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Membrane-Mediated Decrease in Root Exudation Responsible for Phorphorus Inhibition of Vesicular-Arbuscular Mycorrhiza Formation

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ABSTRACT

The mechanism responsible for phosphorus inhibition of vesicular-arbuscular mycorrhiza formation in sudangrass (*Sorghum vulgare* Pers.) was investigated in a phosphorus-deficient sandy soil (0.5 micrograms phosphorus per gram soil) amended with increasing levels of phosphorus as superphosphate (0, 28, 56, 228 micrograms per gram soil). The root phosphorus content of 4-week-old plants was correlated with the amount of phosphorus added to the soil. Root exudation of amino acids and reducing sugars was greater for plants grown in phosphorus-deficient soil than for those grown in the phosphorus-treated soils. The increase in exudation corresponded with changes in membrane permeability of phosphorus-deficient roots, as measured by K^+ (^{86}Rb) efflux, rather than with changes in root content of reducing sugars and amino acids. The roots of phosphorus-deficient plants inoculated at 4 weeks with *Glomus fasciculatus* were 88% infected after 9 weeks as compared to less than 25% infection in phosphorus-sufficient roots; these differences were correlated with root exudation at the time of inoculation. For plants grown in phosphorus-deficient soil, infection by vesicular-arbuscular mycorrhizae increased root phosphorus which resulted in a decrease in root membrane permeability and exudation compared to nonmycorrhizal plants. It is proposed that, under low phosphorus nutrition, increased root membrane permeability leads to net loss of metabolites at sufficient levels to sustain the germination and growth of the mycorrhizal fungus during pre- and postinfection. Subsequently, mycorrhizal infection leads to improvement of root phosphorus nutrition and a reduction in membrane-mediated loss of root metabolites.

During the past 20 years, there has been a growing appreciation of the importance of VAM[1] in the improvement of plant growth through increased uptake of phosphorus (P) and other mineral nutrients, especially in soils of low fertility (5, 9). As evidence has mounted for the role of VAM in the enhancement of P uptake from P-deficient soils, it has also been recognized that high soil P levels severely limit VAM infection (9, 10).

Early work failed to distinguish whether P was inhibiting the activity of the mycorrhizal fungus in the soil or during the host-fungus interaction (10, 16). Later, Sanders (15) found that foliar application of P inhibited VAM infection of onion, demonstrating that P concentration within the host plays a more important role. Using a split-root technique, Menge *et al.* (8) confirmed this finding when they established that the portion of the root system of sudangrass inoculated with *Glomus fasciculatus* (Thaxt.) Gerd. and Trappe only became heavily infected if the host was not receiving adequate P from the other half of the root system. It is now clear that the P content of the host plant is the critical factor controlling the mycorrhizal symbiosis.

Ratnayake *et al.* (12) proposed that the mechanism of P control of VAM formation was associated with a membrane-mediated decrease in root exudation. They were able to correlate low P content of sudangrass and citrus roots with a decrease in phospholipid levels and a large increase in permeability of root membranes, which results in a greater net leakage of amino acids and sugars from the root. They suggested that under P-sufficient conditions, metabolites required to initiate mycorrhizal formation were not exuded from roots in large enough quantities to sustain the infection process. They did not, however, present direct evidence which correlated the condition of high and low exudation with development of VAM.

In this report, we seek to demonstrate the interdependence of root membrane permeability, root exudation, and VAM infection by identifying the condition of the host at different P contents before and after inoculation with the mycorrhizal fungus, *Glomus fasciculatus.*

MATERIALS AND METHODS

Plant Material. A sandy soil containing 0.5 μg available P per g drysoil, as determined by Olsen analysis (2), was sterilized by autoclaving twice (121 C; 1.1 kg/cm^2) for 1 h with a 24-h interval between treatments. P in the form of finely ground superphosphate ($Ca[H_2PO_4]_2 \cdot H_2O$) was incorporated into the soil at rates of 0, 28, 56, and 228 μg/g (μg P per g dry soil). During the experiments, levels of available P (Olsen analysis) in the soils receiving P rates of 0, 28, 56, and 228 μg/g decreased from 0.5 to 0.4, 20.8 to 10.9, 38.6 to 24.5, and 146.3 to 66.5 μg/g, respectively.

