m*M* $CaCl_2$. Salination caused greater reduction ($P < 0.05$) in *P. acutifolius* shoot dry weight (35 and 43% for two lines) than *P. vulgaris* [<25% for Linden and Ferry Morse 53 (FM 53) cultivars]. Root growth was unaffected in both species. Salt reduced the capacity of leaves of *P. acutifolius* to incorporate $NaH^{14}CO_3$ but not [^{14}C]citrulline into arginine plus urea ($\Sigma A + U$) per gram fresh weight tissue (80% for both lines, $P < 0.05$). Arginine de novo synthesis in *P. vulgaris* was unaffected. Incorporation of $NaH^{14}CO_3$ into $\Sigma A + U$ was increased by added ornithine but remained lower ($P < 0.05$) in salinized *P. acutifolius* leaves than in control leaves, suggesting that ornithine availability was not the single factor limiting arginine de novo synthesis during salination. Salination reduced activities of glutamine synthetase and carbamylphosphate synthetase per milligram protein in *P. acutifolius* (both lines, 50%) and FM 53 (20–40%) but not Linden. Thus, enzyme reaction(s) leading to the formation of citrulline in arginine de novo synthesis in leaves of *P. acutifolius* are more affected by salt than those in *P. vulgaris*.

DURING STRESS, arginine may occupy a pivotal role in the synthesis of both proline and putrescine. In glycophytes, L-[^{14}C]arginine and L-[^{14}C]ornithine yield L-[^{14}C]proline via the pathway arginine → ornithine → 2-oxo-5-aminovaleric acid → pyrroline-2-carboxylic acid → proline or the pathway arginine → ornithine → glutamic semialdehyde → pyrroline-5-carboxylic acid → proline (Thompson, 1980). Water-deficit stress enhanced proline labeling (Boggess and Stewart, 1976; Stewart and Boggess, 1977; Wrench et al., 1977a, b). Calculations based on the specific activity of the precursors, the pool sizes of free arginine and free glutamate, and the transfer rates of radioactivity to proline indicated that arginine may be more important than glutamate as the precursor for the synthesis of proline in bean (*P. vulgaris*) leaves during water-deficit stress (Stewart and Boggess, 1977) and in Jerusalem artichoke (*Helianthus tuberosus* L.) tubers during osmotic stress (Wrench et al., 1977a). In addition, putrescine accumulating in cereals subjected to water-deficit or osmotic stress appears to be synthesized predominantly from arginine via the arginine decarboxylase pathway. Arginine decarboxylase activity, but not ornithine decarboxylase, increased during water-deficit stress induced by various osmotica (Galston, 1989) or by withholding water (Flores and Galston, 1984b). Experiments employing α-difluoromethyl-ornithine, an analogous specific inhibitor of ornithine decarboxylase, did not inhibit the stress-induced accumulation of putrescine (Flores and Galston, 1984a), whereas those employing α-difluoromethylarginine, the enzyme-activated irreversible inhibitor of arginine decarboxylase, did (Flores and Galston, 1984b).

Despite these data, changes in the capacity of plant cells to synthesize arginine de novo during abiotic stresses, other than phosphorus deficiency (Rabe and Lovatt, 1984, 1986a, b), have not been reported previously. In this study, we assessed the effect of salinity on the activity of the pathway for the de novo biosynthesis of arginine by measuring the incorporation of $NaH^{14}CO_3$ into the total combined pool of arginine plus urea ($\Sigma A+U$) in intact cells of young, fully expanded (YFE) leaves of two cultivars of *P. vulgaris* and two lines of *P. acutifolius* hydroponically cultured for 16 d with and without 40 m*M* NaCl plus 20 m*M* $CaCl_2$. In an effort to identify the salt-sensitive site(s) in de novo arginine synthesis, we quantified the effect of the salt treatment on the incorporation of [^{14}C]citrulline into $\Sigma A+U$ by intact cells and on the activities of glutamine synthetase (GS) and carbamylphosphate synthetase (CPSase) in cell-free extracts of YFE leaves from salinized and control plants of both species.

We selected these species because *P. acutifolius* is successfully cultivated where saline soils, high temperatures, and drought are common (Nabhan and Felger, 1978); furthermore, *P. acutifolius* is of interest because through interspecific hybrids it might serve as a source of stress-tolerance traits for *P. vulgaris* (Thomas et al., 1983; Haghigi and Ascher, 1988). Research to determine whether *P. acutifolius* tolerates salinity stress has been limited (Marcarian, 1981; Goertz and Coons, 1989). Thus, the present study examined the relative ability of *P. acutifolius* and *P. vulgaris* to tolerate salinity during the vegetative growth phase.

