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# Quantitative and Qualitative Effects of Phosphorus on Extracts and Exudates of Sudangrass Roots in Relation to Vesicular-Arbuscular Mycorrhiza Formation

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## ABSTRACT

**A comparison was made of water-soluble root exudates and extracts of *Sorghum vulgare* Pers. grown under two levels of P nutrition. An increase in P nutrition significantly decreased the concentration of carbohydrates, carboxylic acids, and amino acids in exudates, and decreased the concentration of carboxylic acids in extracts. Higher P did not affect the relative proportions of specific carboxylic acids and had little effect on proportions of specific amino acids in both extracts and exudates. Phosphorus amendment resulted in an increase in the relative proportion of arabinose and a decrease in the proportion of fructose in exudates, but did not have a large effect on the proportion of individual sugars in extracts. The proportions of specific carbohydrates, carboxylic acids, and amino acids varied between exudates and extracts. Therefore, the quantity and composition of root extracts may not be a reliable predictor of the availability of substrate for symbiotic vesicular-arbuscular mycorrhizal fungi. Comparisons of the rate of leakage of compounds from roots with the growth rate of vesicular-arbuscular mycorrhizal fungi suggest that the fungus must either be capable of using a variety of organic substrates for growth, or be capable of inducing a much higher rate of movement of specific organic compounds across root cell membranes than occurs through passive exudation as measured in this study.**

Vesicular-arbuscular mycorrhizal (VAM)[2] fungi apparently are obligately dependent on living plant roots for their supply of fixed carbon. Under some conditions, nearly the entire length of a root cortex may be colonized by VAM fungi (11). However, variables such as host species, light, temperature, and host nutrition affect the proportion of root inhabited by the fungal symbiont. Several studies have shown that high levels of P in plant tissue typically reduce the extent of VAM formation (13, 19, 27). In some experiments, the total length of root colonized by VAM fungi was not decreased with P application, but the proportion of root colonized was reduced due to the stimulation of root growth in high P treatments (4, 29). However, other studies have shown that total fungal growth, as well as proportion of colonized root, was reduced with P application (4, 28). In comparisons of VAM formation in P-deficient and P-amended roots of sudangrass, the major effect of P on VAM was expressed after initial penetration of the root by the fungus had occurred (28). However, there was no morphological evidence of an active defense against the fungus in high P roots. Instead, the overall growth rate of the fungus was reduced in the P-amended plants, as would be predicted if increasing concentrations of P reduced the availability of metabolites within the root to the fungus (28). Because the fungus normally does not disrupt the plasma membrane of host cells (6), host products must cross this barrier before they are available to the endophyte. Increasing concentrations of P in plant tissue reduced the rate of leakage of metabolites from roots of Monterey pine (*Pinus radiata* D. Don.), sour orange (*Citrus aurantium* L.), and sudangrass (3, 7, 23). Based on correlations between reduced leakage of organic material from roots and reduced VAM formation as tissue P increases, it has been hypothesized that the supply of organic nutrients leaked from cortical cells is a critical factor in determining the extent of VAM formation in a plant (7, 23, 28). Studies of the effects of light intensity, temperature (8), photoperiod (15), flower bud initiation (14), and ozone stress (17) on VAM formation and root exudation indicate that modification of the supply of nutrients leaked from the root may be a general means of regulating VAM formation by the host plant, rather than a phenomenon specific to VAM-P interactions.

Most studies of the relationship between leakage of metabolites from roots and VAM formation have been directed toward quantitative, but not qualitative, measurements of root exudation. However, the concentrations of sucrose and fructose in the extracts of short roots of loblolly pine (*Pinus taeda* L.) did respond differently to variations in soil fertility, and ectomycorrhiza formation was more closely correlated with sucrose than fructose concentrations (16). It is not known, however, if the proportions of individual compounds within root extracts are a reliable indicator of the composition of material that is leaked across the plasma membrane and hence available to the fungus. To define the role of exudation in modifying VAM formation more precisely, information is needed on specific compounds leaked from roots and how environmental variation affects the composition, as well as quantity, of material that may act as a substrate for the fungal symbiont. This paper reports the results of experiments designed to determine the effect of P nutrition on the composition of carbohydrate, amino acid, and carboxylic acid fractions of soluble material within root cells and material leaked from roots of sudangrass.

