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Relationship between Relative Water Content, Nitrogen Pools, and Growth of *Phaseolus vulgaris* L. and *P. acutifolius* A. Gray during Water Deficit

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ABSTRACT

***Phaseolus acutifolius* A. Gray is a potential source of stress-tolerant traits for *Phaseolus vulgaris* L. through interspecific hybrids. The objective of this study was to quantify the effects of water-deficit stress on vegetative growth, shoot relative water content (RWC), and leaf concentrations of proline, polyamines, and related metabolites in *P. vulgaris* compared with *P. acutifolius*. Stress-induced changes in N metabolism putatively related to stress tolerance have not been investigated previously in *P. acutifolius*. Replicate pots, each containing three 5-d-old plants in 18.9 L of soil with 4 L of available water, were subjected to water deficit by withholding water (terminal drought) or were maintained under well-watered (control) conditions. Compared with controls, stressed plants of both species accumulated approximately 55% less shoot dry matter. Root dry matter accumulation was inhibited to a greater degree in *P. acutifolius* (≈70% for two genotypes) than in *P. vulgaris* (14 and 27% for two genotypes). *P. acutifolius* maintained greater shoot RWC than *P. vulgaris*. In droughted plants of *P. acutifolius*, leaf arginine and proline concentrations did not change, total polyamine (Σ agmatine + putrescine + spermidine + spermine) concentrations decreased, and ammonia increased compared with controls. In *P. vulgaris*, water deficit increased concentrations of arginine (>30%) and proline (>300%), whereas total polyamine and ammonia concentrations did not change compared with controls. In all four genotypes examined, proline concentration was inversely related to RWC ($R^2 \geq 0.90$). Leaf proline concentration is an indicator of plant water status in *Phaseolus* but not of tolerance or sensitivity of vegetative growth to water deficit.**

THE AMINO ACID PROLINE and the diamine putrescine accumulate in the leaves of diverse species in response to environmental stresses, especially water deficit and osmotic stresses (Rabe, 1990). Despite extensive research to elucidate the roles of proline and putrescine in ameliorating the detrimental effects of abiotic stresses on plant growth and yield, the physiological significance of their accumulation remains equivocal. Proposed roles in stress tolerance common to both proline and polyamines include serving as osmotica, stabilizing macromolecules and membranes, and detoxifying tissues of excess N (Rabe, 1990; Hung et al., 1994). Mounting evidence supports the hypothesis that proline or putrescine accumulation might be associated with excess NH_3 production during stress (Lovatt, 1990; Slocum and Weinstein, 1990).

Biosynthesis of proline and putrescine is metabolically linked through the common substrates ornithine and arginine. Arginine is the more important precursor during stress for both the synthesis of proline and putrescine in a number of plant species (Rabe, 1990), including *Phaseolus vulgaris* L. (Stewart and Boggess, 1977). Two questions arise: does arginine concentration increase during stress and drive the synthesis of both proline and putrescine, or does one pathway successfully compete for available arginine at the expense of the other? Few studies have quantified changes in the concentrations of both proline and putrescine simultaneously in the same plant species. The results of these limited studies suggest that the relative accumulation of proline compared with putrescine in response to stress is likely to vary between plant species. In barley (*Hordeum vulgare* L.) leaves, both proline and putrescine accumulated in response to osmotic stress. Putrescine concentration increased with small changes in leaf water potential. The increase in proline concentration was delayed, but was 10-fold greater than putrescine after 24 h (Turner and Stewart, 1988). In contrast, excised rice (*Oryza sativa* L.) leaves accumulated only proline under water-deficit stress and only putrescine under osmotic stress (Chen and Kao, 1993). Ornithine and arginine were incorporated into putrescine, but not proline.

The objective of our study was to examine the relationship between N metabolism (specifically, arginine, proline, putrescine, and related compounds) within and between *Phaseolus* species, and the maintenance of plant water status and vegetative growth during terminal drought. Two cultivars of *P. vulgaris* and two breeding lines of *P. acutifolius* were used. *P. vulgaris* is a principle food crop worldwide, the productivity of which is severely limited by water deficit (Singh et al., 1991), and *P. acutifolius* is cultivated successfully where high temperatures and drought are common (Federici et al., 1990). Furthermore, *P. acutifolius* is a potential source of stress-tolerant traits for *P. vulgaris* through interspecific hybrids (Haghigi and Ascher, 1988). *P. acutifolius* possesses both morphological and physiological characteristics that enable it to complete its life cycle and yield well under hot, arid conditions (Castonguay and Markhart, 1991, 1992; Miklas et al., 1994; Lin and Markhart, 1996). Tolerance of *P. acutifolius* to drought may have a biochemical as well as morphological basis (Castonguay and Markhart, 1991, 1992). In *P. acutifolius*, water is conserved during drought, so dehydration is postponed. The contribution of proline or putrescine to dehydration postponement in *P. acutifolius* has not been investigated; no studies have quantified changes in con-

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Abbreviations: FW, fresh weight; RWC, relative water content; TCA, trichloroacetic acid; TW, turgid weight; YFE, young, fully expanded leaves.

centrations of nitrogenous pools in leaves of *P. acutifolius* during any stress. In a previous study, we demonstrated that salinity inhibited both vegetative shoot growth and enzyme reactions leading to the formation of citrulline in the arginine de novo pathway more in *P. acutifolius* than in *P. vulgaris*. However, we did not quantify the effect of salt-stress on concentrations of arginine or its metabolites (Lazcano-Ferrat and Lovatt, 1997). Furthermore, few studies have contrasted the vegetative growth of *P. acutifolius* to that of *P. vulgaris* under water deficit in the absence of high temperature. Recent results suggest that *P. acutifolius* is more tolerant to heat stress than water-deficit stress (Lin and Markhart, 1996). Our study examined the relative tolerance of *P. acutifolius* and *P. vulgaris* to a terminal water deficit imposed during vegetative growth.