MATERIALS AND METHODS

Chemicals

All radiolabeled chemicals were purchased from ICN Pharmaceuticals, Inc., Costa Mesa, CA. Liquiscint (liquid scintillation cocktail) was purchased from National Diagnostics, Sommerville, NJ. Mineral salts for Shive's nutrient solution (Shive

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Abbreviations: $\Sigma A+U$, total combined pool of arginine plus urea; YFE, young fully expanded; ΣUMP, sum of the uridine nucleotides converted to UMP by acid hydrolysis at 100°C; ψs, osmotic potential; CPSase, carbamylphosphate synthetase; CP, carbamylphosphate; GS, glutamine synthetase; OCTase, ornithine carbamyltransferase; ACTase, aspartate carbamyltransferase.

and Robbins, 1938) were of analytical reagent quality from Fisher Scientific, Pittsburgh, PA. All other chemicals were purchased from Sigma Chemical Company, St. Louis.

Plant Material

Seeds of *P. vulgaris*, cvs Linden and Ferry Morse 53 (FM 53) (courtesy of California Certified Seed, University of California, Davis), and *P. acutifolius*, tepary bean lines PI-321638 and PI-319443 (provided by the University of California, Riverside), were used in this study. Linden is a determinate, large-seeded, red kidney bean (Type I) and represents the Andean gene pool, whereas FM 53 is an indeterminate, white, small-seeded, semi-vine (Type IIIA) and represents the MesoAmerican gene pool (Singh et al., 1991). *Phaseolus acutifolius* PI-321638 is a white landrace bought at a market in Newfields, AZ, and PI-319443 is a white landrace from Movas, Sonora, Mexico. Both are indeterminate, and both are MesoAmerican in origin. Seeds were imbibed in distilled H_2O overnight at room temperature, rinsed three times with distilled H_2O, and spread evenly between two sheets of paper towel in a plastic box. The covered box was placed in an incubator, and the seeds were allowed to germinate at 30°C in the dark. At the end of 2 d, seedlings were transferred to 4.5-L breadboxes and hydroponically cultured in aerated Shive's (Shive and Robbins, 1938) nutrient solution [5 *M* $Ca(NO_3)_2$, 2 m*M* $MgSO_4$, 2 m*M* K_2SO_4, 1 m*M* KH_2PO_4, 1 mg Fe L^{-1}, 1 mg Mn L^{-1}, 0.13 mg Cl L^{-1}, 0.1 mg Zn L^{-1}, 0.1 mg B L^{-1}, 0.01 mg Cu L^{-1}, 0.01 mg MoL^{-1}, and 0.01 mg Na L^{-1}] (pH 4.7) at 30°C under continuous light (500 µmoles m^{-2} s^{-1}). After 3 d, the plants, which were now 5 d old and had two true leaves, were transferred to the following treatments: (i) fresh Shive's nutrient solution; or (ii) Shive's nutrient solution amended with 40 m*M* NaCl plus 20 m*M* $CaCl_2$ (electrical conductivity = ≈9 dSm^{-1} at 30°C) (Shannon et al., 1984). To avoid shocking the plants, salt was added at the rate of one-third of the final concentration every other day. This reduced the ψs of the nutrient solution by approximately 0.1 MPa every other day. Water lost through evaporation and transpiration was replaced daily. Solutions were changed every 3 d. When solutions were changed, the salt concentration specified by the treatment date was supplied. The experimental unit consisted of 15 plants per treatment and three replications per experiment using a completely randomized design. Most experiments were repeated at least three times. All analyses were made on YFE trifoliate leaves located at the third and fourth node below the apex of the plants. Shoot and root dry weight were determined at the end of 16 d of treatment. Plants were dried at 60°C for 72 h.

Measurement of the Incorporation of Radiolabeled Precursors into the Combined Pool of Arginine Plus Urea or Total Uridine Nucleotides in Intact Cells

Samples consisting of 300 mg fresh weight of YFE leaf tissue (cut into 5- by 5-mm pieces) were routinely preincubated for 2 h at 30°C in 5 mL Shive's nutrient solution adjusted to pH 7.4. At the end of the preincubation period, the nutrient solution was decanted, and the leaf tissue was immediately transferred to fresh Shive's nutrient solution supplemented with 5 m*M* $NaH^{14}CO_3$, 1,650 dpm $nmol^{-1}$, or 4 m*M* [ureido-^{14}C]citrulline, 220 dpm $nmol^{-1}$ (both supplied at a saturating concentration and uniform specific radioactivity, respectively), and any other additives in a final volume of 5 mL, pH 7.4. Reaction mixtures were incubated for 3 h at 30°C in a water bath-shaker. Incubations were carried out in 25-mL Erlenmeyer flasks sealed with rubber stoppers that held a plastic center well (Kontes Glassware, Vineland, NJ) containing a filter paper wick above the medium in the flask. At the end of the incubation period, 0.5 mL of 6 *M* KOH was injected into the plastic center well, and the reaction was terminated by injecting 1 mL of 1.5 *M* $HClO_4$ into the main chamber of the flask. The unreacted radioisotope was allowed to distill from the acidified incubation mixture into the KOH in the center well for an additional 10 min incubation at 30°C. The contents of the main chamber were homogenized with a Polytron homogenizer (PCU-2, Brinkman Instruments, Westbury, NY) at speed 6. The Polytron probe was rinsed with 2 mL of 0.25 *M* $HClO_4$ which was added to the homogenate. The insoluble material was removed by centrifugation at 10 000 *g* for 10 min at 0°C.

