Ammonia accumulation: A key response of plants to phosphorus deficiency

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Abstract

Investigations of nitrogen metabolism during phosphorus deficiency of several plant species provided evidence that arginine accumulated during P deficiency due to increased activity of the pathway for the biosynthesis *de novo* of arginine. Concurrently, there was increased degradation of most species of amino acids, including arginine. Arginase and arginine decarboxylase activities during P deprivation were normal or slightly increased. The reduction in plant height and biomass during P deprivation, without a concomitant decrease in nitrate uptake, resulted in the accumulation of nitrate in the leaves of P-deficient plants. Increased availability of nitrate, normal or accelerated nitrate reductase activity, and the increased turnover of amino acids, which occurred during P deficiency, resulted in increased generation and in some cases, significant accumulation of ammonia-ammonium. Increased activity of the arginine *de novo* biosynthetic pathway was demonstrated to be positively correlated with endogenous leaf ammonia-ammonium content and exogenous ammonium supply.

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Introduction

L-arginine and the arginine pathway intermediates, L-ornithine and L-citrulline, have been demonstrated to accumulate during phosphorus deficiency in a variety of higher plant species (Freiberg and Steward, 1960; Gelieter and Parker 1957; Nemec and Meredith, 1981). Under conditions of low P fertilization, free arginine levels were 10 to 50 times higher in leaves of four commercially important citrus rootstocks than in control plants receiving an adequate supply of P (Nemec and Meredith, 1981; Rabe and Lovatt, 1984) or in plants also grown with low P fertilization, but in symbiosis with vesicular-arbuscular mycorrhizae (Nemec and Meredith, 1981). In addition, the arginine content of the protein fraction increased 2- to 4-fold in P-deficient leaves of the citrus rootstocks (Rabe and Lovatt, 1984). P deficiency also caused an increase in the free arginine content of leaves of banana (4-fold)(Freiberg and Steward, 1960), alfalfa (4- to 20-fold)(Gelieter and Parker, 1957), and summer squash (6-fold)(Rabe and Lovatt, 1986a). It is important to note that for squash and for several species of citrus rootstocks, the total amino acid content of the leaves, excluding arginine, decreased during P deficiency to a level below that of the P-sufficient control plants (Rabe and Lovatt, 1984, 1986a). These results are consistent with the earlier report of Achituv and Bar-Akiva (1978), which provided evidence that during P deficiency, arginine was preferentially synthesized at the expense of other proteinaceous amino acids and did not accumulate as a result of protein degradation.

Thus, the goal of this communication is to demonstrate, using unpublished and selected previously published data from the work of Rabe and Lovatt (1984, 1986a, b),(1) that the arginine that accumulates in the leaves of P-deficient plants is, in fact accumulating due to increased activity of the pathway for the *de novo* biosynthesis of arginine; (2) is not accumulating due to increased protein degradation and/or inhibition of arginase or arginine decarboxylase activities; and (3) that the increased activity of the arginine *de novo* biosynthetic pathway during P deficiency is a direct result of the increased NH_3-NH_4content of the P-deficient leaves.

Materials and Methods

Chemicals. All radiolabelled chemicals were purchased from ICN Pharmaceuticals. Liquiscint (liquid scintillation cocktail) was purchased from National Diagnostics. Mineral salts for Hoagland's nutrient solution (Hoagland and Arnon, 1950) were of analytical reagent quality from Fisher. All other chemicals were purchased from Sigma.

Plant materials. Seed of *Citrus limon* (L.) Burm. f cv. rough lemon, *Poncirus trifoliata* (L.) Raf. cv. Australian trifoliate orange, and *C. sinensis* (L.) Osbeck x *P. trifoliata* cv. Carrizo and cv. Troyer citrange were planted in sterile sand containing P applied as $Ca(H_2PO_4)_2$ at the range of 5 µg/g (P deficient; -P) or 100 µg/g (P sufficient; + P) and watered with half-strength Hoagland's solution as previously described (Rabe and Lovatt, 1984). Analyses were carried out when plants were 6, 12 and 28 weeks old. All analyses employed the youngest, fully expanded leaves harvested and pooled from 5 to 20 plants. The youngest, fully expanded (YFE) leaves of the citrus rootstocks were approximately 21 days old. Experiments were initiated at seed germination, no two experiments were initiated in the same week, and several seed lots were used in the many experiments. Experiments consisted of single treatments.

Leaf tissue preparation. Excised leaves were immediately frozen in liquid N, lyophilized, and ground with a Wiley mill to a size fine enough to pass through a 40-mesh screen.