Soil from each P treatment was potted into 150 Leach tubes (Leach Cone-Tainer Nursery, Aurora, OR), each with a volume of 65 cm^3. Seed of sudangrass (*Sorghum vulgare* Pers.) was sown and seedlings thinned to one per tube. Completely randomized seedlings were grown in the glasshouse under a maximum light intensity of 2400 μE/m^2·s at 400 to 700 nm and 32/23 C day/night temperatures. Seedlings were fertilized weekly with a nutrient solution minus P (7).

After 3 weeks, 60 seedlings from each P treatment were selected for transplant into 500-cm^3 clay pots. For VAM inoculation, 40 seedlings per P treatment were potted into the same P level soil mixed with *Citrus aurantium* L. pot culture inoculum of *Glomus fasciculatus,* containing a mixture of chlamydospores (300 spores per g soil), hyphae, and infected roots, at the rate of 10 g inoculum per pot. For nonmycorrhizal treatments, 20 seedlings per P level

[1] Abbreviation: VAM, vesicular-arbuscular mycorrhizae.

were potted into soil at the same P level. As a control, an inoculum water extract, prepared by leaching inoculum (an amount equivalent to that received by mycorrhizal treated pots) on a 38 μm sieve to exclude mycorrhizal propagules, was added to nonmycorrhizal treatments to establish the associated pot culture microflora. Transplanted seedlings were grown under the conditions described above for another 4 to 5 weeks until harvest (VAM harvest). Beginning at 4 weeks, the remainder of the untransplanted seedlings were harvested (pre-VAM harvest).

Phosphorus Content of Roots. Phosphorus content of root tissue was determined by magnesium nitrate-nitric acid digestion followed by colorimetric assay using the molybdenum blue method (2). Results were expressed relative to dry root weight (70 C; 24 h).

Mycorrhiza Formation. For the VAM harvest, mycorrhiza formation was assessed for 10 plants per treatment by sampling a cross-section of the root mass and staining the sample in trypan blue-lactophenol (11). The stained root segments were randomly distributed under a grid of 1-mm^2 divisions and examined for the presence or absence of mycorrhizal arbuscules, vesicles, hyphae, and spores in 100 1-mm^2 sections of root tissue.

Root Exudation. One or five plants, depending on harvest time, were removed from their soil containers and carefully washed free of soil. Each batch of plants was immediately placed in a beaker with the roots completely covered with aerated 0.5 mM $CaCl_2$ solution containing 0.05 g/l rifampicin and 0.025 g/l tetracycline and incubated for 2 h. During the subsequent period of exudate collection, this pretreatment with antibiotics was found to reduce bacterial populations 100-fold compared to untreated roots. Bacterial populations were monitored by periodically sampling the solution during exudate collection and plating the sample dilution on media containing 10 g casein, 5 g yeast extract, 4 g K_2PO_4, and 15 g agar (Difco) per liter distilled H_2O.

After antibiotic pretreatment, the roots were rinsed in one volume of aerated 0.5 mM $CaCl_2$ solution for 5 min and then allowed to stand in fresh 0.5 mM $CaCl_2$ solution for 22 h under continuous low light at 23 to 24 C. The plants were removed from the beakers and the dry weight of the roots determined. The exudate solution of 200 to 250 ml was immediately passed through a 0.45-μm filter to remove root debris and microorganisms. The filtered solution was stored at 5 C until rotoevaporated to a 20- to 25-ml volume and then kept frozen until analyzed.

The exudate solution was tested for total amino acid and reducing sugar content by the standard procedures using ninhydrin (18) and sulfonated α-naphthol (4) reagents, respectively. These procedures were performed in duplicate for each replicate sample. The concentrations of amino acids and reducing sugars were expressed as μg or mg equivalents of leucine and glucose, respectively, per gram dry weight of root.