To quantify the incorporation of ^{14}C-precursors into $\Sigma A+U$ and into ΣUMP, an aliquot of 1.5 mL was removed from the acid-soluble supernatant fraction and neutralized to pH 7.5 with KOH. From the neutralized fraction, a 1.0-mL aliquot was removed and made 50 m*M* with $Ba(OH)_2$ to precipitate any residual $^{14}CO_3^{-2}$, which was removed by centrifugation prior to quantitation of [^{14}C]arginine and [^{14}C]urea. The amounts of [guanido-^{14}C] arginine and [^{14}C]urea synthesized by the leaf tissue were determined with commercial arginase and urease, as described previously by Lovatt and Cheng (1984). The remainder of the acid-soluble fraction was boiled for 1 h at 100°C to convert the uridine nucleotides to UMP. [^{14}C]UMP was isolated from the neutralized acid-soluble fraction by cocrystallization with carrier disodium UMP (Lovatt et al. 1979).

For both assays, samples were brought to a final volume of 4 mL, diluted with 13 mL of Liquiscint, and the content of radioisotope measured with a Beckman LS 9000 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, CA). Quenching was determined by an automatic external standardization system of quench analysis. A standard quench curve was determined by means of a series of samples containing a known quantity of ^{14}C radioactivity and increasing amounts of chemical quencher.

Leaf Protein Content

YFE leaves (1-g fresh weight) were homogenized in 10 mL of Shive's nutrient solution, pH 7.4. The protein content of the supernatant fraction was determined according to Bradford (1976) with BSA as the standard. The relationship between absorbance and protein content was linear for protein concentrations from 0.2 to 1.0 mg mL^{-1}. Samples were diluted to give values within this range.

Measurement of Enzymatic Activities in Cell-Free Extracts

Enzymatic activities were measured in cell-free extracts of YFE leaves homogenized in a Duall all-glass conical tissue grinder (Kontes Glassware, Vineland, NJ) in the specified extraction medium.

Glutamine Synthetase. GS activity was assayed by the formation of γ-glutamyl hydroxamate according to the method of McCormack et al. (1982). Addition of 0.5 mL of cell-free homogenate (20%) prepared in 0.2 *M* Tris-maleic buffer (pH 7.2) initiated the reaction that proceeded in a 1-mL reaction mixture of the following composition: 50 m*M* Tris-maleic buffer (pH 7.2), 10 m*M* $MgCl_2$, 10 m*M* ATP (pH 7.0), 40 m*M* L-glutamate, and 10 m*M* hydroxylamine. Reactions were incubated for 60 min at 30°C and terminated by the addition of 0.3 mL of a solution containing equal volumes of 150 g L^{-1} ferric chloride in 0.2 *M* HCl, 240 g L^{-1} trichloroacetic acid (TCA), and 6 *M* HCl. Reaction mixtures were centrifuged at 10 000 *g* for 10 min, and the OD_{540nm} of the supernatant

fractions measured. An OD_{540nm} of 0.1 corresponded to 0.225 µmol γ-glutamyl hydroxamate.

Carbamylphosphate Synthetase. Activity of CPSase was assessed by three separate methods: (i) by trapping the carbamylphosphate (CP) synthesized, or the cyanate released from the breakdown of CP, as hydroxyurea by reaction with hydroxylamine as described by Levine and Kretchmer (1971); (ii) in the presence of excess ornithine and commercial ornithine carbamyltransferase (OCTase) to convert all the CP synthesized by the plant enzyme to citrulline, which was isolated as described by Lovatt et al. (1979); and (iii) in the presence of excess L-aspartate and commercial aspartate carbamyltransferase (ACTase) to convert all the CP synthesized by the plant enzyme to carbamylaspartate. The following conditions for the extraction and assay of the enzyme are based on those of Ong and Jackson (1972) and O'Neal and Naylor (1976). A homogenate of 1 g of YFE leaves was prepared in 5 mL 50 m*M* Tris-glycine buffer (pH 8.4) containing 25 m*M* KCl, 25 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 300 mL L^{-1} dimethyl sulfoxide, and 200 mL L^{-1} glycerol. The homogenate was centrifuged at 10 000 *g* at 0°C for 20 min, and the supernatant liquid was used as the source of the enzyme.