MATERIALS AND METHODS

Chemicals

Mineral salts for modified half-strength Hoagland's (Hoagland and Arnon, 1950) and Shive's (Shive and Robbins, 1938) nutrient solutions were of analytical reagent quality (Fisher Scientific, Pittsburgh, PA). All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

Plant Material

Seeds of *P. vulgaris* L., 'Linden' and 'FM 53' (courtesy of California Certified Seed, University of California, Davis), and *P. acutifolius* A. Gray, lines PI-319443 and PI-321638 (provided by the University of California, Riverside), were used in this study. Linden is a determinate, large-seeded, red kidney bean (Type I) representing the Andean gene pool; FM 53 is an indeterminate, white, small-seeded, semivine (Type IIIA) representing the Mesoamerican gene pool (Singh et al., 1991). *P. acutifolius* PI-319443 is a white landrace from Movas, Sonora, Mexico, and PI-321638 is a white landrace bought at a market in Newfields, AZ. Both are indeterminate and Mesoamerican in origin. Seeds were dusted with the fungicide Captan (cis-*N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) 22 g kg^{-1} + Zineb (zinc ethylenebisdithiocarbamate) 21 g kg^{-1} (Patterson Chemical Co., Kansas City, MO) according to the manufacturer's directions, 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 germinated at 30°C in the dark. After 2 d, three uniform seedlings were planted per pot containing 18.9 L of sterile University of California Soil Mix III (Baker, 1957). Pots filled with soil mix were watered once to full capacity with 4 L of modified half-strength Hoagland's complete nutrient solution, to prevent mineral nutrient deficiencies (Hoagland and Arnon, 1950). Plants were grown under a 12-h day and photosynthetic photon flux density of 550 μmol m^{-2} s^{-1} at 27°C and 12-h nights at 23°C for the duration of each experiment. Five days after plants were transferred to pots, the soil was saturated to full capacity with 4 L of H_2O. Thereafter, plants were subjected to water-deficit stress by withholding water (terminal drought) or were maintained under well-watered (control) conditions. Three plants grown in each of three pots in three separate growth chambers were pooled to produce one replication. Each experiment was arranged in a factorial completely randomized design: two water levels by four cultivar-lines, and by three sampling dates in some experiments, with the number of experiments as replications. Shoot, root, and leaf fresh and dry weights, and shoot RWC were determined at 10, 14, or 18 d after water was withheld. Leaf RWC, concentrations of endogenous nitrogenous pools, and metabolism of exogenous N compounds were quantified at the 18-d sampling date. For determination of dry weight, plant tissues were oven dried at 72°C for 48 h. Leaf area was measured at 18 d with a leaf area meter (Li-Cor Model 3100, Lincoln, NE). All leaf analyses were made on young, fully expanded (YFE) trifoliolate leaves located at the third and fourth nodes below the apex of the plant. Leaves were collected between 900 to 1000 h.

Relative Water Content

Shoots or individual YFE leaves of the plants were collected and immediately weighed (fresh weight, FW). Intact shoots or leaves were transferred to sealed amber flasks, rehydrated in 1 L of water for 5 h until fully turgid at 4°C, surface dried, and reweighed (turgid weight, TW). The shoot or leaf samples were then oven dried at 72°C for 48 h and reweighed (dry weight, DW) (Silva et al., 1996). The RWC was calculated by the following formula:

$$RWC\ (\%) = (FW - DW)/(TW - DW)100$$

Leaf Protein Concentration

Leaves (500 mg FW) were homogenized in 10 mL of Shive's nutrient solution (Shive and Robbins, 1938) [5 m*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}, 1mg Mn L^{-1}, 0.13 mg Cl^- L^{-1}, 0.1 mg Zn^{2+} L^{-1}, 0.1 mg B L^{-1}, 0.01 mg Cu^{2+} L^{-1}, 0.01 mg Mo L^{-1}, and 0.01 mg Na^+ L^{-1}], pH 7.4 (Lazcano-Ferrat and Lovatt, 1997). The protein content of the supernatant fraction was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. The relationship between absorbance and protein content of the extract was linear for protein concentrations from 0.2 to 1.0 mg mL^{-1}. Samples were diluted to give values within this range.

Leaf NH_3–NH_4^+, Arginine, and Polyamine Concentrations

Leaf sections (500 mg FW, midvein removed and cut into 5 by 5 mm pieces) were immediately homogenized in 10 mL of trichloroacetic acid (TCA) at 100 g L^{-1} using a Polytron tissue homogenizer (PCU-2, Brinkman Instruments, Westbury, NY) at speed 6 and then centrifuged at 10 000 *g* for 10 min at 4°C. The NH_4^+ concentration of the acid soluble supernatant fraction, containing the combined pool of NH_3–NH_4^+ as NH_4^+, was determined using an Alltech Ammonia Analyzer (Alltech, Deerfield, IL) (Carlson, 1978). The assay was linear for NH_4^+ concentrations from 0 to 100 μg mL^{-1}. Samples were diluted to give values in this range.

