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Communication

Citrate, Malate, and Succinate Concentration in Exudates from P-Sufficient and P-Stressed *Medicago sativa* L. Seedlings[1]

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ABSTRACT

Under certain stress conditions roots exude organic molecules, which may facilitate the uptake of nutrients. The objective of this research was to identify and measure the effect of low P upon the exudation of organic acids by roots of alfalfa (*Medicago sativa* L.) seedlings. Surface-sterilized alfalfa seeds were grown aseptically in sterile sand using an apparatus specially designed for the addition of ±P nutrient solutions and for the collection of root exudates. Citric, malic, and succinic acids were detected in the root exudates of 24-day-old alfalfa seedlings. Citrate exudation from the roots of P-stressed alfalfa was 182% that of plants receiving a complete nutrient solution. The increased release of citrate may provide a mechanism by which P-stressed plants enhance the availability of P in the rhizosphere.

The rhizosphere is a dynamic microenvironment continually renewed by root growth and the substances released by the root. Vancura (16) referred to these exudates as the "determining factor of the rhizosphere effect." Since the early 1950s investigators have demonstrated the ability of organic acids *in vitro* to increase the availability of phosphorus from aluminum and iron phosphates (2, 7, 15) and from calcium phosphates (6). Acetic, aconitic, citric, fumaric, glycolic, malic, malonic, oxalic, and succinic acids have all been identified in the root exudates of a variety of vegetables (17) and pine trees (12, 13). Recently, Gardner *et al.* (3) have identified citrate in *Lupinus albus* root exudates and proposed that citrate reacts in soil to form ferric hydroxyphosphate chelates which diffuse to the root surface.

Under P deficiency amino acids in the root exudates increased from 104.5 to 248.5 μmol/plant, which contrasted sharply with the almost 4-fold decrease under N deficiency (104.5–25.1 μmol/plant; 1). Graham *et al.* (5) observed P deficiency to result in a 3-fold increase in amino acid exudation with only a concentration increase of 60% within the root.

The effective study of the rhizosphere has been limited in the past due to the difficulty of isolating root effects from general soil and microbiological effects and quantifying those effects. The objective of this research was to identify and measure the effect of P deficiency upon the exudation of organic acids by the growing aseptic roots of alfalfa (*Medicago sativa* L.) seedlings.

MATERIALS AND METHODS

Design of Culture Apparatus for Exudate Measurement. The main body of the apparatus consisted of a 135 mm long × 21 mm diameter tube (Analytichem International, Harbor City, CA) wrapped in Al foil, and connected via a rubber stopper to a 250 ml Buchner flask (Fig. 1). Glass wool was placed at the bottom of the tube and the tube filled with quartz sand (0.6–0.9 mm) which had been heated at 500°C for 30 min, allowed to cool, and rinsed with deionized distilled water. The Buchner flask served as the collection vessel for root washings and was protected from the outside environment by an 8 to 10 cm piece of tygon tubing stuffed with sterile washed cotton and attached