Activity of CPSase was measured in a 1-mL reaction mixture of the following composition: 10 m*M* Tris-HCl (pH 8.0), 15 m*M* $MgCl_2$, 10 m*M* L-glutamine, 10 m*M* ATP, 20 m*M* $NaH^{14}CO_3$ (1000 dpm $nmol^{-1}$), and 0.5 mL of enzyme preparation. The reaction was terminated at the end of a 30-min incubation at 30°C by the addition of hydroxylamine hydrochloride (pH 7.0) at a final concentration of 0.2 *M*. The vials were transferred to a boiling water bath for 10 min. After cooling, 0.2 mL of 500 g L^{-1} TCA was added to acidify the mixture, and 1 g of granulated dry ice was added to remove residual $^{14}CO_2$. The reaction mixture was diluted to 4 mL with distilled H_2O and 13 mL of Liquiscint scintillation cocktail was added. The activity of the radioisotope in each sample was determined by using a Beckman LS 9000 liquid scintillation spectrometer.

When CP synthesized by the plant enzyme was to be isolated as citrulline, the reaction mixture contained 10 m*M* L-ornithine and 1.5 units OCTase (Sigma) (1 unit catalyzes the formation of 1 µmol of citrulline min^{-1} at pH 8.5 at 37°C). After 30-min incubation at 30°C, the reaction was terminated by the addition of 3 mL of ethanol-isopropyl alcohol (1:1, v v^{-1}), and the precipitated protein was removed by centrifugation. The [^{14}C]citrulline synthesized from $NaH^{14}CO_3$ was isolated from the supernatant fluid by cocrystallization with carrier citrulline according to the procedure described by Lovatt et al. (1979).

When CP synthesized by the plant enzyme was to be isolated as carbamylaspartate, the reaction mixture contained 10 m*M* L-aspartate and 1.5 units ACTase (Sigma) (1 unit catalyzes the formation of 1 µmol of *N*-carbamyl-L-aspartate min^{-1} at pH 8.5 at 37°C). After 30-min incubation at 30°C, the reaction was terminated by the addition of 0.25 mL of 1 *M* HCl, and the acidified reaction mixture was baked to dryness over a boiling water bath. The residue, containing the acid-stable carbamylaspartate, was extracted with 4 mL of distilled H_2O and diluted with 13 mL of Liquiscint scintillation fluor (Lovatt et al., 1979). The content of acid-stable radioactivity was determined using a Beckman LS 9000 liquid scintillation spectrometer.

Statistical Analyses

Duncan's Multiple Range Test was used to analyze the effects of the salt treatment on shoot and root growth (dry weight) and leaf protein content. Student's *t*-test was used to determine the effect of the salt treatment on incorporation of radiolabeled precursors into $\Sigma A+U$ and into ΣUMP. Mean ± SE is reported for two replicate samples in two separate experiments assessing each enzyme activity in cell-free extracts. Unless otherwise stated, conclusions are based on situations in which the probability of the differences being due to chance is less than 5%.

RESULTS

Effect of Salination on Plant Growth

After 16 d of salt treatment, the *P. vulgaris* cultivars exhibited a decrease (<25%) in shoot growth (dry weight); root growth was unaffected (Table 1). The *P. acutifolius* lines exhibited a greater decrease in shoot dry weight (35 and 42% reduction for PI-321638 and PI-319443, respectively). Root growth of the two *P. acutifolius* lines was also unaffected by the salt treatment. Salt had no effect on the ratio of fresh weight to dry weight of *P. vulgaris* relative to *P. acutifolius*.

YFE leaves of all salt-treated plants exhibited symptoms of salinity stress. YFE leaves at the third and fourth nodes below the apex were smaller than the same leaves on the control plants. These leaves exhibited slight yellowing along the margin of the leaf blade. Mature leaves at lower nodes exhibited burning of the leaf margin and necrotic areas on the leaf blade at the end of 16 d of salination.

De Novo Arginine Biosynthesis in Intact Cells of Leaves of Control and Salinized Plants

The effect of salinity on the capacity of intact cells of leaves to synthesize arginine de novo was assessed by measuring the incorporation of $NaH^{14}CO_3$ into $\Sigma A+U$. The 16-d salination treatment had no significant effect on the capacity of leaves of either *P. vulgaris* cultivar to synthesize arginine de novo (Table 2). In contrast, the $NaCl:CaCl_2$ treatment decreased by 77 and 82% the incorporation of $NaH^{14}CO_3$ into $\Sigma A+U$ in intact cells of YFE leaves of the *P. acutifolius* lines PI-321638 and PI-319443, respectively ($P < 0.05$) (Table 2).

Changes in the amount of $NaH^{14}CO_3$ incorporated into $\Sigma A+U$ during salination were not due to changes in the protein content of YFE leaves from salt-treated plants. At the end of 16 d of salination, the protein content of YFE leaves from the two cultivars of *P. vulgaris* and the two lines of *P. acutifolius* was unchanged (milligrams protein per gram fresh weight) (data not shown).