The arginine concentration of the acid-soluble supernatant fraction was determined in a 1-mL sample, after dilution to 50 g TCA L^{-1}, according to the modified method of Sakaguchi as described in Van Pilsum et al. (1956). The sample in a 13 by 100 mm test tube set on ice was reacted with 1 mL of 1 *M* NaOH, 0.5 mL of 1:1 (v/v) mixture of NaOH at 100 g L^{-1} containing 20 mg thymine mL^{-1} (prepared just before use) and napthol at 0.4 g L^{-1} in ethanol at 950 mL L^{-1}, and 0.2 mL of a 1:5 (v/v) dilution of commercial bleach in distilled H_2O prepared just before use. After 1 min, 0.2 mL of aqueous sodium thiosulfate at 20 g L^{-1} was added and mixed. After 5 min at room temperature, the optical density at 500 nm was determined. The assay was linear for arginine concentrations

from 0 to 100 nmol mL^{-1}. Samples were diluted with TCA at 50 g L^{-1} to give values within this range. Interferences from 100 nmol mL^{-1} of ornithine, citrulline, urea, or putrescine were insignificant. Similarly, a sample of pure protein at a concentration greater than the protein concentration of the leaf tissue employed in these assays showed no significant absorbance at 500 nm.

The free polyamine content of the acid-soluble supernatant fraction was determined, after benzoylation, by high performance liquid chromatography. A 1-mL sample of the acid-soluble supernatant fraction diluted to 50 g TCA L^{-1} was reacted with 2 mL of 2 *M* NaOH and 10 μL benzoylchloride, vortexed for 10 s and incubated at room temperature for 20 min. The reaction was terminated with 2 mL of saturated NaCl and the benzoylpolyamines extracted in 2 mL of diethylether (anhydrous, HPLC grade). After centrifugation at 3000 *g* for 5 min, 1 mL of the ether phase was collected, evaporated to dryness in a stream of air, and the resulting precipitate redissolved in 100 μL of pure methanol (HPLC grade). HPLC was carried out at room temperature through a 4.6 by 250 mm, 5-μm particle size C18 column (octadecylsilane; Alltech) eluted with methanol at 600 ml L^{-1} at a flow rate of 1 mL min^{-1} (Flores and Galston, 1982). The benzoylpolyamines were detected at 254 nm at a sensitivity of 0.04 absorbance units full scale. This method does not separate agmatine from putrescine. Therefore, the data represented are the combined pool of agmatine plus putrescine. The assay was linear for concentrations of agmatine, putrescine, spermidine, and spermine from 0.01 to 1 nmol mL^{-1}. Samples were diluted with TCA at 50 g L^{-1} to give values in this range. The limit of detection is 2 nmol g^{-1} FW tissue.

Leaf Proline Concentration

Proline was quantified spectrophotometrically by the method of Chinard (1952). Leaf sections (500 mg FW, midvein removed and cut into 5 by 5 mm pieces) were homogenized in 10 mL of aqueous sulfosalicylic acid at 30 mL L^{-1}, and the homogenate was filtered through Whatman no. 2 filter paper. A 2-mL aliquot of the sample filtrate was reacted with 2 mL of acid ninhydrin (1.25 g of ninhydrin in 30 mL of glacial acetic acid plus 20 mL of phosphoric acid stirred on low heat and stored in the dark) and 2 mL of glacial acetic acid in a 20-mL glass test tube and boiled for 1 h at 100°C. The samples were put on ice to stop the reaction, diluted with 4 mL of toluene, vortexed vigorously to extract the color into the organic phase and swirled to insure uniform mixing of the organic phase. After 20 min, the OD of the toluene fraction at 520 nm was determined. Proline concentration was linear from 2.5 to 80 μg mL^{-1}. Samples were diluted with sulfosalicylic acid at 30 ml L^{-1} before reaction with acid ninhydrin to give values within this range.

This method (using toluene or benzene to extract the chromophore or the elimination of this step altogether) does not detect most other amino acids under conditions that detect proline (pH 1.0) with the exception of ornithine, which yields a red product with an absorption spectrum identical to that produced by proline, and lysine, which forms a black product (Chinard, 1952). Lysine was not detected in the samples of YFE leaves of *P. vulgaris* or *P. acutifolius*. The method of Karlin et al. (1976) was used to determine the relative amounts of proline and ornithine in YFE leaves from *P. vulgaris* and *P. acutifolius* under well-watered (control) and water-deficit stress conditions. Proline was separated from ornithine by fractionation of a 5-mL sample of the 50 g L^{-1} TCA-soluble fraction prepared as described in the preceding section, on an Dowex-50W Na^{+} column (200–400 mesh, 80% cross linking, 0.7 by 7.5 cm) eluted with 25 mL of 0.116 *M* sodium citrate buffer (pH 5.3). In two separate recovery experiments employing commercial standards, the first 10 mL of eluant (including the 5-mL void volume) contained 88% of the proline and the second 15 mL of eluant contained 97% of the ornithine standard.