For pre-VAM harvest (plants harvested before inoculation with mycorrhizal fungi), five 4-week-old plants from each P treatment were used for each exudate collection, and this procedure was repeated five times. For VAM harvest, one 8-week-old inoculated or uninoculated plant from each P treatment was used for each exudate collection, and this was repeated three times.

Root Extracts. One g of freshly harvested root tissue was homogenized in 10 ml absolute ethanol, and the resulting extract was centrifuged for 15 min at 10,000*g*. Aliquots of the supernatant were tested for total soluble amino acids and reducing sugars, as described above. The concentrations were expressed on a root fresh weight basis.

For pre-VAM and VAM harvests, replicate root tissue samples were obtained by subsampling five plants (each 5 weeks old) and three plants (each 9 weeks old) per treatment, respectively. This procedure was repeated five times for pre-VAM harvest and three times for VAM harvest.

Root Membrane Permeability. Procedures for study of root membrane permeability were performed according to Ratnayake *et al.* (12), with minor modifications. Briefly, 2-g samples of roots from each treatment were cut into 2-cm segments and incubated for 3 h in a 0.25 mM KCl solution labeled with ^{86}Rb (100,000 cpm/μmol K^+) with the addition of 0.5 mM $CaCl_2$. Samples were then transferred to unlabeled efflux solution with the same concentration of salts, and efflux solutions were periodically drained into scintillation vials and replaced over a 6-h period. Samples were counted by Cerenkov radiation in aqueous solution, and the counts per minute for each root sample were expressed relative to the corresponding total counts at the beginning of efflux.

For pre-VAM and VAM harvest, root samples were obtained as described for root extracts. The procedure was repeated for each treatment at least twice with the same results. The results for one trial are reported.

RESULTS

Pre-VAM Harvest. After 4 weeks of growth, significant differences in the P content of sudangrass roots occurred, and these differences were correlated with the amounts of P supplied as superphosphate in the soil (Table I). There were obvious growth differences between plants grown in unamended soil with respect to P (zero P) and the P-treated plants, an indication that zero P plants were P deficient (Table I).

Roots of P-deficient plants exuded over 3 times more amino acids than did roots supplied with increasing levels of soil P (Table I). Reducing sugar exudation followed the same trend, but differences were smaller and, therefore, not significant. The correlation between P deficiency and increased root exudation has also been observed by Ratnayake *et al.* (12), for sudangrass as well as for citrus, and by Bowen (1), for *Pinus radiata.*

The magnitude of differences in exudation of amino acids and reducing sugars between zero P and P treatments might partially have been a reflection of root contents of these compounds. Bowen (1) suggested that higher amino acid exudation from P-deficient plants was due to a doubling of the amide and amino nitrogen in pine roots. Amino acid content of zero P sudangrass roots was about 60% higher than it was in roots receiving P (Table I), a difference that was not nearly large enough to account for the 3-fold increase in amino acid exudation. Moreover, the trend for reducing sugar content was opposite that for root exudates and, perhaps, was responsible for the lack of significant differences in reducing sugar exudation between zero P and P treatments. Similar trends for the effect of P nutrition on reducing sugar and amino acid content of sudangrass roots were reported by Ratnayake *et al.* (12). The increase in exudation of P-deficient roots,

Table I. *Total Amino Acid and Reducing Sugar Content of Root Exudates and Extracts of Sudangrass*

The plants were grown for 4 weeks at a range of soil P concentrations (pre-VAM harvest).

Soil P	P in Dry Root	Plant Dry Weight	Root Exudate		Root Extract	
			Reducing sugars per dry root	Amino acids per dry root	Reducing sugars in fresh root	Amino acids in fresh root
μg/g	%	g	mg/g	μg/g	mg/g	μg/g
0	0.095a[a]	0.17a	3.49a	597a	4.14a	1,504a
28	0.131b	1.18b	3.00a	186b	10.90b	904b
56	0.190c	1.29bc	2.88a	157bc	12.37bc	919b
228	0.279d	1.44c	2.69a	116c	14.40c	841b

[a] Column means followed by the same letter are not significantly different at 0.05 level, according to Duncan's multiple range test.

therefore, cannot be adequately explained on the basis of root contents alone, an indication that some other factor controlled net loss of these compounds from roots.

