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De Novo Arginine Biosynthesis in Leaves of Phosphorus-Deficient *Citrus* and *Poncirus* Species[1]

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ABSTRACT

Young, fully expanded leaves from 7-month-old P-deficient citrus rootstock seedlings had levels of nonprotein arginine that were 10- to 50-fold greater than those from P-sufficient control plants. Arginine content of the protein fraction increased 2- to 4-fold in P-deficient leaves. Total arginine content, which averaged 72 ± 6 micromoles per gram dry weight of P-sufficient leaf tissue (mean ± SE, n = the four rootstocks) was 207, 308, 241, and 178 micromoles in P-deficient leaves from *Citrus limon* cv rough lemon, *Poncirus trifoliata* × *C. sinensis* cv Carrizo citrange and cv Troyer citrange, and *P. trifoliata* cv Australian trifoliate orange, respectively. For each rootstock, the accumulation of arginine paralleled an increase in the activity of the pathway for the *de novo* biosynthesis of arginine. The ratio of the nanomoles $NaH^{14}CO_3$ incorporated into the combined pool of arginine plus urea per gram fresh weight intact leaf tissue during a 3-hour labeling period for P-deficient to P-sufficient plants was 91:34, 49:11, 35:11, and 52:41, respectively. When P-deficient plants were supplied with P, incorporation of $NaH^{14}CO_3$ into arginine plus urea was reduced to the level observed for the P-sufficient control plants of the same age and arginine ceased to accumulate. Arginase and arginine decarboxylase activity were either unaffected or slightly increased during phosphorus deficiency. Taken together, these results provide strong evidence that arginine accumulation during phosphorus deficiency is due to increased activity of the *de novo* arginine biosynthetic pathway.

Most macro- and many micronutrient deficiencies in vascular plants result in altered amino acid metabolism (16). The accumulation of L-arginine, and the arginine pathway intermediates L-ornithine and L-citrulline, is associated with a number of mineral nutrient deficiencies in a variety of higher plant species: K (13), P (3, 5, 14), Fe (6, 7, 10, 17), Cl (4), and Zn (17). Two hypotheses regarding the etiology of the observed accumulation of arginine pathway metabolites have emerged from studies employing plants in symbiosis with mycorrhizal fungi and uninoculated control plants. It has been proposed that the one or more mineral nutrient deficiencies that develop in nonmycorrhizal plants (a) prevent the further metabolism of arginine pathway substrates including arginine resulting in the accumulation of these compounds (2, 9), or (b) cause a reorganization of N metabolism which results in greater activity of the arginine pathway (14). Recently, Nemec and Meredith (14) provided evidence that arginine accumulation in the leaves of nonmycorrhizal citrus plants is due to P deficiency. Under conditions of low P fertilization, total arginine levels were 10 and 13 times higher in leaves of two commercially important citrus rootstocks, *Citrus limon* and *C. aurantium*, respectively, than in control plants receiving an adequate supply of P or in plants also grown with low P fertilization but in symbiosis with vesicular-arbuscular mycorrhizae.

Using two species and two cultivars of a single hybrid, all commercially important citrus rootstocks, we confirmed that arginine accumulated in the leaves of −P[3] *C. limon* and extended this observation to a second species and a *Citrus* hybrid. In this communication, we also report the results of a comparison of the capacity of +P and −P leaf tissue to synthesize arginine and urea *de novo*. Since catabolism opposes synthesis, we determined the specific activity of arginase and arginine decarboxylase in cell-free extracts prepared from +P and −P leaves for the four rootstocks. Finally, we examined the utilization of arginine in protein synthesis during the two states of P nutrition.

MATERIALS AND METHODS

Chemicals. All radiolabeled chemicals were purchased from ICN Pharmaceuticals, Inc. Liquiscint (liquid scintillation cocktail) was purchased from National Diagnostics. Mineral salts for Hoagland and Shive nutrient solutions were of analytical reagent quality from Fisher Scientific. All other chemicals were purchased from Sigma Chemical Company.