Proline Accumulation in Excised Young Fully Expanded Leaves

Leaf sections (500 mg FW, midvein removed and cut into 5 by 5 mm pieces) were immediately transferred to a 25-mL Erlenmyer flask containing 5 mL of Shive's nutrient solution, supplemented with 10 m*M* arginine, 10 M*M* glutamate, or 50 m*M* NH_4Cl (pH 7.4), and incubated for 3 h at 30°C in a water-bath shaker. At the end of the incubation period, the reactions were terminated by adding 5 mL of sulfosalicylic acid at 60 mL L^{-1} into the main chamber of the flask. The samples were stored at −25°C for subsequent analysis of proline.

A negative effect on proline recovery was observed when leaf tissue was incubated in the presence of added NH_4NO_3. To determine the cause, NH_4NO_3 was replaced with NH_4Cl or $Ca(NO_3)_2$ in the above reaction mixture. To determine if the interference was nonenzymatic, a known of amount of proline was incubated with increasing concentrations of NH_4Cl or $Ca(NO_3)_2$ with and without adding tissue to the incubation mixture. Based on the results, NH_4Cl was used as the source of ammonium in all subsequent experiments as indicated above.

Statistical Analyses and Presentation of Data

Duncan's Multiple Range Test or Student's *t* Test was used to compare means of two to five separate experiments. The value for *n*, the number of experiments, is given in the table legends. Regression analyses were used to determine the relationships between leaf RWC and leaf proline concentrations and between the ratio of nitrate/proline concentration and the percentage of reduction in optical density at 520 nm. Unless otherwise stated, conclusions are based on differences between means significant at $P \leq 0.05$.

RESULTS

Plant Growth

Withholding water for 18 d reduced shoot dry weight in both *P. vulgaris* cultivars approximately 55% compared with control plants (Table 1); shoot growth of *P. acutifolius* lines PI-319443 and PI-321638 was reduced 56 and 63%, respectively. At 14 d, the stress treatment did not affect shoot dry weight accumulation of any genotype relative to the control plants (data not shown). Between Days 14 and 18, however, shoot dry mass accumulation was severely inhibited in both species: 94 and 80% for PI-319443 and PI-321638, respectively, and 88 and 63% for Linden and FM 53, respectively. Because 18 d of stress reduced shoot growth rate to a similar degree in both species and significantly decreased shoot dry weight in both species, tissues from plants sampled on this date were used in subsequent analyses.

The surface area of leaves borne at nodes three and four from the apex decreased approximately 50% for both species after 18 d without water (Table 1). Under both control and water-deficit conditions, Linden had significantly larger leaves than the other genotypes; however, under water-deficit stress, Linden exhibited a

Table 1. Leaf area, shoot and root dry weight in control ($+H_2O$) and water deficit ($-H_2O$) plants of *P. vulgaris* (Linden and FM 53) and *P. acutifolius* (PI-319443 and PI-321638). Data are the means of three replicate experiments. Water was withheld for 18 d during vegetative growth ($-H_2O$).

Species	Genotype	Treatment	Leaf area		Dry weight	
			Leaf 3	Leaf 4	Shoot	Root
			cm^2		mg plant^{-1}	
P. vulgaris	Linden	$+H_2O$	44a†	80a	4650a	1720a
		$-H_2O$	24b	35b	2060c	1260b
	FM 53	$+H_2O$	15b	24c	1790c	760cd
		$-H_2O$	7de	12d	830d	650cd
P. acutifolius	PI-319443	$+H_2O$	16c	25c	4300a	1060bc
		$-H_2O$	8de	11d	1900c	300e
	PI-321638	$+H_2O$	9d	12d	3400b	1960a
		$-H_2O$	4c	6e	1300c	560d

† Values in a vertical column followed by different letters are significantly different at $P \leq 0.05$ by Duncan's Multiple Range Test. Leaf area is the mean area of each leaflet of trifoliolate leaves at the third and fourth nodes.

significant reduction in total leaf number per plant and had significantly fewer leaves than the other genotypes (data not shown).

Root dry weight decreased under water-deficit stress in all genotypes except FM 53, which was a smaller plant with less root biomass than the other genotypes even under control conditions (Table 1). Withholding water for 18 d stunted root growth of *P. acutifolius* to a greater degree than it did in *P. vulgaris*. No root nodules were observed on any plants. Since water-deficit stress inhibited shoot dry weight accumulation similarly (55–63%) in both species, differences in the effect of stress on root growth between species is probably the basis for the increase in root to shoot ratio in *P. vulgaris* and the decrease in *P. acutifolius*.

Shoot Relative Water Content

In FM 53 and PI-321638, shoot RWC decreased significantly when water was withheld for 14 d (Table 2). Shoot RWC decreased significantly in Linden and PI-319443 4 d later. Under stress, *P. acutifolius* had a greater shoot RWC than did *P. vulgaris*. Compared with control plants, shoot RWC decreased only 5% in *P. acutifolius* after 18 d of water deficit, whereas shoot RWC for Linden and FM 53 decreased approximately 10%.