The permeability of root membranes, as measured by K^+ efflux characteristics, was influenced significantly by P nutrition (Fig. 1). There was a large increase in K^+ loss from P-deficient roots. The rate constant for K^+(^{86}Rb) efflux from the slowest exchanging cell compartment (probably the vacuole) was larger for the zero P-treated roots than it was for those roots from seedlings grown at the higher P levels (Table II). Ratnayake *et al.* (12), likewise, found that P deficiency increased the rate constant for K^+ loss through root cell membranes. Furthermore, they noted that a parallel relationship existed between changes in reciprocal half-times ($1/t_{1/2}$) as a function of P content of roots and the pattern of variation in amino acid and reducing sugar exudation. They found that a steep decline in these parameters occurred with increasing root P levels below 0.1% P and a small linear decrease over a range of higher root P levels. We observed similar differences in root membrane permeability and exudation between roots below 0.1% P and roots with higher P contents (Fig. 2). These studies confirm that the rate of exudation is directly related to fundamental changes in root membrane permeability controlled by P.

VAM Harvest. Having identified changes in membrane-mediated root exudation as a function of P, we expected differences in VAM formation to occur after inoculation with *G. fasciculatus.* Nine-week-old sudangrass seedlings inoculated at 4 weeks with *G. fasciculatus* showed dramatic differences in VAM formation (Table III). Almost 90% of the root length of zero P plants contained mycorrhizal structures. As a result of VAM infection, there were significant increases in P content of roots and total dry weight compared to nonmycorrhizal controls (Table III). By contrast, VAM levels of less than 25% for P treatments were insignificant with respect to enhancement of P uptake and growth of sudangrass.

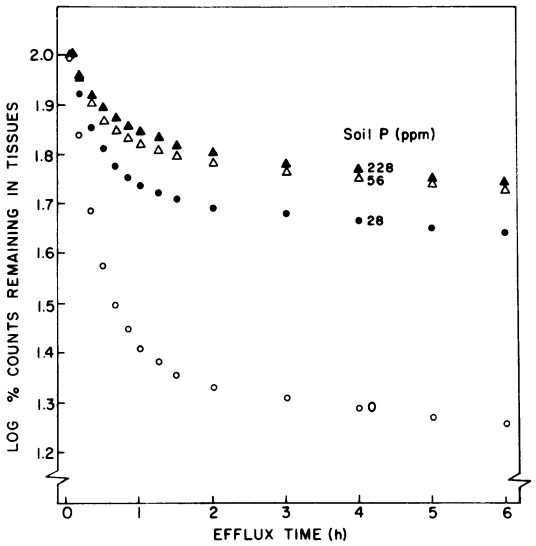

FIG. 1. Semilogarithmic plot of K^+ (^{86}Rb) loss from excised roots of sudangrass seedlings grown at a range of soil P concentrations.

Table II. *Rate Constants and Halftimes for K^+ (^{86}Rb) Efflux from Excised Roots of Sudangrass*

The plants were grown for 4 weeks at a range of soil P concentrations (pre-VAM harvest).

Soil P	Rate Constant[a]	Halftime[b]
$\mu g/g$	h^{-1}	h
0	0.040	17.3
28	0.031	22.4
56	0.030	23.1
228	0.033	21.0

[a] Slope determined by regression analysis from Figure 1 over the period of time between 2 and 6 h.

[b] Calculated according to Cram (3), using the equation $t_{1/2} = \frac{0.693}{K}$, where K is the rate constant.