Plant Materials. Seeds of *Citrus limon* (L.) Burm f cv rough lemon, *Poncirus trifoliata* (L.) Raf cv Australian trifoliate orange, and *P. trifoliata* × *C. sinensis* (L.) Osbeck cv Carrizo and cv Troyer citrange were germinated in sterile sand moistened with H_2O in a growth chamber under 16 h light (310 $\mu E/m^2 \cdot s$) at 32°C and 8 h dark at 21°C. After 2 weeks, seedlings of uniform size and appearance were transplanted in 10-cm square plastic pots with sterile sand containing 1 μg extractable P/g. At the time of transplanting, P at the rate of 100 μg $Ca(H_2PO_4)_2$/g sand was added to the +P control plants. Twice a week, plants were watered with half-strength Hoagland solution without P. When the seedlings were 7 months old, height and stem diameter 5 cm above the soil line were measured. All analyses employed the youngest fully expanded leaves from each of eight plants (usually two to five leaves were excised per plant; midveins were removed).

Leaf Content of Amino Acids and Phosphorus. Excised leaves were immediately frozen in liquid nitrogen, lyophilized, and ground with a Wiley mill to a size fine enough to pass through a 40-mesh screen.

(a) Nonprotein Amino Acids. A 200-mg sample of ground leaf tissue was rehydrated with 7 ml H_2O for 30 min with stirring

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[3] Abbreviations: +P, 100 μg phosphorus/g planting medium, phosphorus-sufficient; −P, phosphorus withheld, the planting medium contained 1 μg of extractable phosphorus/g medium, phosphorus-deficient.

and then extracted with 26 ml $CHCl_3$:CH_3OH (1:2, v/v) for 30 min with stirring. The extract was partitioned with water and the aqueous phase was collected after centrifugation at 3,000 rpm for 10 min. The extract was prepared for amino acid analysis as described by Labanauskas and Handy (11).

(b) Total Amino Acids. A 20-mg sample of the ground leaf tissue was subjected to acid hydrolysis in 4 ml 6 N HCl under vacuum (samples were purged with N_2 during evacuation) at 110°C. Hydrolysis of protein was complete in 22 h (11). Samples were prepared for amino acid analysis (11). The amino acid content of the nonprotein and total amino acid samples was quantified with a Beckman 120C amino acid analyzer previously calibrated with commercial amino acid standards at 570 nm.

(c) Phosphorus Content. Phosphorus content of leaves was determined in a 50-mg sample of the ground leaf tissue by a colorimetric assay employing molybdivanadophosphoric acid (8). Absorbance at 420 nm was linear for concentrations of P from 0 to 0.4 mg/100 mg dry weight leaf tissue.

Incorporation of $NaH^{14}CO_3$ into the Combined Pool of Arginine Plus Urea. Samples of 500 mg fresh weight leaf tissue (cut into 5- × 5-mm pieces) from +P and −P seedlings were routinely preincubated in 5 ml Shive nutrient solution [5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 2 mM K_2SO_4, 1 mM KH_2PO_4, 1 mg Fe/l, 1 mg Mn/l, 0.13 mg Cl/l, 0.1 mg Zn/l, 0.1 mg B/l, 0.1 mg Cu/l, 0.1 mg Mo/l, and 0.01 mg Na/l], pH 7.4, for 2 h at 30°C in a water bath shaker. At the end of the preincubation period, the nutrient solution was decanted, and the tissue was immediately transferred to fresh Shive nutrient solution supplemented with $NaH^{14}CO_3$ (5 mM, 37.5 μCi) 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. Incorporations were carried out in a 25-ml Erlenmeyer flask sealed with a rubber stopper, fitted with a plastic center well (Kontes Glassware, Vineland, NJ) containing a filter paper wick. At the end of the incorporation period, 0.3 ml of 4 N KOH was injected into the plastic center well, and the reaction was terminated by injecting 1 ml of 1.5 N $HClO_4$ into the main chamber of the flask. The flasks were returned to the water bath shaker for an additional 10 min to allow the unreacted $^{14}CO_2$ from the acidified incubation mixture to distill into the KOH in the center well. The contents of the main chamber of the flask were homogenized with a Polytron tissue homogenizer (PCU-2, Brinkman Instruments). The polytron probe was rinsed with 2 ml of 0.25 N $HClO_4$ which was added to the homogenate. The insoluble material was removed by centrifugation at 10,000*g* for 10 min at 0°C. The supernatant solution was diluted to 10 ml with H_2O and the amount of [*guanido*-^{14}C]arginine and [^{14}C] urea synthesized by the leaf tissue from $NaH^{14}CO_3$ was determined in the entire 10-ml sample using commercial arginase and urease as described previously by Lovatt and Cheng (12).