## MATERIALS AND METHODS

**Plant Material.** P-deficient sudangrass (*Sorghum vulgare* Pers.) seedlings were grown in a glasshouse in 10-cm clay pots filled with a sandy loam soil containing 3 μg available P/g soil. Soil was autoclaved before use to kill indigenous VAM fungi. P-amended seedlings were grown at the same time in the same soil

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[2] Abbreviation: VAM, vesicular-arbuscular mycorrhiza (l).

with 200 μg P/g soil added in the form of finely ground $Ca(H_2PO_4)_2 \cdot H_2O$. Half the soil in each treatment was inoculated with 10 g of soil from pot cultures of *Glomus fasciculatus* (Thaxter) Gerd. and Trappe on sour orange, containing hyphae, infected root pieces, and about 3500 spores of the fungus. Roots from five inoculated plants in each treatment were harvested 6 weeks after sowing and stained in trypan blue-lactophenol (22). The per cent of root length colonized by the fungus was estimated by counting the proportion of 100 1-mm root segments containing VAM fungi structures. Plant tissue P concentrations were measured after ashing samples using the vanadate-molybdate-yellow method (5).

**Root Exudates.** Root exudates of P-deficient and P-amended plants grown in the absence of VAM fungi were collected 6 weeks after sowing. After carefully washing away soil, roots of five replicate noninoculated plants in each treatment were submerged in 250 ml of an aerated aqueous solution of 0.5 mM $CaCl_2$ containing 0.05 mg/ml rifampicin and 0.025 mg/ml tetracycline to reduce bacterial contamination of exudates. After 2 h in the antibiotic solution, roots were rinsed in sterile water, then reimmersed in aerated sterilized water containing 0.5 mM $CaCl_2$ for 6 h, followed by a transfer to a second flask of water for another 6 h. Contents of the two flasks were pooled for each plant, evaporated to dryness under reduced pressure at 45°C on a rotary evaporator, and resuspended in 10 ml of distilled $H_2O$. Samples were stored at −20°C until analysis.

**Root Extracts.** Roots of five replicate noninoculated plants in each treatment were oven dried at about 80°C for 2 d. Extracts were prepared by grinding 0.1 g dry root in a tissue homogenizer in 15 ml of 70% aqueous ethanol. Samples were centrifuged at 2000 rpm for 5 min to remove insoluble root material. The precipitated root material was washed in 70% ethanol and recentrifuged twice. The supernatants from the three centrifugations for each sample were pooled, evaporated to dryness, and resuspended as for the exudate samples.

**Analyses.** Samples of exudates and extracts were passed through a 10-ml bed volume column of Bio-Rad Ag 50W-X8 cation exchange resin. Neutral and acidic compounds were washed from the resin with 100 ml of water. Basic compounds were eluted with 200 ml of 2.0 N HCl. The neutral-acidic mixtures were passed through a 10-ml bed volume column of Bio Rad Ag 1-X8 Formate-form anion exchange resin. Neutral compounds were washed from the resin with 100 ml of water, and acidic compounds were eluted with 80 ml of 6.0 N formic acid. Each neutral fraction was resuspended in water, each basic fraction in 0.2 N Na-citrate buffer (pH 2.2), and each acidic fraction in 0.1 N $H_2SO_4$.