Leaf P and N content. Phosphorus content of leaves was determined for a 50-mg sample by a colorimetric assay employing molybdivanadophosphoric acid (Kitson and Mellon, 1944). Absorbance at 420 nm was linear for concentrations of P from 0 to 0,4 mg per 100 mg leaf tissue (dry weight).

Total N was determined for a 25-mg sample using the conventional micro-Kjeldahl method; nitrate for a 100-mg sample extracted in 100 ml of 16 mM $CaSO_42H_2O$ using a Technichon Autoanalyzer (Technicon, 1973); and NH_3-NH_4^+ for a 200-mg sample extracted in 50 ml of 1 N KCl using a Technicon Autoanalyzer (Technicon, 1987). Amino acid content

rmined using a Beckman 120C Amino Acid Analyzer ...ated with commercial standards in the appropriate buffer at 570 nm (Labanauskas and Handy, 1970). Soluble protein content of the leaf tissue was determined according to Bradford (1976).

Incorporation $NaH^{14}CO_3$ into the combined pool of arginine plus urea.

Activity of the *de novo* arginine biosynthetic pathway was assessed in the intact cells of the youngest, fully expanded leaves (500 mg fresh leaf tissue cut into 5 x 5 mm pieces, midvein removed) by measuring the incorporation of $NaH^{14}CO_3$ (5 mM, 37,5 µCi) into arginine and urea during a 3-hour incubation period in Shive's nutrient solution. The amounts of [guanido-^{14}C]arginine and [^{14}C]urea synthesized by the leaf tissue from $NaH^{14}CO_3$ was determined using commercial arginase and urease as described previously by Lovatt and Cheng (1984).

Nitrate reductase activity in cell-free extracts of +P and –P leaves. Cell-free extracts were prepared from 1 g fresh leaf tissue homogenized with a Duall all-glass tissue grinder (Kontes Glassware) (citrus leaves were frozen in liquid N_2 and ground with a mortar and pestle before homogenization) in 5 ml 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA, 1 mM cysteine, and 3% (w/v) casein and centrifuged at 10,000 g for 10 min at 0°C. The supernatant fraction served as the source of enzyme. The activity of nitrate was measured according to the method of Scholl, Harper and Hageman (1974) in a 2-ml reaction mixture of the following composition: 50 µmol K-phosphate (pH 7,5), 20 µmol KNO_3, 0,8 µmol NADH, and 50 to 200 µl enzyme. After incubation at 30°C for 30 min, the reaction was stopped with the addition of 50 µmol/ml zinc acetate. Phenazine methosulfate (14 nmol/ml) was added as a postassay treatment to oxidize excess NADH.

Results

Withholding P resulted in a reduction in total biomass and plant height which became significantly greater as the deprivation continued (Table 1). Consistent with previous reports in the literature (Freiberg and Steward, 1960; Gelieter and Parker, 1957; Nemec and Meredith, 1981; Rabe and Lovatt, 1984, 1986a, b), arginine accumulated (Table 1). The amount of arginine that accumulated increased with the severity of the stress. While the reduction in plant biomass, including inhibition of shoot development and leaf expansion, may have a concentrating effect and thus contribute to the increase in leaf arginine content, the activity of the pathway for the *de novo* biosynthesis of arginine increased in a manner that also parralleled the severity of P deprivation and arginine accumulation (Table 1).

TABLE 1 Effects of phosphorus deprivation on plant growth and nitrogen metabolism expressed as P-deficient/P-sufficient x 100%[z]

Plant (Species)	Treatment duration	Plant growth	Total N[y]	NO_3[x]	NH_3–NH_4[+v]	*De novo* Arginine biosynthesis[u]	Total arginine[t]	Total amino acids excluding arginine[s]
Rough lemon	6 wk	55	88	146	119	993	194	86
(Citrus limon)	12 wk	24	129	337	177	1 300	425	77
Carrizo citrange	6 wk	81	97	147	237	300	115	97
(Citrus sinensis x	12 wk	59	82	152	106	740	295	73
Poncirus trifoliate)	28 wk	15	131	300	216	445	495	86
Troyer citrange	28 wk	15	128	319	107	318	370	93
(C. sinensis x P. trifoliata)								
Trifoliate orange	12 wk	48	91	161	60	271	150	
(P. trifoliata)	28 wk	20	116	421		127	202	92