Detection of Enzymic Activities in Cell-Free Extracts of +P and −P Leaves. In all cases, enzymic activities were measured in cell-free extracts prepared from 1 g fresh weight leaf tissue (midveins removed) homogenized in 5 ml of the specified buffer with a Duall all-glass tissue grinder (Kontes Glassware). The homogenate was centrifuged at 10,000*g* at 0°C for 10 min, and the supernatant fraction served as the source of enzyme. In each case, the protein content of this fraction was determined by the Bradford method (1) using BSA as the standard. Protein content was linear from 0.2 to 1.0 mg/ml. Samples were diluted to give values in this range.

(a) Arginase. The activity of arginase was measured in the presence of excess urease to convert all the urea synthesized by the plant enzyme to CO_2 and NH_4^+. The activity of arginase was measured in a 1.5-ml reaction mixture of the following composition: 0.1 M glycine-NaOH buffer, pH 9.0; 2.5 mM $MnCl_2$; 60 mM [*guanido*-^{14}C]arginine (1 μCi); 2 units of urease (Sigma type III, from jack beans; 1 unit liberates 1.0 μmol ammonia from urea per min at pH 7.0 at 25°C); and 0.5 ml enzyme preparation in a scintillation vial sealed with a rubber stopper from which was suspended a plastic center well containing 0.3 ml 4 N KOH and a filter paper wick. Samples were incubated at 37°C in a water bath shaker for 1 h. Reactions were terminated by injecting 1.0 ml of 1.5 N $HClO_4$ into the main chamber of the flask. Samples were returned to the water bath shaker for 10 min, and then 0.2 ml of 0.1 N $NaHCO_3$ was injected into the main chamber of the flask to drive the residual $^{14}CO_2$ from the acidified reaction mixture. Samples were returned to the water bath shaker for 30 min. The plastic center well and its contents were transferred to scintillation vials containing 3.7 ml H_2O and diluted with 13 ml Liquiscint.

(b) Arginine Decarboxylase. Since [*carboxy*-^{14}C]arginine was not available to us, we measured the nmol $^{14}CO_2$ generated from [U-^{14}C]arginine by the action of the plant arginine decarboxylase and combined activity of the plant arginase and urease. From this value we subtracted the nmol of $^{14}CO_2$ released from [*guanido*-^{14}C]arginine by the combined activity of the plant arginase and urease. The difference between these two values was the $^{14}CO_2$ released from [U-^{14}C]arginine by arginine decarboxylase. The reaction mixture contained in a final volume of 1.5 ml: 0.1 M maleate buffer, pH 6.0; 30 mM [U-^{14}C]arginine (1.0 μCi) or 30 mM [*guanido*-^{14}C]arginine (0.1 μCi); and 0.5 ml enzyme preparation. Incubation conditions and procedures were the same as those described for arginase.

RESULTS

Effects of Phosphorus Deprivation on Plant Growth. Withholding P from young seedlings from germination through their early development severely limited their growth. Average height of −P plants for all four cultivars was 15 cm compared to an average height of 94 cm for the +P control plants of the same age (7 months) ($P < 0.001$ by Student's *t* test). Stem diameter 5 cm above the soil line was also reduced in −P plants ($P < 0.05$ by Student's *t* test) (Table I).

Influence of Phosphorus Nutrition on Leaf Phosphorus. For an individual species or hybrid, the level of P in leaves from −P plants was lower than that of the corresponding +P control plants. The reduction in P content for the four rootstocks ranged from as little as 10% to greater than 60% (Table I). When

Table I. *Effects of Phosphorus Deprivation on Plant Growth*

Seeds of the four cultivars were germinated in sterile sand moistened with H_2O. After 2 weeks, seedlings were transplanted in 10-cm plastic pots of sterile sand containing 1 μg extractable P/g. P-sufficient plants were treated with 100 μg P/g sand. All plants were grown in a growth chamber under 16 h light (310 $\mu E/m^2 \cdot s$) at 32°C and 8 h dark at 21°C and watered twice a week with one-half strength Hoagland solution without P. Data are the average values for eight or more plants.