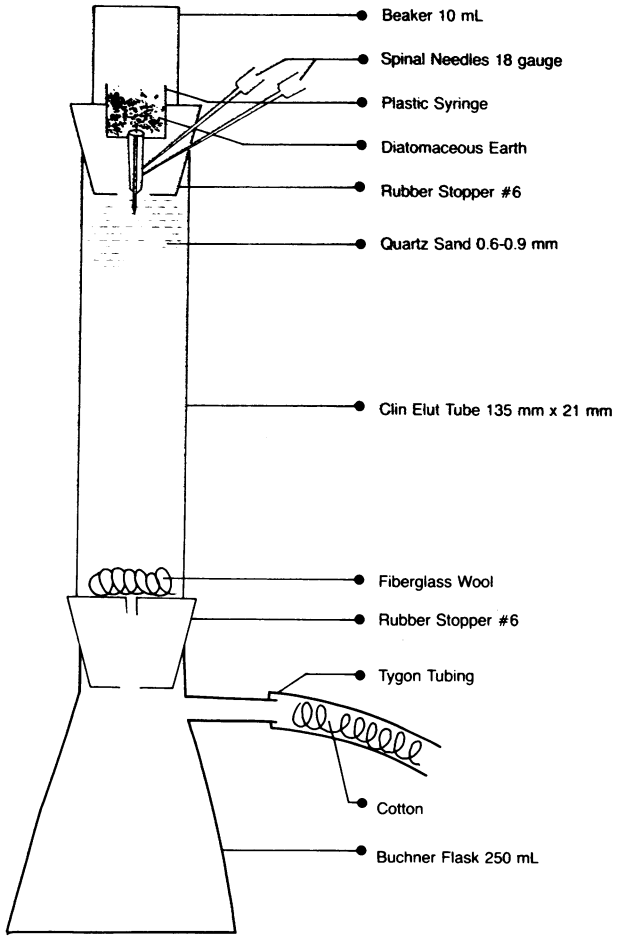

FIG. 1. Apparatus for the growth of alfalfa and the aseptic collection of root exudates.

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to its side arm.

The top part of the apparatus, into which the seedling was transplanted, consisted of the bottom 3 ml of a 12 ml Clin-Elut tube (Fig. 1). This syringe was chosen to hold the alfalfa seedling because of the 1 cm protrusion at its end which fitted around the radicle without allowing the cotyledon to fall through. The 1 cm nipple served also as a buffer between the sterile media and the outside environment, minimizing the invasion of microorganisms into the system.

Fertilization and final washing of the roots were facilitated by two 18-gauge spinal needles inserted into and through the upper rubber stopper. The nutrient solution was passed to the Clin-Elut tube through one spinal needle attached by tygon tubing, and metal-plated gang valves (Fritz Chemical Co.) to two 1-L Erlenmeyer flasks, containing 500 ml of the appropriate nutrient solution. The flasks were sealed with rubber stoppers with vents of sterile washed cotton to serve as an air intake and to inhibit the entry of microorganisms. The top of the culture tube and the unattached spinal needle were covered with Al foil. Eight replicates and one control were placed in a wooden rack and autoclaved for 30 min at 121°C. The flasks were autoclaved in the upright position and then placed in the growth chamber in an inverted position.

Seeds of alfalfa (*Medicago sativa* var "Buffalo) were soaked in ethyl alcohol for 10 min while under vacuum to facilitate the entry of the sterilizing solution into the seed coat. The alcohol was decanted, then the seeds shaken in Clorox (5.25% hypochlorite) for 45 min. Seeds were washed with two rinses of sterile water and immediately deposited into sterile Petri dishes containing nutrient yeast extract agar. Seeds were germinated in the dark at room temperature (23–25°C) for 48 h or until they had attained a length of at least 1 cm.

Sterile seedlings were transferred to the culture tubes and 3 g of a sterilized, inert, hydrophilic substance (greater than 90% diatamaceous earth, Fisher Scientific Co.) were quickly poured into the top of each culture tube and watered with 2 ml of sterilized, filtered, deionized distilled water. The entire system was placed in a growth chamber at temperatures of 23°C for the 14 h day and 19°C for the 10 h night. Light intensity was 450 μE m^{-2} s^{-1} for the photoperiod.

The plants were watered with 2 ml every 24 h for 2 d. After the first day a sterilized 10 ml beaker replaced the Al foil on top of the culture apparatus. Fertilization of plants began after the first watering. Daily aliquots of 2 to 3 ml of nutrient solution were dispensed to each culture.

The nutrient solution consisted of: 6 mM KNO_5, 4 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 2 mM $MgSO_4$, 0.3 mM $FeSO_4$, 2.3 μM H_3BO_3, 0.455 μM $MnSO_4$, 0.6 μM $ZnSO_4$, 0.15 μM $CuSO_4$, 0.10 μM $NaMoO_4$, and brought to a pH of 6.5 with 1 M NaOH. The low P medium contained one-tenth the amount of original (0.01 mM) $NH_4H_2PO_4$ and 0.1 mM $(NH_4)_2SO_4$, the remaining nutrients were the same.