Leaf Protein Concentration

Water-deficit stress significantly decreased protein concentrations in leaves for all genotypes compared with control plants, although the decrease was not significant in *P. acutifolius* line PI-319443 (Table 3). Under control conditions, this line had low protein concentrations, which declined only 33% in response to stress treatment. Protein concentrations were similar for both species after 18 d without water. However, the percentage of decrease was greater in *P. vulgaris* (≈60%) because it had higher protein concentrations than *P. acutifolius* under well-watered conditions.

Leaf Concentrations of Proline, Putrescine, and Related Metabolites

Water-deficit stress did not affect leaf NH_3–NH_4^+ concentrations in *P. vulgaris* but increased leaf NH_3–NH_4^+ concentrations 52 and 235% in *P. acutifolius* lines PI-319443 and PI-321638, respectively. For both species, concentrations of NH_3–NH_4^+ in YFE leaves of control plants were similar for both species (Table 4).

Concentrations of free arginine increased 33 to 40% in leaves of *P. vulgaris*, but not in *P. acutifolius*, after 18 d of water deficit. Free arginine concentrations in leaves of control plants of both species were not statistically different. For both species, concentrations of proline were similar in leaves from control plants (Table 4). Leaf proline concentrations in the two *P. acutifolius* lines did not change significantly during the water-deficit stress treatment; proline concentrations increased four- and threefold in leaves of *P. vulgaris* Linden and FM 53, respectively.

Leaf proline concentration was inversely related to leaf RWC in all four genotypes (Fig. 1). The relationship between proline concentration and RWC differed between Linden and FM 53. Proline concentration remained relatively stable in Linden until RWC decreased

Table 2. Shoot relative water content of genotypes of *P. vulgaris* (Linden and FM53) and *P. acutifolius* (PI-319443 and PI-321638) after withholding water for 10, 14, and 18 d. Data are the means of four replicate experiments.

Species	Genotype	Shoot relative water content					
		10 d		14 d		18 d	
		$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
		%					
P. vulgaris	Linden	82def†	79def	79def	79efg	82c	73d
	FM53	93a	88ab	86bcd	77efg	83b	71d
P. acutifolius	PI-319443	83bcd	83cde	84bcd	81def	83b	78c
	PI-321638	84a	93a	88abc	81def	86ab	81c

† Values in a vertical column or in a horizontal row followed by different letters are significantly different at $P \leq 0.05$ by Duncan's Multiple Range Test.

Table 3. Protein concentration of young, fully expanded leaves from well watered ($+H_2O$) and water-deficit stressed ($-H_2O$) genotypes of *P. vulgaris* (Linden and FM 53) and *P. acutifolius* (PI-319443 and PI-321638). Data are the means of three replicate experiments.

Species	Genotype	Protein content	
		$+H_2O$	$-H_2O$
		mg protein g^{-1} fresh wt	
P. vulgaris	Linden	18.0a†	7.3d
	FM 53	18.3a	6.6d
P. acutifolius	PI-319443	13.0bc	8.6cd
	PI-321638	16.6ab	9.1cd

† Values in a vertical column or a horizontal row followed by different letters are significantly different at $P \leq 0.05$ by Duncan's Multiple Range Test.

to approximately 75%, then accumulated rapidly. In FM 53, proline accumulated slowly across the same range of RWC. Leaf proline concentrations in the two *P. acutifolius* lines were not significantly different from control plants across the range of RWC values quantified during the 18 d of water-deficit stress (Table 4). The negative quadratic relationship between leaf proline concentration and RWC for all four genotypes combined was significant ($R^2 = 0.7$, $P \leq 0.001$). Leaf concentrations of ammonia or arginine showed a negative quadratic relationship with RWC for both species, but the R^2 values were 0.4 or less ($P \leq 0.05$).

The proline values reported above represent the combined pool of proline plus ornithine quantified by the Chinard (1952) method. Ornithine constituted 40% of this pool in leaves from control plants of both *P. acutifolius* lines. For the *P. vulgaris* cultivars Linden and FM 53, ornithine comprised <30% of the combined pool. At Day 18 of the stress treatment, the contribution made by ornithine to the combined pool was 20% in all genotypes except FM 53, for which it was at 15% of the total as it was under the control conditions. Thus, proline was the greater component of the pool under both control and water-deficit stress conditions.

Both genotypes of *P. vulgaris* (a known drought-sensitive species) exhibited greater capacity for proline accumulation than did the lines of *P. acutifolius* (a reported drought-tolerant species). To test whether variation in proline accumulation during water-deficit stress was a response to excess N, leaves were excised from *P. vulgaris* and *P. acutifolius* control and water-deficit stressed plants and incubated in the presence of 50 m*M* NH_4NO_3. In all cases, proline concentrations were less than in leaves not receiving additional NH_4NO_3 (data not shown). This result suggested that excess N promoted proline catabolism or that NH_4^+ or NO_3^- interfered with the reaction between proline and the ninhydrin reagent. Adding NH_4Cl or $Ca(NO_3)_2$ to a known concentration of proline identified NO_3^- as the interfering agent. Experiments with and without leaf tissue confirmed that the interference was nonenzymatic and that NO_3^- interfered with the ninhydrin reaction. The interference was highly dependent on the ratio of the nitrate/proline concentration. A ratio of 8:1 reduced color formation 50% under conditions used in the Chinard (1952) method.