[z] Data used to calculate P-deficient/P-sufficient x 100% were taken from Rabe and Lovatt (1984, 1986a, b, and unpublished results).
[y] Average value ± STD.DEV. for the P-sufficient control plants was 28,6 ± 2,8 mg N per g dry wt leaf tissue for all experiments.
[x] Values for P-sufficient control plants ranged from 200 to 1 000 µg NO_3^- per g dry wt leaf tissue for all experiments.
[v] Average value for P-sufficient control plants was approximately 750 µg NH_3–NH_4^+ per g dry wt leaf tissue for all experiments.
[u] For all experiments, incorporation of $NaH^{14}CO_3$ into arginine plus urea per g fr wt leaf tissue during a 3-h incubation at 30°C ranged from 14 to 40 nmol for leaves from P-sufficient control plants.
[t] Total arginine content of leaves from the P-sufficient control plants for all experiments was approximately 75 µmol per g dry wt leaf tissue.
[s] Leaf content of total amino acids, excluding arginine, was 1 371 ± 129 (X±SDT.DEV.) dry wt leaf tissue from P-sufficient control plants.

To determine the potential contribution of arginine from degradation or inhibition of protein synthesis, we compared the protein content of healthy P-sufficient control plants with that of citrus rootstock species deprived of P for 28 weeks. The protein content of leaves from the P-deficient plants was 30% less: compare 14,5 ± 1,3 mg protein/g fr wt for –P leaves to 21,5 ± 2,9 ($\bar{X}$±STD.DEV., N = the four rootstock species) for the leaves of +P plants. Further, we evaluated the significance of the increased availability of arginine released by protein degradation or reduced protein synthesis by comparing the activity of the arginine metabolizing enzymes arginase and arginine decarboxylase in the leaves of the same plants. The activities of the two enzymes were very low in leaves from P-sufficient citrus rootstocks. These activities were either unaffected or accelerated up to 2-fold in the leaves from citrus rootstocks deprived of P for 28 weeks.

In light of the relatively small decrease in protein content and the lack of inhibition of argininase and arginine decarboxylase activity during P deprivation, it must be concluded that arginine accumulates during P deficiency because the rate of *de novo* synthesis is stimulated to a greater degree than arginine catabolizing reactions. In light of the data demonstrating that the total leaf content of all other amino acids was unchanged or less at all stages of P deficiency (Table 1), the results of our research provide strong evidence that arginine is preferentially synthesized over most other amino acids during P-deprivation.

While total N content of leaves did not increase until the growth of the plants was severely restricted by prolonged P deprivation, the NO_3^- content of leaves increased significantly with only a 20% decrease in plant biomass (Table 1). A re-

shoot growth and leaf expansion would be expected to have a concentrating effect on leaf NO_3^- content. With a 20% decrease in biomass, the effect should be minimal. The increase in leaf nitrate content even during this early stage of P deficiency suggests that nitrate uptake, at least early in P deprivation, was not adversely affected. Concomitant with the increase in leaf nitrate content during P deficiency, there was an increase in leaf NH_3–NH_4 content and/or an increase in the activity the arginine *de novo* biosynthetic pathway in a manner that paralleled the severity of the stress (Table 1). The reduction in plant biomass would be expected to also have a concentrating effect on leaf NH_3–NH_4 content and on leaf amino acid content. The fact that the pool of total amino acids excluding arginine remains the same or decreases during P deprivation suggests that amino acid turnover may contribute to the increase availability of NH_3–NH_4. The specific activity of nitrate reductase was unaffected in leaves from P-deficient plants. Thus, given the significant increase in available nitrate during P deprivation, nitrate reduction probably also contributed to the increasing pool of free NH_3–NH_4.

To confirm that the activity of the pathway for the *de novo* biosynthesis of arginine increased in response to an increased availability of NH_3–NH_4 in the P-deficient leaves, responsiveness and sensitivity of the *de novo* arginine biosynthetic pathway to changing leaf NH_3–NH_4 levels was tested. The petioles of detached leaves were immersed in aerated solutions of NH_4Cl at final concentrations ranging from 10 to 50 mM for 3 to 15 hours. *De novo* arginine biosynthesis increased with increasing concentration and duration of exposure (Table 2). The results of this experiment also demonstrated that the symptoms of P deficiency could be induced by NH_3–NH_4^+.

TABLE 2 De novo *arginine biosynthesis in ammonia-treated, young, fully expanded leaves from P-sufficient control plants expressed as NH‡ -treated/control x 100%*

NH_4Cl concentration	Duration of treatment	*De novo* arginine biosynthesis[z]
30	3	278
10	15	425
50	3	512
50	15	600

[z] Average value ± STD.DEV. (N = 5 experiments) for basal incorporation of $NaH^{14}CO_3$ in arginine plus urea per g fr wt leaf tissue during the 3-h incubation at 30°C for detached leaves of P-sufficient rough lemon plants immersed in aerated solutions for up to 15 h was 9.3 ± 1.4 nmol (Rabe and Lovatt, 1986b).