Plants (7 Months Old)	Treatment	P/Leaf Tissue Dry Wt	Plant Height	Stem Diameter at 5 cm above the Soil Line
		mg/g	*cm*	*mm*
Rough lemon	+P	1.9	85	5.0
	−P	1.7	10	1.5
Carrizo citrange	+P	2.2	93	5.4
	−P	1.0	14	2.0
Troyer citrange	+P	2.6	96	5.4
	−P	1.0	14	2.0
Trifoliate orange	+P	3.1	104	4.9
	−P	1.9	21	1.8

averaged for the four rootstocks, the P content of +P and −P leaves was significantly different ($P < 0.05$ by Student's t test).

Amino Acid Content of +P and −P Leaves. Withholding P during seedling development altered amino acid metabolism in all four rootstocks. The accumulation of L-arginine was the most significant change observed for all four rootstocks when the amino acid content of both the nonprotein and protein fractions of −P leaves were compared with +P control plants (Tables II and III). The arginine peak was confirmed to be arginine by enzymic removal. In four separate samples treated with commercial arginase, 91.5 ± 3% (mean ± SE) of the material absorbing at 570 nm and eluting at the same retention time as commercial arginine was removed from the putative arginine peak. Content of nonprotein lysine increased in all four rootstocks during P deprivation (Table II). We have subsequently demonstrated that both ornithine and citrulline are detected at the same retention time as lysine. Whether the greater size of the lysine peak during P deficiency was due to an increase in lysine or to the presence of the arginine pathway intermediates ornithine or citrulline is not known.

With the exception of rough lemon, total μmol amino acid per g dry weight (obtained by adding total nonprotein and protein amino acids from Tables II and III) did not change significantly in −P plants. However, the amount of arginine in both the nonprotein and protein fractions increased dramatically, 10- to 50-fold and 2- to 4-fold, respectively (Tables II and III). Total amino acid content of the leaves did not change, with the exception of rough lemon, but the accumulation of arginine was significant (Table IV), and probably at the expense of other amino acids.

Protein content of −P leaves was only 30 ± 2% (mean ± SE, n = 8) less than that of the +P control plants (Table IV), but the amount of arginine incorporated into protein was 3- to 7-fold greater per mg protein for the four rootstocks after 7 months of P deprivation (Table IV).

Arginine Metabolism. Despite the increased levels of arginine that had already accumulated in the leaves of −P plants by the time that they were harvested as young, fully expanded leaves, the activity of the *de novo* arginine biosynthetic pathway was greater in this tissue than in leaves from +P control plants (Table V). The difference in the activity of the *de novo* arginine pathway during the two states of P nutrition is accentuated by expressing the activity per mg protein since protein content is reduced during P deprivation. The relationship between P nutrition and the activity of the *de novo* arginine pathway was investigated further: (a) by growing *C. limon* at three levels of P (5, 50, and 100 μg P/g potting medium) as described in "Materials and Methods"; and (b) by supplying −P plants with P (50 μg P/g potting medium). In young, fully expanded leaves harvested just 6 weeks after the seeds were planted, both arginine content and the activity of the *de novo* arginine pathway increased in direct response to the degree of P deprivation (Table VI). Plants that initially received half of the optimal level of P exhibited an increase in both arginine content and activity of the *de novo* arginine pathway during the subsequent 6-week period as P became more limiting. When plants that initially received 5 μg P/g potting medium were given 50 μg P/g potting medium during the second 6-week period, activity of the *de novo* arginine pathway was reduced to a level below that of plants that had received 50 μg P/g potting medium for the full 12 weeks and comparable

Table II. *Nonprotein Amino Acid Content of Young, Fully Expanded Leaves Excised from 7-Month-Old +P and −P Plants*

Young, fully expanded leaves were excised from 7-month-old seedlings initially treated with 100 μg P/g sand (+P) or 1 μg P/g sand (−P) at age 2 weeks. Plants were watered twice a week with one-half strength Hoagland nutrient solution without P. All plants were grown in a growth chamber under 16 h light (310 μE/$m^2 \cdot s$) at 32°C and 8 h dark at 21°C. Each value represents the mean of two separate experiments. The average difference between experiments was ± 20%.