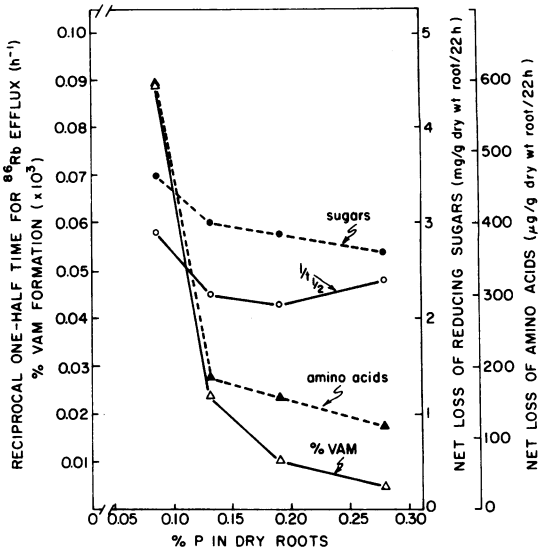

FIG. 2. Relationship between efflux of K^+ (^{86}Rb) and exudation of reducing sugars and amino acids from sudangrass roots of varying P contents and subsequent formation of VAM.

Table III. *VAM Formation of Nine-Week-Old Sudangrass*

The plants were grown at a range of soil P concentrations (VAM harvest).

	Soil P	VAM Formation	P in Dry Root	Total Plant Dry Weight
	$\mu g/g$	%	%	g
Nonmycorrhizal	0		0.065a[a]	0.26a
	28		0.093bc	12.89c
	56		0.110d	14.11d
	228		0.202e	15.88d
Mycorrhizal	0	89a	0.084b	0.94b
	28	24b	0.085b	12.58c
	56	10c	0.105cd	14.65cd
	228	5c	0.191c	15.25d

[a] Column means followed by the same letter are not significantly different at 0.05 level, according to Duncan's multiple range test.

The large differences in VAM infection were highly correlated with the previously discussed pattern of variation in membrane permeability and root exudation among P treatments prior to inoculation with *G. fasciculatus* (Fig. 2). This correlation is indicative of the importance of membrane-mediated root exudation in the control of the VAM infection process.

The higher rates of amino acid and reducing sugar exudation in P-deficient plants were still evident after 8 weeks (Table IV). In general, however, the levels of exudation for 8-week-old plants were less than those for 4-week-old plants. This relationship between increasing plant age and decreasing quantities of root exudation has also been reported for peas, oats (14), and sugar maple (17). Reducing sugar exudation in the zero P treatments provided an exception to the trend towards decreased exudation in older seedlings (Table IV).

Comparison of 8-week-old nonmycorrhizal plants with 4-week-

Table IV. *Total Amino Acid and Reducing Sugar Content of Root Exudates and Extracts of Nonmycorrhizal and Mycorrhizal Sudangrass*

The plants were grown for 8 weeks at a range of soil P concentrations (VAM harvest).

		Root Exudate		Root Extract	
	Soil P	Reducing sugars per dry root	Amino acids per dry root	Reducing sugars in fresh root	Amino acids in fresh root
	μg/g	mg/g	μg/g	mg/g	μg/g
Nonmycorrhizal	0	5.75a[a]	372a	10.06a	306a
	28	1.59c	60c	24.16b	134b
	56	1.47c	38c	22.57b	112b
	228	1.51c	25c	26.23b	95b
Mycorrhizal	0	3.89b	271b	9.35a	241a
	28	1.22c	60c	17.97ab	112b
	56	1.08c	50c	22.33b	97b
	226	1.19c	40c	26.57b	111b

[a] Column means followed by the same letter are not significantly different at 0.05 level, according to Duncan's multiple range test.

Table V. *Rate Constants and Halftimes for K^+ (^{86}Rb) Efflux from Excised Roots of Nonmycorrhizal (NM) and Mycorrhizal (VAM) Sundangrass Grown for 8 Weeks at a Range of Soil P Concentrations (VAM Harvest)*

See Table II and Figure 1 for calculation of K and $t_{1/2}$ values.