Taken together, the changes in nitrogen metabolism induced by P deprivation strongly suggest the following sequence of biochemical events leading to the accumulation of arginine: reduced plant growth, NO_3^- accumulation, increased NH_3–NH_4^+ production, and accelerated arginine *de novo* biosynthesis.

Discussion

The results of this research provided evidence that increased NH_3–NH_4^+ production during phosphorus deprivation is a key response of plants to this mineral nutrient deficiency. The data strongly suggest that it is this metabolic event that causes the acceleration *de novo* arginine biosynthesis and the accumulation of arginine during P deficiency. In light of the fact that the activities of the arginine catabolizing enzymes arginase and arginine decarboxylase were not inhibited in leaves from P-deficient plants, accelerated *de novo* biosynthesis of arginine is the only viable explanation for arginine accumulation.

Comparison of the pool size of free NH_3–NH_4^+ and/or rate of *de novo* arginine biosynthesis for the four citrus rootstocks studied provides a good indication of the relative P dependency of the rootstocks. Rough lemon is a P-dependent rootstock, while the trifoliate orange is P-independent (Embleton *et al.*, 1973). The two citranges, hybrids between *Citrus* and *Poncirus*, are intermediate in their dependence on P. The increased production of NH_3–NH_4^+, as evidenced by the accumulation of NH_3–NH_4^+ in leaf tissue or by the degree of stimulation of the activity of the *de novo* arginine biosynthetic pathway, occurred sooner and to a greater degree during P deprivation of rough lemon than trifoliate orange. The two citranges exhibited an intermediate response (Table 1).

Phosphorus became limiting to the growth of the more P-dependent rootstock sooner and to a greater degree, causing a reduction in growth that eventually resulted in the accumulation of N in the leaves. Failure of the plant to regulate N (NO_3^-) uptake is consistent with the fact that land plants evolved under conditions in which N was limiting. Thus, there was little selection pressure for mechanisms regulating NO_3^- uptake or reduction (Miflin and Lea, 1980). In light of this, it is clear that ranking rootstocks for their tendency to maintain a given level of N in scion leaves under field conditions (Embleton *et al.*, 1973) would be compromised by the fact that P deficiency, or probably any other stress that limits canopy growth (except N deficiency), would result in N accumulation in the leaves.

A number of considerations logically follow from the results of our research. First, the N:P ratio, rather than the actual levels of these nutrients, may be more useful in evaluating the severity of P deficiency. Whenever N:P ratios >20 were observed (Table 3), *de novo* arginine biosynthesis was increased significantly (Table 1), and significant amounts of excess arginine accumulated (Table 1). These changes are consistent with NH_3 detoxification. The N:P ratio for P-sufficient plants was always <15 (Table 3); arginine metabolism was normal.

TABLE 3 Nitrogen: phosphorus ratio in the leaf tissue of four citrus rootstocks during P deficiency and P sufficiency

	Sand culture					
	Duration					
	6 weeks		12 weeks		28 weeks	
Rootstock	+P	–P	+P	–P	+P	–P
Rough lemon	14	19	14	38	13	23
Carrizo citrange	13	12	14	26	13	34
Trifoliate orange	—	—	11	32	11	20

From Rabe and Lovatt (1986b).

J. leaf nutrient analysis is used routinely to design fertilizer programmes for citrus cultivars and most other crops. Our results suggest that leaf total N content, which is currently the basis for N fertilizer recommendations, may not be as valuable as a measurement of leaf NH_3–NH_4^+ and arginine concentrations. For example, during the early stages of P deficiency, leaf total N content did not increase, but nitrate and NH_3–NH_4^+ were significantly increased (Table 1).

Finally, the results of this research suggest that the P dependency of citrus rootstocks used commercially and the P nutritional status of the scions should be taken into consideration when designing a fertilization programme, especially with regard to the application of N when soil P levels are marginal.

The relative P dependency of the rootstocks determined in this study paralleled their dependency on vesicular-arbuscular mycorrhizae (VAM), as determined by Menge, Johnson and Platt (1978). In addition, relative P dependency appears to be inversely related to the hydraulic conductivity of roots (Syvertsen and Graham, 1985). Trifoliate orange and Carrizo citrange have higher conductivities (resulting in higher mass flow of water and minerals to the shoots) relative to sour orange, a rootstock that ranked very similar to rough lemon for both P dependency (Embleton *et al.*, 1973) and VAM dependency (Menge *et al.*, 1978).