Table 1. Effect of 16 d of salination with 40 m*M* NaCl plus 20 m*M* $CaCl_2$ on plant growth. Data are the means of three separate experiments.

Species	Genotype	±Salt	Shoot	Root	Plant
			mg dry weight $plant^{-1}$		
P. vulgaris	Linden	−	1270 A†	249 B	1519 A
		+	1012 B	211 ABC	1223 B
	FM 53	−	1004 B	286 A	1290 B
		+	770 C	234 AB	1004 D
P. acutifolius	PI-321638	−	907 BC	185 BC	1092 CD
		+	586 D	136 C	735 E
	PI-319443	−	561 D	141 C	735 E
		+	326 E	167 BC	493 F

† Values in the same vertical column followed by different letters are statistically different at $P < 0.05$ by Duncan's Multiple Range Test.

Table 2. Effect of 16 d of salination with 40 m*M* NaCl plus 20 m*M* $CaCl_2$ on the incorporation of precursors into the combined pool of arginine plus urea in intact cells of YFE leaves of *P. vulgaris* and *P. acutifolius*. Data are the means of three separate experiments.

Species	Genotype	[^{14}C]Precursor	±Orn 10 m*M*	nmol precursor incorporated into ΣA + U g^{-1} fresh wt leaf tissue during a 3-h incubation period – Salt	+ Salt
P. vulgaris	Linden	$NaHCO_3$	–	1.2 A†	1.6 A
		$NaHCO_3$	+	17.9 A	15.0 A
		Citrulline	–	60.5 A	64.9 A
	FM 53	$NaHCO_3$	–	1.6 A	0.7 A
		$NaHCO_3$	+	25.7 A	10.5 B
		Citrulline	–	63.0 A	115.0 B
P. acutifolius	PI321638	$NaHCO_3$	–	18.3 A	4.2 B
		$NaHCO_3$	+	59.6 A	28.1 B
		Citrulline	–	60.7 A	60.0 A
	PI-319443	$NaHCO_3$	–	5.6 A	1.0 B
		$NaHCO_3$	+	65.8 A	18.1 B
		Citrulline	–	289.0 A	275.0 A

† Values within the same horizontal row followed by different letters are statistically different at $P < 0.05$ by Student's *t*-test.

The activity of the arginine de novo pathway was examined after six additional days of salination (22 d total) to further test the potential sensitivity of *P. vulgaris* cultivar FM 53 to salinity and to further contrast the effect of salination on arginine metabolism in *P. vulgaris* and *P. acutifolius*. In two separate experiments, the incorporation of $NaH^{14}CO_3$ into ΣA+U in leaves from either Linden or FM 53 salinized for the additional 6 d was not significantly different from that of the control plants but was reduced 83 and 86% for the *P. acutifolius* lines PI-321638 and PI-319443, respectively (data not shown).

Site(s) of Salt Sensitivity in Arginine De Novo Biosynthesis in *P. acutifolius*

In an attempt to localize the salt-sensitive rate-limiting steps in the de novo synthesis of arginine, the effect of salination on the incorporation of intermediates of the pathway was quantified. Whereas the salt treatment decreased the incorporation of $NaH^{14}CO_3$ into ΣA+U in *P. acutifolius*, the incorporation of [^{14}C]citrulline was not reduced by salination. Ornithine added to both the preincubation and incubation mixtures at a final concentration of 10 m*M* dramatically increased the amount of $NaH^{14}CO_3$ incorporated into ΣA+U in YFE leaves of both species. However, for both *P. acutifolius* lines and *P. vulgaris* FM 53, the incorporation of $NaH^{14}CO_3$ in the presence of ornithine during salination did not increase to a level equal to that of the control plants treated with ornithine (Table 2). In contrast, added ornithine did increase the incorporation of $NaH^{14}CO_3$ in YFE leaves from the salt-treated *P. vulgaris* cultivar Linden to an amount equal to that observed for the ornithine-treated control plants (Table 2). These results rule out the possibility that a lack of available ornithine is the cause of the reduction in de novo arginine synthesis during salt stress. The results indicate that the second substrate of OCTase (CP) may be limiting or that OCTase is salt sensitive in *P. acutifolius* and *P. vulgaris* FM 53. CP was not used as a substrate to test the ability of OCTase to utilize it during salt stress, because it is spontaneously and enzymatically hydrolyzed (Herzfeld and Knox, 1971; Grisolia and Hood, 1974), making the results equivocal.

Table 3. Effect of 16 d of salination with 40 m*M* NaCl plus 20 m*M* $CaCl_2$ on glutamine synthetase activity in cell-free extracts of YFE leaves of *P. vulgaris* and *P. acutifolius*.