Amino Acid[a]	Rough Lemon		Carrizo Citrange		Troyer Citrange		Trifoliate Orange	
	−P	+P	−P	+P	−P	+P	−P	+P
				μmol/g dry wt				
Try	1.25	0.27	0.60	0.68	0.52	0.53	1.15	0.41
Lys	59.33	2.76	25.76	4.56	18.37	4.79	11.31	4.45
His	2.70	0.61	2.07	0.85	1.23	0.93	2.04	0.72
Arg	40.01	1.00	39.17	0.82	25.28	0.98	16.90	1.74
Asp	22.68	12.41	13.85	11.34	11.88	11.69	14.39	11.22
Thr	9.52	4.38	5.03	7.57	5.87	8.38	10.43	6.57
Ser	164.73	14.72	21.08	23.84	34.13	24.41	67.71	33.16
Glu	2.73	0.59	0.55	0.40	0.41	0.50	0.61	0.68
Pro	181.90	95.64	128.19	112.91	112.62	104.47	99.67	84.07
Gly	17.41	7.65	4.34	14.84	6.28	11.96	13.57	10.49
Ala	23.43	7.97	7.94	11.69	9.74	13.05	19.59	12.08
Cys	ND[b]	ND	ND	ND	ND	ND	ND	ND
Val	11.62	3.95	5.25	6.03	6.04	6.72	9.96	5.53
Met	2.03	0.87	1.60	1.62	0.88	2.18	2.03	1.65
Ile	6.87	2.38	2.83	2.85	2.99	4.58	5.93	2.89
Leu	11.46	5.31	6.23	8.11	7.06	9.93	13.06	5.87
Tyr	5.53	1.84	2.01	2.29	2.30	2.66	3.81	1.76
Phe	7.15	2.50	3.44	3.86	3.32	4.57	5.08	2.96
Total nonprotein amino acids	570.35	164.85	269.94	214.26	248.92	212.33	297.24	186.25

[a] During extraction, asparagine and glutamine are converted to aspartate and glutamate, respectively.
[b] Not detectable.

Table III. *Amino Acid Composition of the Protein Fraction (μmol/g Dry Wt) and Protein Content (mg/g Fresh Wt) of Young, Fully Expanded Leaves Excised from 7-Month-Old +P and −P Plants*

Young, fully expanded leaves were excised from 7-month-old seedlings initially treated with 100 μg P/g sand (+P) or 1 μg P/g sand (−P) at age 2 weeks. Plants were watered twice a week with one-half strength Hoagland nutrient solution without P. All plants were grown in a growth chamber under 16 h light (310 μE/m^2·s) at 32°C and 8 h dark at 21°C. The amino acid content of the protein fraction is the difference between the total amino acid content for each amino acid in a sample obtained by acid hydrolysis and the content of each free amino acid in the sample before acid hydrolysis. Each value represents the mean difference from two separate experiments. The difference between values did not exceed 30%.

Amino Acid[a]	Rough Lemon		Carrizo Citrange		Troyer Citrange		Trifoliate Orange	
	−P	+P	−P	+P	−P	+P	−P	+P
Lys	94.23	80.55	69.60	72.95	75.23	78.89	86.45	93.71
His	22.79	26.03	21.99	24.83	21.90	26.73	26.26	30.10
Arg	166.84	69.77	268.39	61.56	216.10	63.87	161.35	86.59
Asp	289.85	125.44	104.66	126.67	145.46	123.64	216.23	183.75
Thr	60.77	66.60	49.04	65.33	60.27	58.64	77.47	80.59
Ser	73.06	67.75	49.63	63.12	41.56	59.54	43.60	91.78
Glu	141.53	140.14	104.56	130.38	118.34	130.93	163.69	107.82
Pro	61.36	59.34	15.54	6.83	35.23	80.12	18.86	112.58
Gly	129.59	133.08	109.95	128.01	129.24	123.74	152.02	162.27
Ala	99.96	104.29	84.44	107.61	96.39	114.67	120.27	137.25
Cys	ND[b]	2.85	ND	3.31	ND	2.88	0.97	4.97
Val	50.20	74.99	55.98	70.65	69.40	76.90	92.07	91.98
Met	11.95	16.22	9.86	15.79	9.60	15.96	13.97	17.23
Ile	49.30	54.14	40.30	53.11	50.43	55.62	62.25	66.03
Leu	109.66	125.69	91.03	116.65	111.04	122.33	138.51	129.98
Tyr	30.97	32.21	27.85	35.40	35.63	34.77	39.33	45.35
Phe	49.78	56.02	45.67	57.30	60.14	57.93	69.01	73.45
Total protein amino acids	1441.84	1235.11	1148.49	1139.50	1275.96	1227.16	1482.31	1515.43

[a] Asparagine and glutamine are converted to aspartate and glutamate, respectively, during extraction; degradation of tryptophan occurs during the acid hydrolysis necessary to obtain the total amino acid content of a sample.