Duplicate 1-ml aliquots of the neutral fractions were analyzed for soluble carbohydrate content using anthrone reagent (20). A portion of each neutral fraction was dried and converted to trimethylsilyl derivatives using Tri-Sil Reagent (Pierce Chemicals) (31). Separation of individual carbohydrate derivative was done on a GLC fitted with a 200-cm glass column packed with 3% OV-210 on 80- to 100-mesh Chromsorb. The flow rate of the carrier gas ($N_2$) was 35 ml/min, and the oven temperature was programmed to increase from 125°C to 250°C over a 20-min period. Peaks were tentatively identified by cochromatography with known sugar standards. The values reported in this paper are pooled values for the α and β isomers of each sugar. Further evidence for the identity of sugars in the neutral fractions was obtained from paper chromatography of underivatized samples, using ethylacetate, pyridine, and water (8:2:1, v/v/v) as the solvent (26) and 5% *p*-anisidine (12) as the developing agent.

The quantity and composition of amino acids in the basic fractions were determined using a dual column Beckman 120°C amino acid analyzer, with elution from the columns with sodium citrate buffers.

The carboxylic acid composition of the acidic fraction was determined using an HPLC fitted with a Bio-Rad HPX-87 column, with 0.01 N $H_2SO_4$ flowing at a rate of 0.5 ml/min at ambient temperature as the solvent (32). Peaks were detected with an UV detector at 210 nm and tentatively identified by cochromatography with known standards.

## RESULTS

Plant growth and tissue P concentrations were increased 3- to 4-fold and per cent of root length colonized by VAM fungi was decreased nearly 5-fold in the P-amended compared to P-deficient treatment (Table I). The inhibitory effect of P on VAM formation was similar to that reported in several other studies (7, 13, 19, 27).

There was no significant difference in the concentration of soluble carbohydrates or amino acids in extracts of roots of P-deficient and P-amended sudangrass, but the concentration of carboxylic acids was 50% higher in the extracts from the P-deficient roots (Table II). In contrast, there were significantly lower concentrations of all three classes of compounds in the exudates of P-amended roots compared to the P-deficient roots (Table II). Values for carboxylic acid concentrations do not include oxalic acid, which was present in all samples but was not quantified because of precipitation of oxalate with $Ca^{2+}$ in the exudate solutions. The carboxylic acid fraction comprised less than half the material detected in the extracts but made up nearly 80% of the exudates from both P-deficient and P-amended roots. P amendment had no effect on the relative proportions of carbohydrates, amino acids, and carboxylic acids in root exudates, but the per cent of the total root extract composed of amino acids increased 10%, with a corresponding decrease in the portion composed of carboxylic acids in the P-amended roots.

Of the five sugars detected in the extracts or exudates of sudangrass, fructose and glucose were the major components of the extracts and P-deficient exudates, and arabinose was the major component of exudates from roots of the P-amended plants. Sucrose made up about 10% of the carbohydrate fraction of extracts but was not detected in exudates, and arabinose also did not occur in the same proportion in extracts as in exudates. The process of drying roots prior to extraction may have caused some qualitative changes in sugar composition. Specifically, release of invertase during drying may have resulted in lower readings for sucrose and higher readings for glucose and fructose than would be found in extracts from fresh tissue. If such conversion of sucrose did occur, then the values reported in this paper underestimate the differences in the composition of extracts and exudates. An unidentified compound comprised 30% of the neutral fraction of exudates from P-amended roots. This compound eluted between glucose and sucrose and did not cochromatograph with galactose, maltose, mannose, or myoinositol. No corresponding unknown was detected on paper chro-

Table I. *Effect of Soil P Amendment on Growth, Tissue P Content, and VAM Formation in Sudangrass*

Means of five replicate plants grown under glasshouse conditions for 6 weeks.

| P Amendment | Noninoculated Plants | | | Inoculated (Mycorrhiza Formation) |
|---|---|---|---|---|
| | Shoot | Root | Shoot P | |
| μg/g | g dry wt | g dry wt | % dry wt | % of root |
| 0 | 0.06a[a] | 0.07a | 0.10a | 48a |
| 200 | 0.23b | 0.19b | 0.36b | 10b |

[a] Numbers in same column followed by same letter are not significantly different at P = 0.05 using Student's *t* test.