The NO_3^- concentration in the Shive's nutrient solution used as the incubation medium in the feeding experiments described below was diluted during preparation of samples for proline analysis. The final nitrate to proline ratio did not interfere with the determination of proline. This is confirmed by the fact that leaf proline concentrations in control plants harvested at the end of the stress treatment and analyzed immediately (Table 4) or after a 3-h incubation in Shive's nutrient solution (Table 5) were not significantly different.

Despite that rehydration of leaves from water-deficit stressed plants during the incubation period, significant differences in the metabolism of precursors to proline were quantified in leaves from stressed and control plants. In general, ammonium had little effect on leaf proline concentrations in both species (Table 5). Only leaves from water-deficit stressed FM 53 and water-deficit stressed and control *P. acutifolius* PI-321638 supplied with ammonium had higher proline concentrations than leaves from their respective controls not supplied with ammonium. Glutamate increased proline concentrations in leaves from three of the four genotypes compared with their respective controls and affected stressed and control plants similarly. Only *P. acutifolius* line PI-321638 exhibited greater proline accumulation under stress conditions. Stressed leaves of all genotypes, except FM 53, accumulated more proline when N was supplied as glutamate rather than ammonium. A similar trend was observed in leaves of PI-319443. Arginine was the most effective source for increasing proline accumulation. Stressed leaves from both lines of *P. acutifolius* supplied with arginine accumulated proline to concentrations comparable to those of FM 53 but not

Table 4. Concentrations of NH_3–NH_4^+, arginine and proline in young, fully expanded leaves of well watered ($+H_2O$) and water-deficit stressed ($-H_2O$) genotypes of *P. vulgaris* (Linden and FM 53) and *P. acutifolius* (PI-319443 and PI-321638). Data are the means of five replicate experiments. Water was withheld for 18 d during vegetative growth.

Species	Genotype	Treatment	NH_3–NH_4^+	Arginine	Proline
			μmol g^{-1} fresh wt		
P. vulgaris	Linden	$+H_2O$	2.6bc†	1.5bcd	0.4d
		$-H_2O$	2.9bc	2.1a	1.7a
	FM 53	$+H_2O$	2.1c	1.2cd	0.4d
		$-H_2O$	2.7bc	1.6b	1.3ab
P. acutifolius		$+H_2O$	2.3c	1.4bcd	0.7bc
		$-H_2O$	3.5b	1.5bcd	1.0bc
	PI-321638	$+H_2O$	2.0c	1.1d	0.6cd
		$-H_2O$	6.7a	1.5bcd	0.9bc

† Values in a vertical column followed by different letters are significantly different at $P \leq 0.05$ by Duncan's Multiple Range Test.

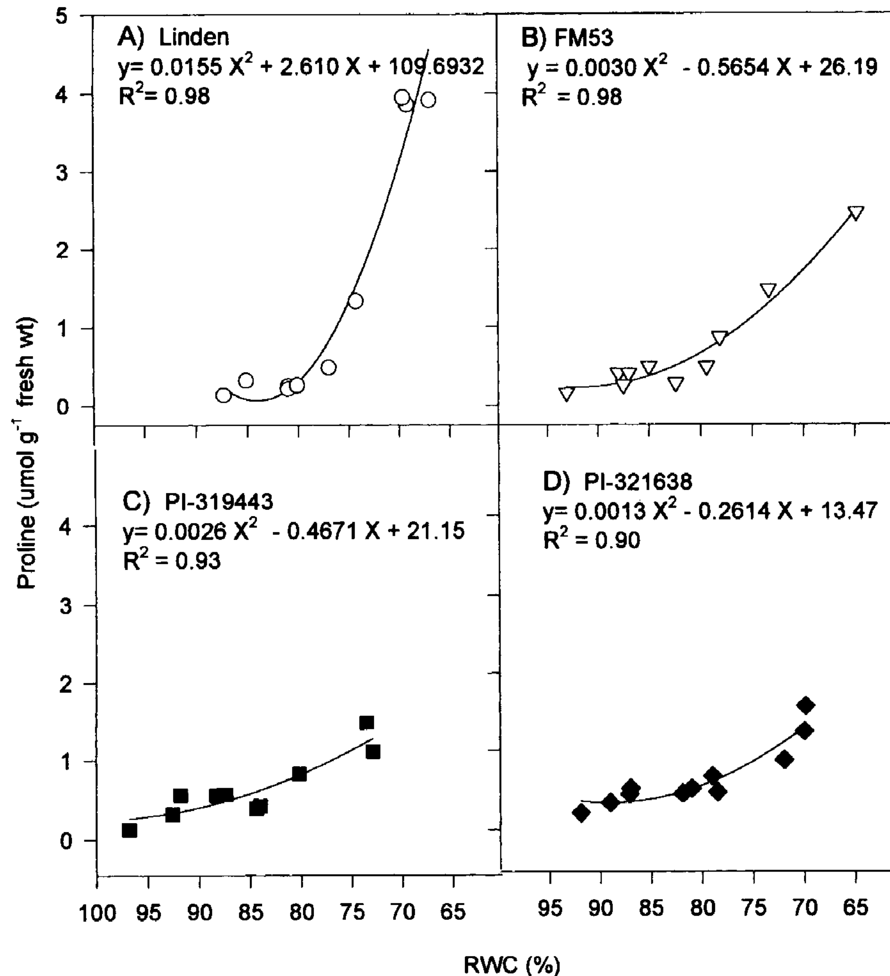

Fig. 1. Relationship between proline concentration and relative water content (RWC) in young, fully expanded (YFE) leaves of *P. vulgaris* cultivars (A) Linden and (B) FM 53, and *P. acutifolius* lines (C) PI-319443 and (D) PI-321638 at 18 d of treatment; $P \leq 0.05$.