Finally, the relative P dependency appears to be indicative of the susceptibility of citrus rootstocks to citrus nematode [*Tylenchulus semipenetrans* (Cobb)]. Sour orange and rough lemon, which are highly P-dependent (Embleton *et al.*, 1973), were demonstrated to be highly susceptible to root infection by citrus nematode. Troyer citrange was moderately susceptible, and trifoliate orange was resistant to *T. semipenetrans* (Ibrahim, Taha and Hassaw, 1985).

References

ACHITUV, M. & BAR-AKIVA, N., 1978. Metabolic pathway of α-keto glutarate in citrus leaves as affected by phosphorus nutrition. *Plant Physiol.* 61, 703-705.

BRADFORD, M.M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

EMBLETON, T.W., JONES, W.W., LABANAUSKAS, C.K. & REUTHER, W., 1973. Leaf analysis as a diagnostic tool and guide to fertilization. In W. Reuther (ed.). The citrus industry, Ch. 6, Vol. III. Univ Berkley Press, Berkley, Calif.

FREIBERG, S.R. & STEWARD, F.C., 1960. Physiological investigations on the banana plant. III. Factors which affect the nitrogen compounds of the leaves. *Ann. Bot.* 24, 147-157.

GELIETER, M.E. & PARKER, H.E., 1957. Phosphorus deficiency and arginine accumulation in alfalfa. *Arch. Biochem. Biophys.* 71, 430-436.

HOAGLAND, D.R. & ARNON, D.E., 1950. The water-culture method for growing plants without soil. Univ. Berkley Press, Berkley, Calif, Circ. 347.

IBRAHIM, I.K.A., TAHA, M.W. & HASSAW, M.W.A., 1985. Resistance of 7 citrus rootstocks to *Tylenchulus semipenetrans* and *Meloidogyne* ssp. *Egypt. J. Nematol.* 17, 499 (Abstr.).

KITSON, R.E. & MELLON, M.O., 1944. Colorimetric determination of phosphorus as molybdivanadophosphoric acid. *Ind. & Eng. Chem. Anal.* 16, 379-383.

LABANAUSKAS, C.K. & HANDY, M.F., 1970. The effects of iron and manganese deficiencies on accumulation of nonprotein and protein amino acids in macadamia leaves. *J. Amer. Soc. Hort. Sci.* 95, 218-223.

LOVATT, C.J. & CHENG, A., 1984. Application of commercial enzymes to measure the activity of the arginine pathway-urea cycle in intact cells. *Anal. Biochem.* 142, 305-311.

MENGE, J.A., JOHNSON, E.L.V. & PLATT, R.G., 1978. Mycorrhizal dependency of several citrus cultivars under three nutrient regimes. *New Phytol.* 81, 553-559.

MIFLIN, B.J. & LEA, P.J., 1980. Ammonia assimilation. In P.K. Stumpf and E.E. Conn (eds), p. 169-202. The biochemistry of plants, Vol. 5. Academic Press, New York.

NEMEC, S. & MEREDITH, F.I., 1981. Amino acid content of leaves in mycorrhizal and nonmycorrhizal citrus rootstocks. *Ann. Bot.* 47, 351-358.

RABE, E. & LOVATT, C.J., 1984. *De novo* arginine biosynthesis in leaves of phosphorus-deficient *Citrus* and *Poncirus* species. *Plant Physiol.* 76, 747-752.

RABE, E. & LOVATT, C.J., 1986a. Increased arginine biosynthesis during phosphorus deficiency: a response to the increased ammonia content of leaves. *Plant Physiol.* 81, 774-779.

RABE, E. & LOVATT, C.J., 1986b. Relative phosphorus dependency of citrus rootstocks is reflected in leaf nitrogen metabolism. *J. Amer. Soc. Hort. Sci.* 111, 922-926.

SCHOLL, R.L., HARPER, J.E. & HAGEMAN, R.H., 1974. Improvements of the nitrate color development in assays of nitrate reductase by phenazine methosulfate and zinc acetate. *Plant Physiol.* 53, 825-828.

SYVERTSEN, J.P. & GRAHAM, J.H., 1985. Hydraulic conductivity of roots, mineral nutrition and leaf gas exchange of citrus rootstocks. *Hort. Science* 20, 588 (Abstr.)

TECHNICON INDUSTRIAL METHODOLOGY (Jan. 1973), AA II, Industrial method No. 100-70W.

TECHNICON INDUSTRIAL METHODOLOGY (Nov. 1978), AA II, Industrial method No. 329-74W/B.