Table II. *Effect of P Amendment on Total Soluble Carbohydrates, Amino Acids, and Carboxylic Acids in Extracts and Exudates of Sudangrass Roots*

Means of five replicate plants grown under glasshouse conditions for 6 weeks.

| P Amendment | Extracts | | | Exudates | | |
|---|---|---|---|---|---|---|
| | Carbohydrate | Amino acid | Carboxylic acid[a] | Carbohydrate | Amino acid | Carboxylic acid[a] |
| μg/g | mg/g dry root | | | mg/g dry root · 12 h | | |
| 0 | 7.4a[b] | 6.7a | 12.9a | 1.1a | 0.7a | 7.3a |
| 200 | 5.7a | 7.5a | 8.1b | 0.3b | 0.2b | 1.8b |

[a] Does not include oxalic acid.

[b] Numbers in same column followed by same letter are not significantly different at P = 0.05 using Student's *t* test.

Table III. *Effect of P Amendment on Soluble Carbohydrate Composition of Exudates and Extracts of Sudangrass Roots*

Means of five replicate plants grown under glasshouse conditions for 6 weeks.

| Sugar | Extracts | | Exudates | |
|---|---|---|---|---|
| | 0 P | 200 P | 0 P | 200 P |
| | % of total soluble carbohydrate | | | |
| Arabinose | 19.0b[a] | 22.8b | 4.4a | 29.9b |
| Fructose | 40.5bc | 31.8ab | 57.0c | 15.5a |
| Glucose | 25.0a | 24.1a | 38.6a | 20.5a |
| Sucrose | 8.9a | 16.0a | NDnd[b] | ND |
| Unknown | 6.6a | 5.3a | ND | 34.1b |

[a] Numbers in same row followed by same letter are not significantly different at P = 0.05 using Duncan's Multiple Range test.

[b] ND, not detected.

Table IV. *Effect of P Amendment on Amino Acid Composition of Extracts and Exudates of Sudangrass Roots*

Means of five replicate plants grown under glasshouse conditions for 6 weeks.

| Amino Acid | Extracts | | Exudates | |
|---|---|---|---|---|
| | 0 P | 200 P | 0 P | 200 P |
| | % of total amino acids | | | |
| Ala | 15.1c[a] | 9.7b | 7.5ab | 4.3a |
| Arg | 2.0a | 1.9a | ND[b] | 1.3a |
| Asp | 9.3a | 19.8b | 12.8a | 19.2b |
| Glu | 14.2a | 14.1a | 10.8a | 19.5a |
| Gly | 2.3a | 1.8a | 11.5c | 5.5b |
| His | 2.8a | 2.8a | 3.0a | 3.0a |
| Ile | 3.2b | 4.0b | 1.3a | 2.8b |
| Leu | 4.0a | 3.3a | 2.9a | 4.1a |
| Lys | 4.4a | 3.3a | 15.1c | 9.1b |
| Met | 0.2a | 0.5a | ND | ND |
| Phe | 2.0b | 2.5b | 0.6a | ND |
| Pro | 1.6a | 1.0a | ND | 1.3a |
| Ser | 28.0c | 22.6ab | 25.3bc | 20.4a |
| Thr | 2.4a | 3.3a | 5.3b | 5.7b |
| Tyr | 2.9b | 2.9b | 0.7a | ND |
| Val | 5.6bc | 6.5c | 3.2a | 3.8ab |

[a] Numbers in same row followed by same letter are not significantly different at P = 0.05 using Duncan's Multiple Range test.

[b] ND, not detected.

matograms of root exudates. With the exception of sucrose, P amendment did not have a significant effect on the composition of the neutral fraction of root extracts but did affect the proportion of arabinose, fructose, and the unidentified compound in root exudates (Table III).