Soil P	Rate Constant		Halftime	
	NM	VAM	NM	VAM
μg/g	h^{-1}		h	
0	0.058	0.026	11.9	26.7
28	0.031	0.037	22.4	18.7
56	0.034	0.038	20.4	18.2
228	0.032	0.034	21.7	20.4

old seedlings shows that a greater difference in root exudation between zero P and P treatments occurred in older plants (*cf.* Tables I and IV). The increased exudation by 8-week-old plants grown without added P was a function of decreasing root P content in older seedlings, which resulted in an even greater increase in root membrane permeability (as measured by rate constant and $t_{1/2}$ values for K^+ efflux) than was observed for P-deficient 4-week-old plants (*cf.* Tables I and II with Tables III and V). By contrast, there was an increase in P content of mycorrhizal plants grown without added P, and, as a result, amino acid and reducing sugar exudation dropped significantly, compared to nonmycorrhizal controls (Tables II and IV).

Although the root P content of mycorrhizal plants grown without added P was comparable to that of mycorrhizal or nonmycorrhizal plants grown with 28 μg/g added P, the level of exudation of mycorrhizal zero P plants was still much greater (Table IV). This disparity could be attributed to differences in plant growth and development. P-treated plants were flowering at 8 to 9 weeks, whereas zero P plants were not. The stage of plant growth may have a considerable effect on levels of root exudation (13, 14, 17), which makes comparisons between zero P and P-treated plants at 8 weeks less valid.

The magnitude of decrease in root exudation and increase in root P content for mycorrhizal and nonmycorrhizal plants grown without added P was not consistent with the efflux characteristics of mycorrhizal roots (Table V). The halftime for K^+ efflux from VAM zero P roots was greater than that observed for any P treatments. In heavily colonized roots containing hyphae, arbuscules, and vesicles, the proportion of fungal to root tissue was apparently large enough to influence significantly K^+ efflux characteristics. That is, apparent efflux from the tissue may represent loss of tracer from both the root and fungal cells, hence K^+ efflux rate of mycorrhizal roots may not be directly comparable to that for roots with little or no mycorrhizal infection. When the level of VAM was less than 25%, the presence of the fungus appeared to have no effect on the efflux characteristics of the roots, since differences in halftime values between VAM and nonmycorrhizal plants were insignificant (Table V).

DISCUSSION

Ratnayake *et al.* (12) proposed that the mechanism for P inhibition of VAM formation was associated with membrane-mediated decrease in root exudation. They found that the amount of exudation was correlated with a P-induced decrease in phospholipid levels and associated changes in permeability properties of root membranes. We confirmed that the rate of exudation is directly related to fundamental changes in root membrane permeability controlled by P and, furthermore, demonstrated that subsequent mycorrhizal infection was highly correlated with initial differences in root exudation.

Jasper *et al.* (6) suggested that higher VAM infection in low P status subterranean clover was correlated with an increase in the root content of soluble carbohydrates. In spite of higher levels of reducing sugar exudation, we found lower levels of sugars in roots of P-deficient sudangrass. Our studies, therefore, indicate that the dramatic increase in VAM infection at low P status was due to the increase in membrane-mediated root exudation rather than to higher concentrations of reducing sugars or amino acids in roots.

The frequency of hyphal penetration of roots has been observed to increase in P-deficient subterranean clover (6). Presumably, root exudates in the rhizosphere stimulate the growth and development of mycorrhizal fungus propagules, in the form of spores, hyphae, or infected root pieces, which leads to greater root penetration and formation of vesicles and arbuscules.

Following VAM infection of sudangrass, P nutrition improved significantly, and, as a result, membrane permeability and root exudation decreased. Nevertheless, the lower levels of exudation may be adequate to sustain high levels of mycorrhizal activity because of the greatly enhanced surface contact between host cell membranes and the plasmalemma of fungal arbuscules.

To further distinguish the role of root exudation in mycorrhiza formation, we are presently attempting to increase root leakage of sugar and amino acid in plants with sufficient P. In this case, the association should form independently of the P nutrition of the host and directly demonstrate that root exudation is the more important factor affecting fungus activity.

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