Linden. With added arginine, however, only Linden and PI-321638 accumulated more proline under stress than under control conditions.

Concentrations of agmatine plus putrescine in leaves from control plants were significantly greater in *P. acutifolius* than in *P. vulgaris*, whereas spermine concentrations were greater in leaves of *P. vulgaris* (Table 6). Withholding water significantly decreased leaf concentrations of agmatine plus putrescine to a concentrations below the limit of detection (<2 nmol g^{-1} FW) for all genotypes except Linden. Compared with control plants, leaf spermidine concentrations were affected only in PI-321638. Spermine concentrations decreased under water-deficit stress in all genotypes except FM 53. Water-deficit stress significantly reduced leaf total polyamine concentrations in *P. acutifolius*, but not in *P. vulgaris*.

DISCUSSION

P. acutifolius has been of interest to plant physiologists and breeders because it produces greater yields

Table 5. Effect of ammonium, glutamate, or arginine on in vitro proline accumulation in young, fully expanded leaves of water-deficit stressed genotypes of *P. vulgaris* (Linden and FM 53) and *P. acutifolius* (PI-319443 and PI-321638). Data are the means of two replicate experiments. Water was withheld for 18 d during vegetative growth.

Species	Genotype	Treatment	None (control)	NH_4^+ (50 m*M*)	Glutamate (10 m*M*)	Arginine (10 m*M*)
			μmol proline g^{-1} fresh wt			
P. vulgaris	Linden	$+H_2O$	0.73hijklm†	0.74hijklm	1.07ef	1.49bc
		$-H_2O$	0.62klmn	0.64klmn	0.97fg	2.25a
	FM 53	$+H_2O$	0.58lmn	0.71ijklm	0.73hijk	1.39cd
		$-H_2O$	0.55mn	0.82ghijk	0.94fgh	1.53bc
P. acutifolius	PI-319443	$+H_2O$	0.89fghi	0.70ijklmn	1.07ef	1.62b
		$-H_2O$	0.66jklmn	0.83ghijk	1.20de	1.55bc
	PI-321638	$+H_2O$	0.49n	0.77ghijkl	0.87fghi	0.79hi
		$-H_2O$	0.70ijklmn	0.97gh	1.214de	1.53bc

† Values in a vertical column or a horizontal row followed by different letters are significantly different at $P \leq 0.05$ by Duncan's Multiple Range Test.

Table 6. Concentrations of agmatine plus putrescine (Agm + Put), spermidine (Spd), spermine (Spm), and total polyamines (total PA) in young, fully expanded leaves of well-watered (+H_2O) and water-deficit stressed (−H_2O) genotypes of *P. vulgaris* (Linden and FM 53) and *P. acutifolius* (PI-319443 and PI-321638). Data are the means of two replicate experiments. Water was withheld for 18 d during vegetative growth.

Species	Genotype	Treatment	Agm + Put	Spd	Spm	Total PA
			nmol g^{-1} fresh wt			
P. vulgaris	Linden	+H_2O	25.1‡	75.3	153.4	253.9
		−H_2O	11.8	35.5	67.1	114.6
Significance†			NS	NS	*	NS
	FM53	+H_2O	10.6	101.9	154.3	267.0
		−H_2O	<2	79.8	82.3	162.1
Significance			*	NS	NS	NS
P. acutifolius	PI-319443	+H_2O	56.7	54.4	81.7	192.9
		−H_2O	<2	40.5	29.0	69.6
Significance			*	NS	*	*
	PI-321638	+H_2O	50.8	47.7	90.7	189.4
		−H_2O	<2	<2	26.2	26.2
Significance			*	*	*	*

† Values within a vertical column are significant (*) or nonsignificant (NS) at $P \leq 0.05$ by Student's *t*-test.
‡ The limit of the amine detection was <2 nmol g^{-1} fresh weight.

than does *P. vulgaris* under hot, water-limited conditions (Federici et al., 1990). Superior performance of *P. acutifolius*, however, may reflect its greater tolerance to high termperature than to water-deficit stress (Lin and Markhart, 1996). In our study, *P. acutifolius* was not more stress tolerant than *P. vulgaris* with regard to vegetative growth. Water-deficit stress inhibited shoot growth rate (mg dry weight d^{-1}) similarly in both species. Total shoot growth (dry weight $plant^{-1}$) and final leaf area of both *P. vulgaris* and *P. acutifolius* decreased approximately 55% compared with control plants. Root growth of *P. acutifolius* was more sensitive (≈70% net reduction in biomass) to water deficit than in *P. vulgaris* (<30% net reduction in biomass). Whether the 25% reduction in root/shoot ratio in *P. acutifolius* vs. a 69 to 86% increase in root/shoot ratio in *P. vulgaris* would account for the variation in seed production and yield in the two species remains to be determined.