Collection and Analyses of Root Exudates. After 24 d the roots were aseptically rinsed with sterilized, deionized water. This procedure was carried out by attaching an autoclaved 500 ml Erlenmeyer flask to the unused spinal needle. The flask was inverted to rinse the roots with sterilized water. Air was allowed to enter the flask by a glass tube stuffed with sterilized washed cotton. After the rinse was completed the collection flask was removed from the apparatus and sterility checked by transferring 1.0 ml of sample to sterile nutrient yeast extract agar plates which were incubated in the dark at room temperature. Counts were made to estimate contamination after 48 h. Uncontaminated samples were capped with parafilm and refrigerated for further analysis.

Plant tops were collected by cutting them off at the surface of the inert matrix, rinsing, and then placing in paper coin bags. The roots were harvested by cutting the shoot at the surface of the sand, gently rinsing the sand from the roots with deionized distilled water, and then placing them in paper coin bags. Plant parts were dried at 65°C for 24 h, weighed, digested in HNO_3-$HClO_4$, and analyzed for Ca, Mg, and K by atomic absorption spectrometry and for P as described by Murphy and Riley (9).

Uncontaminated exudates were filtered through a 0.8 μm Millipore filter, rotary evaporated at 40°C to approximately 10 ml, and frozen. Exudates were thawed and then passed through cation (Dowex 50 W-X8) and anion (Bio-Rad AG1-X8, formate form) exchange columns. Organic acids were eluted with 10 ml of 8 M formic acid and then 4 ml of filtered deionized water. Samples containing the organic acids were rotary evaporated to dryness at 40°C and resuspended in 1 ml of 7 mM H_2SO_4 and frozen.

Samples were thawed, air dried at 35°C, and then derivatized with tertiary-*N*-butyl-dimethyl-silyl-triflouroacetic acid using procedures similar to those described by Gehrke *et al.* (4). Samples were injected into a Perkin Elmer Gas Chromatogram using the methods developed by T. P. Mawhinney (8). Products were detected by flame ionization and recorded on a Perkin-Elmer recorder. Peaks were integrated and quantities determined by comparing to an internal standard of glutaric acid. Peaks were identified against a set of standard peaks obtained using identical techniques with known organic acids. Comparisons between parameters measured were made using LSD values (14).

RESULTS AND DISCUSSION

Citric, malic, and succinic acids were detected in the root exudates of 24-d-old alfalfa seedlings (Table I). The absence of other types of nonvolatile organic acids may be a reflection of the alfalfa plant itself, since different species of plants have been found to exude varying types of organic acids from their roots (12, 13, 17). Another explanation for the relatively few organic acids detected in the root exudates of alfalfa may be that the derivatization and gas chromatography technique used in our experiment provided superior separation of the organic acids from other unknown acids relative to paper chromatography techniques used by earlier investigators. No organic acids were found in our controls, indicating that the organic acids peaks (Fig. 2) were definitely from plant roots. Some of the unidentified peaks are most likely polystyrene compounds from the exchange resins as the controls also exhibited the same group of peaks. The peaks occurring before the succinate peak were most likely nitrogen, phosphorus, and sulfur oxides from the nutrient solution as these same peaks were present in the controls.

The amount of organic acids found in the root exudates of P-stressed plants suggest differences do exist between plants grown with a full nutrient solution relative to plants receiving one-tenth the amount of P (Table I). The P content of the roots of P-stressed plants was significantly lower than the P content of the roots of plants receiving a full nutrient solution (Table II). No differences were observed in the P content of the tops of the two groups of plants. Potassium, Mg, and Ca concentrations were not different between the two groups of plants for both tops and roots.