Species	Genotype	Experiment I – Salt	Experiment I + Salt	Experiment II – Salt	Experiment II + Salt
		nmol γ-glutamylhydroxamate synthesized mg^{-1} protein during a 60-min incubation			
P. vulgaris	Linden	34 ± 0.1†	43 ± 4.8	44 ± 1.4	21 ± 2.2
	FM 53	43 ± 0.5	27 ± 0.7	68 ± 0.4	46 ± 4.5
P. acutifolius	PI-321638	51 ± 2.5	29 ± 1.7	25 ± 6.8	18 ± 7.3
	PI-319443	51 ± 1.6	24 ± 3.4	77 ± 7.5	20 ± 0.7

† Data are the means of two replicate samples ± SE.

Our method for assessing the incorporation of radiolabeled precursors into ΣA+U does not employ inhibitors to block the subsequent metabolism of arginine or urea. Thus, it actually underestimates the activity of the arginine de novo biosynthetic pathway. The fact that [^{14}C]citrulline was incorporated into ΣA+U to the same degree in YFE leaves from control and NaCl:$CaCl_2$-treated plants (Table 2) rules out the possibility that the reduced incorporation of $NaH^{14}CO_3$ into ΣA+U observed in the YFE leaves from the salt-treated plants was due to more rapid turnover of arginine and urea during salination.

At present, only one CPSase (II, dependent on L-glutamine) is known to exist in plants (Ong and Jackson, 1972; O'Neal and Naylor, 1976; Thompson, 1980; Jacques and Sung, 1981). Thus, this enzyme would provide CP not only to the arginine de novo biosynthetic pathway but also to the orotic acid pathway for the de novo biosynthesis of pyrimidine nucleotides. If CPSase activity is decreased and CP availability is reduced, the incorporation of $NaH^{14}CO_3$ into UMP might also be lower in *P. acutifolius* lines during salination. The incorporation of $NaH^{14}CO_3$ into ΣUMP in YFE leaves from salt-treated *P. acutifolius* line PI-319443 was reduced 49%; for line PI-321638, the 31% decrease was not significant (data not shown).

Effect of Salination on GS and CPSase Activity in Cell-Free Extracts

To determine further whether CP concentration might be limiting arginine de novo synthesis, we quantified the effect of salination on the activity of GS, which supplies the substrate L-glutamine to CPSase and on the activity of CPSase in cell-free extracts prepared from YFE leaves harvested from the *P. vulgaris* and *P. acutifolius* grown with and without salt for 16 d. In two separate experiments, the salt treatment consistently reduced GS activity in *P. acutifolius* and *P. vulgaris* FM 53 (Table 3). The effect of GS activity in Linden was variable.

CPSase activity in cell-free extracts of leaves from salinized *P. acutifolius* plants was reduced more than

Table 4. Effect of 16 d of salination with 40 mM NaCl plus 20 mM $CaCl_2$ on carbamylphosphate synthetase activity in cell-free extracts of YFE leaves of *P. vulgaris* and *P. acutifolius*.

Species	Genotype	Experiment I		Experiment II	
		− Salt	+ Salt	− Salt	+ Salt
		nmol [^{14}C]hydroxyurea formed mg^{-1} protein during a 30-min incubation			
P. vulgaris	Linden	98 ± 0.1†	117 ± 8.1	125 ± 4.1	105 ± 1.6
	FM 53	114 ± 0.7	94 ± 13.0	138 ± 0.4	84 ± 1.1
P. acutifolius	PI-321638	–	–	133 ± 4.6	62 ± 2.2
	PI-319443	89 ± 0.5	51 ± 1.5	75 ± 2.3	37 ± 0.5

† Data are the means of two replicate samples ± SE. The activity of CPSase was assessed according to the method of Levine and Kretchmer (1971).

40% (Table 4). CPSase activity in salinized *P. vulgaris* plants was variable. The relative sensitivity of CPSase to salt, PI-321638 > PI-319443 > FM 53 > Linden was confirmed by the results obtained using the two additional assay methods described in the Materials and Methods.

The results indicate that reduced activity of the arginine de novo pathway in intact cells of leaves of both *P. acutifolius* lines during salination might be due either to a lack of CP resulting from reduced GS activity and insufficient supply of L-glutamine or to an effect on CPSase independent of L-glutamine availability, or both. GS and CPSase activities in cell-free extracts of leaves of Linden and FM 53 were less affected by salination, consistent with there being no statistically significant reduction in the incorporation of $NaH^{14}CO_3$ into $\Sigma A+U$ in intact cells of leaves after 16 or 22 d of salination.