For all genotypes except PI-319443, reduced vegetative growth during stress was accompanied by a significant decrease in leaf protein concentration (45–64%). Significant reductions in leaf agmatine plus putrescine concentrations occurred in the three indeterminate genotypes under stress, whereas significant decreases in leaf spermine and total polyamine concentrations were specific to the two *P. acutifolius* lines during stress. The metabolic basis for decreased concentrations of protein or specific polyamines in *P. vulgaris* and *P. acutifolius* during water deficit is presently unknown. Whether lower concentrations of protein and polyamines cause reduced growth, or result from it, remains unresolved. The decrease in protein concentration did not correlate with proline accumulation among genotypes, suggesting that water-deficit induced-proline accumulation was not dependent on inhibition of protein synthesis or increased protein degradation.

Polyamines are obligate growth factors for all living cells, in addition to their putative role as protectants during abiotic stress. A close correlation between meristematic activity in roots, shoots, and leaves and polyamine concentrations has been previously reported for *P. vulgaris* (Palavan and Galston, 1982). Thus, reduced growth rates in these tissues would be anticipated as polyamine concentrations declined under water-deficit stress. Polyethyleneglycol-induced water-deficit stress of two drought-sensitive populations of alfalfa (*Medicago sativa* L.) epicotyl cells in suspension culture significantly reduced both total polyamine content and growth, whereas the two drought-tolerant populations accumulated polyamines and maintained growth (Kuehn et al., 1990).

Rabe and Lovatt (1986) and Lovatt (1990) provided evidence that abiotic stresses that reduce leaf or shoot growth increase production of ammonia, leading to ammonia accumulation or its removal through de novo arginine biosynthesis. Consistent with their results, arginine accumulated in *P. vulgaris*, and NH_3–NH_4^+ accumulated in *P. acutifolius* during water deficit. Arginine is a precursor of both proline and putrescine. Proline accumulation has been proposed as a mechanism for storage of excess N (Stewart and Boggess, 1977); Slocum and Weinstein (1990) proposed that putrescine accumulation was a mechanism to detoxify plant cells of excess ammonia. Accumulation of NH_3–NH_4^+ in leaves of *P. acutifolius* under water deficit is a logical result since agamatine plus putrescine, spermine, and total polyamines concentrations decreased and proline concentrations did not change (Table 4). In contrast, arginine accumulation in leaves of *P. vulgaris* under water-deficit stress was accompanied by increased proline concentration. Failure of leaves of *P. acutifolius* to accumulate proline during water-deficit stress was not due to inhibition of the enzyme machinery necessary for proline synthesis. When leaves from water-deficit stressed PI-319443 and PI-321638 were supplied glutamate or arginine, they accumulated significantly higher concentrations of proline than did leaves of control plants. PI-321638, the line that accumulated the highest concentration of NH_3–NH_4^+ (approximately a threefold increase), was able to utilize exogenous ammonia to synthesize proline (Table 5). This result suggests that endogenous NH_3–NH_4^+ produced during stress was unavailable for the synthesis of arginine or proline. Arginine was the most effective substrate for the synthesis of proline for both species. Accumulation of proline under stress did not improve shoot RWC or dry matter accumulation in *P. vulgaris*, compared with *P. acutifolius*. These results suggest that proline accumulation is a symptom of stress

in *P. vulgaris*, possibly related to increased arginine availability and ammonia detoxification.

The utility of proline accumulation as an indicator of plant tolerance or sensitivity has been compromised by the reliability of its measurement. The Chinard (1952) method has been used to quantify proline for more than 40 yr. Chinard (1952) mentioned that endogenous ornithine interfered with proline quantification but researchers using this method generally ignored this fact. The research presented here is the first to document interference from endogenous NO_3^- in the quantification of proline. Concentrations of both ornithine and NO_3^- increase in response to some of the same abiotic stresses under which proline accumulates (Rabe and Lovatt, 1986; Rabe, 1990). Thus, under stress, proline concentrations might be overestimated because of ornithine accumulation or underestimated because of NO_3^- accumulation if the Chinard (1952) method is used.

The capacity of *P. acutifolius* to maintain higher leaf and shoot RWC than *P. vulgaris* under water-deficit stress is consistent with the ability of *P. acutifolius* to postpone dehydration (Castonguay and Markhart, 1991, 1992). A negative relationship between proline concentration and RWC in leaves was significant for all four genotypes individually ($R^2 \geq 0.90$, $P \leq 0.05$) and combined ($R^2 = 0.7$, $P \leq 0.001$) (Fig. 1). This result is similar to recent studies (Naidu et al., 1992; Ma and Chen, 1992) contrasting various aspects of plant water status with proline accumulation. With increasing vapor-pressure deficits in barley leaves, proline was more closely correlated with decreasing RWC than with leaf water potential (Naidu et al., 1992). For four cultivars of alfalfa, drought resistance and proline accumulation were related to RWC of leaves and stomata (Ma and Chen, 1992).

Taken together, these studies suggest that proline accumulation might be a useful indicator of plant water status (although RWC could be measured directly), but not of the tolerance or sensitivity of plant vegetative growth to stress. The usefulness of putrescine concentration as a biochemical index of plant stress tolerance or sensitivity, especially in regard to *Phaseolus* species, remains to be proven. This study provides additional evidence that changes in N metabolism in response to water-deficit stress are species dependent.

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