Table I. *Amounts of Citrate, Malate, and Succinate in the Root Exudates of 24-d-old Alfalfa Seedlings Receiving a Full Nutrient Solution and a P-deficient Nutrient Solution*

Types of Plants	Citrate	Malate	Succinate	Total
		μmol/g dry root		
Unstressed plants	17.18	18.49	2.85	38.46
P-stressed plants	31.20*	13.39	4.33	48.92
LSD (0.05)	12.7	18.2	3.1	16.0

* Significantly higher at the 5% level.

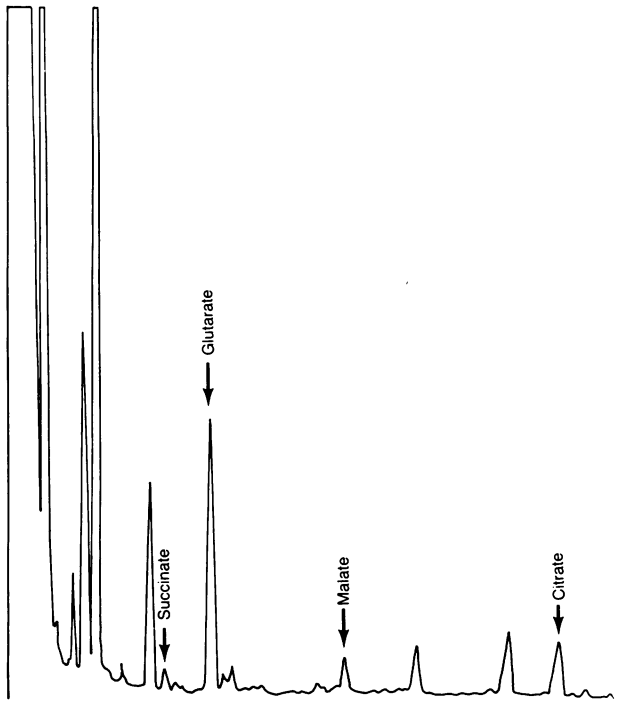

FIG. 2. Chromatograph showing the position of organic acid peaks of the root washings from a P-stressed 24-d-old alfalfa seedling.

Table II. *Phosphorus, Calcium, and Potassium Content in the Tops and Roots of 24-d-old Alfalfa Seedlings Receiving a Full Nutrient Solution and a P-deficient Nutrient Solution*

Type of Plants	Dry weight	P	Ca	K	Mg
	mg	*mg/kg*		%	
Plant tops					
Unstressed plants	10.8	2197	2.37	4.62	0.45
P-stressed plants	9.4	2193	2.24	4.71	0.41
LSD (0.05)	4.0	258	0.93	1.12	0.11
Plant roots					
Unstressed plants	6.6	2043*	1.12	6.44	1.72
P-stressed plants	8.5	1454	0.94	6.15	1.42
LSD (0.05)	3.4	215	0.71	1.33	0.48

* Significantly higher at the 5% level.

Citrate and malate were present in the largest amounts in the exudates of plants receiving a full nutrient solution while citrate concentration was highest in exudates of P-deficient plants. In both groups of plants, succinate constituted the smallest proportion of the organic acids in the root exudates. The variation between plants was relatively large so care must be taken in comparing amounts. The total amount of organic acids released from P-deficient plants was numerically, but not statistically higher than from plants receiving a full nutrient solution (Table I). Citrate, however, was released in significantly greater quantities from P-stressed plants than from plants receiving a full nutrient solution. Similarly, Gardner *et al.* (3) measured high levels of citrate in solutions from around *Lupinus albus* roots. Exudates and extracts from roots of P-deficient sudangrass (*Sorghum vulgare*) contained significantly more carboxylic acid than those from P-amended plants (11). Bowen (1) and Graham *et al.* (5) found a 2-fold and 3-fold increase, respectively, in the quantities of amino acids released from P stressed plants. Graham *et al.* (5) found a correlation between increased exudation and membrane permeability, using potassium-rubidium efflux, and concluded that root exudation is directly related to the permeability of the membrane which is controlled largely by the phospholipid content of the root (10). Depressed phospholipid content in the P-stressed plants may account for the increased release of citrate reported in Table I.