[b] Not detectable.

Table IV. *Effect of Phosphorus Nutrition on Leaf Arginine Content*

Data are calculated from the data presented in Tables II and III.

Rough lemon		Carrizo citrange		Troyer citrange		Trifoliate orange	
−P	+P	−P	+P	−P	+P	−P	+P
ARG expressed as % total nonprotein amino acids							
7.0	0.6	14.5	0.4	10.0	0.5	5.7	0.9
ARG expressed as % total protein amino acids							
12	6	23	5	17	5	11	6
mg protein/g fresh wt							
15	22	13	18	14	21	16	25
μmol protein ARG/mg protein							
11	3	21	3	15	3	10	4

to that of plants receiving 50 μg P/g potting medium for the first 6 weeks (Table VI). In addition, the arginine content of the leaves did not increase during the second 6 weeks.

Arginase and Arginine Decarboxylase Activity. Arginase activity was very low in the leaves of all four rootstocks; in leaves from −P plants, arginase activity was either unaffected or slightly increased. The range of arginase activity observed in +P and −P leaf tissue from all four rootstocks was 0.5 to 1.6 nmol urea formed/mg protein in 1 h. Arginine decarboxylase activity was detected only in leaf tissue from *C. limon*. During P sufficiency, 85 nmol CO_2 were released per mg protein in 1 h. The activity of this enzyme was approximately double in leaves from −P plants.

DISCUSSION

Withholding P during the early development of four commercially important citrus rootstocks, comprising two species and two cultivars of a single hybrid, resulted in a 2- to 4-fold increase in the total arginine content per g dry weight of the youngest, fully expanded leaves. This constituted a net accumulation per g dry weight of 90 to 245 μmol arginine during the development

Table V. *De Novo Arginine Biosynthesis in Young, Fully Expanded Leaves from 7-Month-Old +P and −P Plants*

Young, fully expanded leaves were excised from 7-month-old seedlings initially treated with 100 μg P/g sand (+P) or 1 μg P/g sand (−P) at age 2 weeks. Plants were watered twice a week with one-half strength Hoagland nutrient solution without P. All plants were grown in a growth chamber under 16 h light (310 $\mu E/m^2 \cdot s$) at 32°C and 8 h dark at 21°C. Leaves were cut into 5- × 5-mm sections and preincubated in Shive nutrient solution, pH 7.4, in a water bath shaker. At the end of 2 h, the Shive solution was decanted and fresh Shive solution supplemented with $NaH^{14}CO_3$ (5 mM, 37.5 μCi) was added. Samples were incubated for 3 h at 30°C in a water bath shaker. The amount of [*guanido*-^{14}C]arginine and [^{14}C]urea in the neutralized acid-soluble fraction was determined enzymically.

Rootstock	$NaH^{14}CO_3$ Incorporated into ARG + Urea During the 3-h Incubation[a]	
	+P	−P
	nmol/g fresh wt	
Rough lemon	34	91
Carrizo citrange	11	49
Troyer citrange	11	35
Trifoliate orange	41	52

[a] Values are the average of two separate experiments. The differences between any two replicates did not exceed 20%.

Table VI. *Influence of P Nutrition on Arginine Content and De Novo Arginine Biosynthesis in Young, Fully Expanded Leaves from Citrus limon*

Two-week-old *C. limon* seedlings were transplanted in 10-cm pots containing sterile sand supplemented with P to a final concentration of 5, 50, or 100 μg P/g potting medium. After 6 and 12 weeks, young, fully expanded leaves were excised for analyses. Plants that initially received only 5 μg P/g plotting medium were supplemented with 50 μg P/g potting medium during the second 6 weeks.