DISCUSSION

The fact that the low salination treatment employed in this study had a greater negative effect on the growth and metabolism of *P. acutifolius* than *P. vulgaris* under conditions that prevented the roots from "avoiding" salinity underscores the need for further research to determine whether *P. acutifolius* really tolerates salinity under field conditions or simply avoids it. *Phaseolus acutifolius* has a fast-growing, deep root system (Thomas et al., 1983), which may enable it to "avoid" salinity in the field. Results of an early study (Goertz and Coons, 1989) indicate that the ability of *P. acutifolius* to outyield *P. vulgaris* under field conditions with saline soil and irrigation water and high summer temperatures (Marcarian, 1981) may actually be due to the negative effect of high temperature, which was demonstrated to be more deleterious to the growth of *P. vulgaris* than salinity. Lin and Markhart (1996) have recently confirmed that *P. acutifolius* is more heat tolerant than *P. vulgaris*. The small reduction in growth we observed in the *P. vulgaris* cultivars in response to salination with 40 mM NaCl plus 20 mM $CaCl_2$ is consistent with the conclusion of Neuman et al. (1988) that although mild salination (100 mM NaCl) caused an initial reduction in the rate of leaf growth in *P. vulgaris*, long-term salination (10 d) resulted in an apparent adjustment, which served to counteract reductions in turgor and to sustain leaf growth. We added $CaCl_2$ to prevent sodium-induced calcium deficiency (Maas and Grieve, 1986). In the specific case of *Phaseolus*, LaHaye and Epstein (1969, 1971) demonstrated that high calcium concentrations (3–10 mM) mitigated the adverse effects of 50 mM NaCl on growth by inhibiting sodium uptake.

Research identifying specific enzymatic steps that are sensitive to salinity in vivo has been scarce. Thus, the results of this study, which provide evidence that one or more enzymatic steps involved in the synthesis of arginine de novo in leaves of *P. acutifolius* are affected by salination, is of consequence. It is worthy to note that the affected enzymes are related to the synthesis and subsequent utilization of CP.

The salt treatment had a greater impact on the incorporation of $NaH^{14}CO_3$ into $\Sigma A+U$ than on the incorporation of $NaH^{14}CO_3$ into ΣUMP in *P. acutifolius* leaves. This could be due to OCTase being more sensitive to salt than ACTase or limited to a greater degree than ACTase by the reduced availability of CP. Even when ornithine was added, the incorporation of $NaH^{14}CO_3$ into $\Sigma A+U$ remained lower in *P. acutifolius* grown under saline conditions than the controls, suggesting that OCTase might be salt sensitive or limited by a lack of CP, or both. Under conditions where CP synthesis is low, the CP available to the cell is utilized by the orotic acid pathway for the synthesis of pyrimidine nucleotides due to the greater affinity of ACTase for the existing CP (O'Neal and Naylor, 1976; Jacques and Sung, 1981). The greater affinity of ACTase relative to OCTase for CP is well documented. For example, in soybean, the K_m for CP is three-fold greater for OCTase than ACTase (Acaster et al., 1989). A recent study in which the K_m's of OCTase and ACTase for CP were determined for a number of vascular plant species under identical assay conditions for each enzyme, respectively, revealed that in six of the eight species analyzed, OCTase had a higher K_m for CP than ACTase (Acaster et al., 1989). Thus, the reduced incorporation of $NaH^{14}CO_3$ into ΣUMP in leaves of *P. acutifolius* PI-319443, the more sensitive line, might be due to lack of CP, although an effect of salt on ACTase cannot be ruled out.

The greater sensitivity of the arginine de novo pathway in *P. acutifolius* to the effects of salinity may be a consequence of the fact that the basal rate for the de novo biosynthesis of arginine plus urea was significantly greater in *P. acutifolius* than in *P. vulgaris*. The values for *P. vulgaris* are at the low end of the range of rates that we have determined for other plant species using this same method, whereas that of *P. acutifolius* falls mid-range. Basal rates for the arginine de novo pathway range from a low of 0.6 nmol $NaH^{14}CO_3$ incorporated into $\Sigma A+U$ per g fresh weight YFE leaf tissue during the 3-h incubation period for *Persea americana* Miller (Lovatt and Cheng, 1990), to 5 nmol for *Citrus sinensis* (L.) Osbeck (Lovatt and Cheng, 1990), and up to 35 nmol for *Citrus limon* (L.) Burman f. and *Cucurbita pepo* L. (Rabe and Lovatt, 1984).

Our understanding of the mechanisms of salt tolerance and sensitivity in crop plants is still limited (Serrano and Gaxiola, 1994; Colmer et al., 1995). It is well known that proline and putrescine both accumulate in response to salt stress in a number of plants species (Strogonov

et al., 1972; Priebe and Jager, 1978; Tal et al., 1979; Stewart and Larher, 1980; Shevyakova, 1981; Shevyakova et al., 1981; Galston, 1989). Despite the potential pivotal role of arginine in the synthesis of both proline and putrescine during stress, to our knowledge, the present study is the first to investigate the impact of salinity on the arginine de novo biosynthetic pathway.