In one of the few experiments reporting quantities of individual types of organic acid released from the growing root, Smith (12, 13) found oxalic and acetic acids constituted the largest percentage of the exudates of pine trees. Of the organic acids from the Krebs cycle, citrate, fumarate, and malate were the most abundant with succinate making up a small percentage of the total organic acid content in the root exudates. Similar to our results, Smith (13) reported large variations between different plants of the same species.

The increased release of organic acids, in particular citrate, many provide a mechanism by which a P-stressed plant can enhance the availability of P in the rhizosphere. The release of organic compounds from the root, therefore, may be an adaptive mechanism by which the plant can alter its microenvironment and subsequently affect nutrient availability in the rhizosphere.

LITERATURE CITED

1. BOWEN GD 1969 Nutrient status effects on loss of amides and amino acids from pine roots. Plant Soil 30: 139–142
2. BRADLEY DB, DH SIELING 1953 Effect of organic anions and sugars on phosphate precipitation by iron and aluminum as influenced by pH. Soil Sci 76: 175–179
3. GARDNER WK, DA BARBER, DG PARBERG 1983 The acquisition of phosphorus by *Lupinus albus* L. III. The probable mechanism by which phosphorus movement in the soil/root interface is enhanced. Plant Soil 70: 107–124
4. GEHRKE CW, K KUO, RW ZUMWALT 1971 The complete gas-liquid chromatographic separation of the twenty protein amino acids. J Chromatogr 57: 209–217
5. GRAHAM JH, RT LEONARD, JA MENGE 1981 Membrane-mediated decrease in root exudation responsible for phosphorus inhibition of vesicular-arbuscular mycorrhiza formation. Plant Physiol 68: 549–552
6. JOHNSTON HW 1959 The solubilization of "insoluble" phosphates: V. The action of some organic acids on iron and aluminum phosphates. N Z J Sci 2: 215–218
7. JOHNSTON HW, RB MILLER 1959 The solubilization of "insoluble" phosphate. IV. The reaction between organic acids and tricalcium phosphate. N Z J Sci 2: 109–120
8. MAWHINNEY TP 1983 Separation and analysis of sulfate, phosphate, and other oxyanions as there tertiary butyldimethylsilyl derivatives by gas liquid chromatography and mass spectrometry. J Chromatogr 257: 37–44
9. MURPHY J, JP RILEY 1962 A modified single solution method for the determination of phosphate in natural waters. Anal Chem Acta 27: 31–36
10. RATNAYAKE RT, RT LEONARD, JA MENGE 1978 Root exudation in relation to supply of phosphorus and its possible relevance to mycorrhizal formation. New Phytol 81: 543–552
11. SCHWAB SM, JA MENGE, RT LEONARD 1983 Quantitative and qualitative effects of phosphorus on extracts and exudates of sudangrass roots in relation to vesicular-arbuscular mycorrhiza formation. Plant Physiol 73: 761–765
12. SMITH WH 1969 Release of organic materials from the roots of tree seedlings. For Sci 15: 138–143
13. SMITH WH 1976 Character and significance of forest tree root exudates. Ecology 57: 324–331
14. SNEDECER GW, WG COCKRAN 1980 Statistical Methods, Ed 7. The Iowa State Press, Ames, pp 228–229
15. STRUTHERS PH, DH SIELING 1950 Effects of organic anions on phosphate precipipitation by iron and aluminum as influenced by pH. Soil Sci 69: 205–213
16. VANCURA V 1964 Root exudates of plants. I. Analysis of root exudates of barley and wheat in their initial phases of growth. Plant Soil 21: 231–248
17. VANCURA V, A HOVADIK 1965 Root exudates of plants. II. Composition of root exudates of some vegetables. Plant Soil 22: 21–32