P Treatment/Potting Medium		ARG/Dry Wt Leaf Tissue		$NaH^{14}CO_3$ Incorporated into ARG + Urea During the 3-h Incubation/Leaf Tissue[a]	
1st 6 weeks	2nd 6 weeks	1st 6 weeks	2nd 6 weeks	1st 6 weeks	2nd 6 weeks
μg/g		*μmol/g*		*nmol/g fresh wt*	
5	50	111	113	195	43
50	50	66	89	47	60
100	100	57	62	26	24

[a] $NaH^{14}CO_3$ was provided during the incubation at a final concentration of 5 mM and specific radioactivity of 3300 dpm/nmol.

of a P-deficient leaf, a time span of approximately 4 to 6 weeks. The youngest, fully expanded leaves were chosen for the analyses conducted in this study because P is known to be exported in a number of plant species from older tissue to younger, more metabolically active tissue (15). Thus, the youngest, fully expanded leaves should contain the maximum amount of P available to a developing tissue. Second, by using this tissue, our results were not as likely to be compromised by secondary effects that might result from P export or senescence. Our ability to successfully select young, fully expanded leaves of similar physiological age throughout the study was confirmed by the results of separate experiments employing +P rough lemon seedlings aged 6 and 12 weeks and 7 and 7.5 months. The arginine content of young, fully expanded leaves for all experiments averaged 67 ± 4 μmol (mean ± SE, n = 4) per g dry weight. Similarly, the activity of the *de novo* arginine pathway in this tissue averaged 30 ± 3 nmol (mean ± SE, n = 4) $NaH^{14}CO_3$ incorporated into arginine plus urea per g fresh weight leaf tissue during the 3-h incubation period. Since the levels of these two parameters did not increase as the plants aged, the results confirm that an initial treatment of 100 μg P/g potting medium provided adequate P for the duration of the study.

The results of the present study confirm the previous report (14) that arginine accumulated in the leaves of *C. limon* during P deprivation and demonstrate that this metabolic response is common to plants of other genera. It is interesting to note that the amino acid composition of the nonprotein and protein fractions of the +P control plants is very similar in all four rootstocks, even when two different species, *C. limon* and *P. trifoliata*, are compared. The levels of each amino acid are comparable to those reported for 4-month-old leaves from other *Citrus* species (11). However, the values for nonprotein amino acids reported by Nemec and Meredith (14) for 5-month-old *C. limon* seedlings, from which all the leaves were pooled for amino acid analysis, were very much lower than the values we obtained in the present study. The influence of P deprivation on amino acid metabolism was strikingly similar for all four rootstocks: arginine accumulated in both the nonprotein and protein fractions. With the exception of rough lemon, the increase in arginine content of the leaves were accompanied by a concomitant decrease in the level of most of the other amino acids.

The etiology of the observed increase in leaf arginine content also appeared to be strikingly similar for all four rootstocks: activity of the *de novo* arginine pathway increased during P deficiency, and in each case, the increased activity was proportional to the observed accumulation of arginine. Convincing evidence to support a cause and effect relationship between P nutrition and arginine biosynthesis was provided by experiments that demonstrated: (a) that both arginine content and activity of the arginine pathway increased in parallel to the increasing severity of P deprivation (Table VI); and (b) when −P seedlings were resupplied with P, activity of the *de novo* arginine pathway was reduced and accumulation of arginine ceased (Table VI). Finally, the results of the present study suggest that reduced metabolism of arginine by arginase and arginine decarboxylase or reduced utilization of arginine in protein synthesis are not factors contributing to the accumulation of arginine during P deficiency. These metabolic reactions were either unaffected or slightly enhanced during P deprivation. Taken together, these results provide strong evidence that the accumulation of arginine in leaves during P deficiency is due to increased activity of the *de novo* arginine biosynthetic pathway.

Investigations of the physiological changes brought about by mineral nutrient deficiencies in plants are minimal. In most cases, it is assumed that all metabolic processes requiring the deficient nutrient will be disrupted. Although this may be true in the long run, it is certain that some physiological processes requiring the nutrient are affected earlier than others. In fact, it appears that some metabolic events are specifically spared from the effects of the deficiency until death of the cell is imminent. This appears to be the case for *de novo* arginine biosynthesis during phosphorus deficiency. *De novo* biosynthesis of each arginine molecule requires a phosphorylated precursor, carbamylphosphate, and a total of three ATPs. Thus, the activity of the *de novo* arginine pathway would logically seem prone to inhibition by P deprivation. The increased activity of this pathway during P deficiency, along with reports of increased nitrogen and ammonia levels in the leaves of −P *C. limon* and *C. aurantium* (14), suggest to us the possibility that P deficiency

results in a state of ammonia toxicity.

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