There were significant differences in the proportions of individual amino acids in extracts compared to exudates, and also in P-deficient compared to P-amended treatments (Table IV). Of the six amino acids (alanine, aspartate, glutamate, glycine, lysine, and serine) that each made up at least 10% of the total amino acids in at least one treatment, glycine and lysine showed the greatest differences between extracts and exudates, especially in the P-deficient treatments. Aspartate showed the greatest difference between P-deficient and P-amended treatments, especially in the extracts (Table IV).

Table V. *Effect of P Amendement on Carboxylic Acid Composition of Exudates and Extracts of Sudangrass Roots*

Means of five replicate plants grown under glasshouse conditions for 6 weeks.

| Carboxylic Acid | Extracts | | Exudates | |
|---|---|---|---|---|
| | 0 P | 200 P | 0 P | 200 P |
| | % of total carboxylic acid[a] | | | |
| cis-Aconitic | 0.1a[b] | ND[c] | 3.1b | 3.0b |
| Citric/Isocitric | 26.0b | 28.1b | 6.2a | 2.3a |
| Fumaric | 0.4b | 0.1a | 0.2ab | 0.1a |
| Malic | 19.9b | 15.5b | ND | ND |
| Succinic | 50.5a | 55.9a | 54.3a | 56.0a |
| *trans*-Aconitic | 3.1a | 0.4a | 36.2b | 38.6b |

[a] Does not include oxalic acid.

[b] Numbers in same row followed by same letter are not significantly different at P = 0.05 using Duncan's Multiple Range test.

[c] ND, not detected.

Phosphorus nutrition had no significant effect on the carboxylic acid composition of either root extracts or exudates (Table V). However, the composition of extracts was significantly different from the composition of exudates. Malic and citric/isocitric acids were major components of root extracts but were only detectable in small quantities in exudates. *Trans*-aconitic acid made up only a small proportion of the carboxylic acid pool in root extracts but comprised about one-third of the carboxylic acids detected in root exudates.

## DISCUSSION

Comparisons of VAM formation in P-deficient and P-amended sudangrass indicated that inhibition of VAM formation in P-amended roots occurs after the fungus has come in contact with the host root (28). If P fertilization influences the extent of VAM formation through an effect on the availability of metabolites to the fungus, then it is the supply of organic nutrients within the root, not in the rhizosphere, that appears to be critical. Early theories on the effects of soil fertility on ectomycorrhiza formation suggested that the rate of development of the fungal component was related to the concentration of reducing sugars in host root extracts (9). Similar correlations between VAM formation and carbohydrate concentrations in root extracts were

reported for subterranean clover (*Trifolium subterraneum* L.) grown with different rates of P fertilization (13). However, the results of these experiments show that the composition of soluble root extracts is not necessarily a reliable indicator of the composition of material leaked from root cells. As has been shown previously (7, 23), P amendment altered rates of leakage of organic compounds without a corresponding change in concentrations of material within root extracts. In addition, certain compounds such as sucrose, citric acid, and malic acid made up a substantial portion of root extracts but not exudates, while arabinose, glycine, lysine, and *trans*-aconitic acid were major components of exudates but not extracts. Differences in the composition of extracts and exudates of squash (*Cucurbita maxima* Duchesne) hypocotyls (10) and pea (*Pisum sativum* L.) roots (2) also have been reported. Since the fungus never actually penetrates into the host cytoplasm, the quantity and composition of material leaked from the root may be a more realistic indication of the availability of substrate for the fungus than the concentration or composition of material in root extracts.

Decreases in the rate of leakage of carbohydrates and amino acids from roots with increasing P amendment have been attributed to the effect of P on membrane permeability (7, 23). The differences in composition of extracts and exudates could be the result of subcellular partitioning of metabolites and perhaps selective membrane permeability. Much of the difference, however, may be due to the fact that root extracts are obtained from a sample of the entire root system, while most root exudation is restricted to the root tip and zone of elongation (18), the region which also is apparently most likely to be colonized by VAM fungi (30).