The results of our study demonstrated that the pathway for the de novo biosynthesis of arginine in young leaves is affected by salt in *P. acutifolius*. Furthermore, the results of our study provide evidence identifying GS and CPSase as the enzymatic steps leading to the synthesis of arginine that are affected by salt. The greater sensitivity of these enzymatic activities to salinity in *P. acutifolius* relative to *P. vulgaris* and the fact that the *P. vulgaris* cultivar FM 53 was more affected by salt than Linden probably reflects the genetic origins of the plants. Linden represents the Andean gene pool, whereas FM 53 represents the MesoAmerican gene pool from which the tepary bean originated. Given that reduced CPSase activity could negatively impact the synthesis of both arginine and pyrimidine nucleotides, and might also affect the synthesis of proline and polyamines, it would seem that arginine biosynthesis deserves further examination in future work seeking to identify physiological or biochemical markers related to salt tolerance and sensitivity.

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Transpiration Efficiency, Specific Leaf Weight, and Mineral Concentration in Peanut and Pearl Millet

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ABSTRACT

Transpiration efficiency (TE) is the weight of dry matter produced per unit of transpiration. If mineral nutrients are taken up in proportion to transpiration, then the concentration of minerals in dry matter may be predictive of TE. It has recently been reported that specific leaf weight (SLW) is also correlated with TE. Since both mineral concentration and SLW are easily and inexpensively measured, they may be valuable predictors of TE. Five experiments with 7 to 19 genotypes of peanut (*Arachis hypogaea* L.; C_3 species) and four experiments with 9 or 10 genotypes of pearl millet [*Pennisetum glaucum* (L.) R. Br.; C_4 species] were conducted to assess the predictive value of mineral concentration and SLW for TE. In peanut experiments, samples were also analyzed for C isotope discrimination (Δ). Plants were grown in pots in the greenhouse in sand, soil, and solution culture. Specific leaf weight, Δ, ash, and mineral element concentrations were determined at the end of the experiments. There was a strong negative correlation between Δ and TE in the four peanut experiments in which Δ was determined. Leaf ash concentration was negatively correlated with TE in four out of six comparisons for each of the species (r = −0.55 to −0.88). In peanut, Ca and Mg were negatively correlated with TE. No other elements were consistently related to TE in either peanut or pearl millet. The SLW was positively correlated with TE. Thus, both leaf ash and SLW were predictive of TE, but further research is needed to determine the physiological bases for the relationships and their general applicability.

TRANSPIRATION EFFICIENCY is an important characteristic in environments deficient in water. Variation among cultivars in TE has been demonstrated for several crop species (Hubick et al., 1986; Hubick and Farquhar, 1989; Farquhar and Richards, 1984; Johnson et al., 1990) and the identification of simple traits that predict improved TE would be useful in plant breeding. These studies have shown that differences in TE of C_3 genotypes can be predicted from Δ because both the ratio of CO_2 uptake/transpiration and Δ are partially dependent on the ratio of intercellular to ambient CO_2 concentration (Farquhar et al., 1982). Values of Δ are less likely to be related to TE in C_4 genotypes because Δ is less than in C_3 plants and theory predicts Δ to be either negatively or positively related to the ratio of intercellular to ambient CO_2 concentration, depending on the leakiness of bundle sheath cells to CO_2 (Farquhar, 1983).

Leaf mineral concentration on a dry matter basis is significantly correlated with transpiration ratio (1/TE; Masle et al., 1992) but the theoretical basis for such a relationship is not clear. If mineral nutrient uptake was proportional to transpiration, then a relationship of mineral concentration with TE would be expected as follows (Masle et al., 1992):

$$m = Z/M = Z/E \cdot E/M = xR \quad [1]$$

where m = mineral concentration in the dry matter, Z = mineral mass, M = dry mass, E = water transpired, and R = transpiration ratio (1/TE). If no recycling of minerals from tissue to the xylem stream occurred, then x would represent the average mineral concentration in the transpiration stream during the period represented by m and R, and if x were constant, the relationship of m to R would be linear. Mineral uptake is not a function of transpiration when atmospheric conditions are the cause of variation in transpiration (Masle et al., 1992) and thus x varies with environment. However, genotypic variation in x may be small enough when compared under the same environment to allow prediction of TE from m.

The TE of peanut genotypes has been correlated with specific leaf area (1/SLW) (Wright, 1993; Wright et al., 1994). The entire basis for such a relationship is not known, but it may be related to the fact that genotypes with high SLW often assimilate CO_2 more rapidly per unit of leaf area (Nelson, 1988). Differences in assimilation among genotypes are usually more closely associated with TE than are differences in water loss, thus genotypes

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Abbreviations: TE, transpiration efficiency; SLW, specific leaf weight; Δ, ^{13}C discrimination.