Phosphorus deficiency was associated with a higher proportion of fructose, glycine, lysine, and serine in root exudates compared to metabolites leaked from P-amended sudangrass roots (Tables III and IV). Hence, VAM formation may be associated with leakage of certain specific compounds. However, because VAM occur in a very wide range of plant species, any specific stimulatory compound would be expected to be a component of root exudates of most plant species. Comparisons with other studies on root exudation do not show any single compound that is consistently detected in exudates from different plant species (25), and foliar applications of P did not consistently affect exudation of any single sugar or amino acid among four species studied (1). Therefore, further research is needed on the composition of root exudates under conditions that stimulate VAM formation in other plant species, before VAM-stimulating properties can be attributed to any particular compound.

In a concurrent study comparing specific phases of VAM formation in P-deficient and P-amended sudangrass, the per cent of root length colonized by the fungal symbiont increased from 4.9% to 19.4% over a 10-d period in P-deficient plants (28). Root fresh weight increased from 2.5 to 8.6 g over the same period (Schwab, unpublished). Making corrections for the per cent of the volume of the host root actually occupied by fungal tissue (28), and assuming that the density (weight/volume) of the fungus is about the same as the root, then the rate of fungal growth over the 10-d period was from 1.2% of 2.5 g to 4.9% of 8.6 g, for a total increase of 0.04 g of fungus per day. If the ratio of external hyphal growth to internal hyphal growth is near 1:1, and the fungus is roughly 90% water, than total fungal growth was about 8 mg dry weight/d during the most rapid phase of VAM development. The 'efficiency quotient' for fungal conversion of simple organic componds to hyphae is typically in the range of from 0.2 to 0.4 mg dry weight of hyphae·mg substrate (21). Based on these figures, about 20 to 40 mg substrate would be required per day to support the rate of VAM formation reported for sudangrass.

The results of this study indicate that about 10 mg of combined carbohydrate, amino acid, and carboxylic acid (excluding oxalic acid) are leaked from 1 g dry root of noninoculated low P sudangrass/12-h period. This value represents the amount of substrate lost from the root without any interaction with the fungal symbiont. Over the period of maximum VAM development (28), the total amount of carbohydrate, amino acid, and carboxylic acid lost was in the range of about 5 to 15 mg/12-h period. The rate of exudation of amino acids has been shown to fluctuate diurnally, with much reduced exudation during the dark period (24). Therefore, 15 mg is probably a reasonable estimate of the total amount of these compounds leaked from sudangrass roots per day. That value approaches, but does not meet, the value of about 20 to 40 mg of substrate calculated as necessary to sustain the observed rate of VAM formation in sudangrass. Furthermore, it is highly unlikely that all the material leaked from the roots is available to the fungus.

The discrepancy between the values calculated for rates of leakage necessary to support VAM formation and the observed rates of leakage may be the result of weaknesses in several of the assumptions used in making these calculations, as well as inaccuracies associated with basing rates of leakage in the soil on rates of leakage in a flask of water. Comparisons of the infection process in P-deficient and P-amended sudangrass suggest that the supply of nutrients within the root is more important in regulating VAM formation than the supply of nutrients leaked into the rhizosphere (28). Significant amounts of material leaked from cortical cells may be reabsorbed by cortical and epidermal cells rather than being exuded into the rhizosphere (10), resulting in a higher concentration of metabolites in the apoplast than would be indicated by measurements of material exuded beyond the root surface. However, these values do indicate that it is unlikely that the fungus can be supported on passive leakage of any one specific compound. Either VAM fungi are capable of using a broad spectrum of compounds passively leaked from the root, or they are capable of inducing a much greater rate of movement of compounds across the host plasma membrane once they make contact with the root than occurs by passive leakage alone.

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