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INFLUENCE OF PHOSPHATE SOURCE ON VESICULAR–ARBUSCULAR MYCORRHIZAE OF *BOUTELOUA GRACILIS*

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SUMMARY

Non-mycorrhizal and vesicular–arbuscular mycorrhizal *Bouteloua gracilis* infected with *Glomus fasciculatus* were grown in defined media containing different phosphate sources and compared with respect to phosphate content and form, biomass, chlorophyll concentration, and root phosphatase activity. The phosphate sources were sodium monobasic phosphate, a mixture of sodium monobasic phosphate and calcium phytate, and calcium phytate. Inositol and inositol plus calcium were added to the sodium phosphate medium as additional treatments. Mycorrhizal infection was highest in roots of plants grown in the presence of phytate (75 %). Lower root infection levels were noted in plants from the sodium phosphate (19 %) and mixed phosphate (22 %) media. No penetration by fungi occurred in plants from the sodium phosphate plus inositol or inositol and calcium media. Dry wts of non-mycorrhizal plants were highest when grown in media containing phytate and sodium phosphate plus inositol and calcium followed in decreasing order by sodium phosphate plus inositol, mixed phosphates, and sodium phosphate. Mycorrhizal infection increased leaf dry wt in plants from the sodium phosphate medium and root dry wt from the phytate medium. Phosphate concentrations in the plants were highest when grown in mixed phosphate medium followed by sodium phosphate and phytate. Mycorrhizal infection always increased significantly leaf phosphate concentrations but increased root phosphate concentrations only in the phytate medium. Phosphates were found predominantly as organically-bound compounds in leaves of mycorrhizal plants whereas in leaves of non-mycorrhizal plants, most of the phosphate was inorganic. Chlorophyll concentrations increased significantly with mycorrhizal infection with no change in *a*/*b* ratios. Mycorrhizal plants grown in the phytate medium had substantially higher alkaline phosphatase activity than did non-mycorrhizal plants; acid phosphatase activity was not affected by mycorrhizal condition.

These results suggest that form of the phosphate in the root environment influences mycorrhizal establishment and effect of mycorrhizae on plant growth.

INTRODUCTION

Bouteloua gracilis H. B. K. Lag ex Steud is a dominant grass in the water- and nutrient-stressed shortgrass plains of western United States (Lauenroth, Dodd and Sims, 1978). It may be heavily infected with vesicular–arbuscular (VA) mycorrhizae (Davidson and Christensen, 1977) which might alter numerous physiological processes. For example, VA mycorrhizae can improve plant growth by enhancing phosphorus uptake (Mosse, 1973). Phytates, an abundant form of phosphate in grassland soils (McKercher and Anderson, 1968), are relatively insoluble and may be largely unavailable (Tinker, 1975); thus phosphorus supply might limit primary production in shortgrass communities (Cole, Innis and Stewart, 1977).

Infection of plant roots by VA mycorrhizal fungi may be influenced by phosphate concentration and form (Mosse and Phillips, 1971). Mosse and Hepper

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(1975) obtained mycorrhizal root organ cultures with media containing phytate, potassium dihydrogen phosphate or calcium phosphate, when the medium was sterilized in a rapidly cooling pressure-cooker rather than an autoclave. Allen *et al.* (1979) reported extensive infection of *B. gracilis* using a medium with filter-sterilized phytate. Filtration instead of autoclaving reduced release of inorganic phosphate.

In order to elucidate further the influence of phosphate source on extent of mycorrhizal infection and the subsequent effect of mycorrhizae on plant growth and phosphate uptake, we grew *B. gracilis* with its native symbiont, *Glomus fasciculatus* (Thaxter sensu Gerdemann) Gerdemann and Trappe, in defined media containing various phosphate sources. The *G. fasciculatus* spores were collected from Pawnee National Grasslands soil as previously described (Allen *et al.*, 1979).

MATERIALS AND METHODS

VA mycorrhizal *B. gracilis* seedlings infected with *G. fasciculatus* and non-mycorrhizal seedlings were grown axenically as described by Allen *et al.* (1979). Seeds of *B. gracilis* were surface-sterilized by immersion in 0·5% sodium hypochlorite for 45 min, rinsed in sterile distilled water and germinated on potato-dextrose agar. *G. fasciculatus* spores were separated from soil containing *B. gracilis* roots by centrifugation and handpicking (Allen *et al.*, 1979). The spores were sterilized for 2·5 min in 0·5% sodium hypochlorite, rinsed with sterile, distilled water, and germinated on 2% water agar (Daniels and Graham, 1976). Sterile, germinated seeds and spores were transferred to a medium of the following composition: $Ca(NO_3)_2 . 4H_2O$, 341 mg; KNO_3, 80 mg; KCl, 65 mg; $MnCl_2 . 4H_2O$, 7·07 mg; $ZnSO_4 . 7H_2O$, 2·68 mg; KI, 0·75 mg; $Na_2SO_4 . 10H_2O$, 200 mg; $MgSO_4 . 7H_2O$, 738 mg; H_3BO_3, 1·5 mg; $Na_2MoO_4 . 2H_2O$, 0·00254 mg; $CuSO_4 . 5H_2O$, 0·0201 mg; $FeCl_3 . 6H_2O$, 4·16 mg; Difco Bacto-agar, 10 g; twice-distilled water, 1 litre; and a phosphate source (Table 1). The pH was adjusted to 5·5. Five plants were grown in 100 ml of medium in deep culture dishes; two spores per plant were placed near the roots of sterile seedlings in mycorrhizal treatments. Both mycorrhizal and non-mycorrhizal plants were grown for 30 or 50 days in an environmental chamber adjusted to simulate shortgrass plains growth conditions (15 h light, 32 °C; 9 h dark, 18 °C).

Infection percentage was determined by a modification (Davidson and Christensen, 1979) of the method of Nicolson (1960). Twenty-five 1 mm root segments per plant were surveyed for presence of *Glomus* structures (hyphae, arbuscules, vesicles) within cortical tissue.

Table 1. *Phosphate source and concentrations in the media*

Phosphate source and other amendments	Quantity added (mg l^{-1})	Phosphate concentration (mM)
NaH_2PO_4	167·2	1·4
NaH_2PO_4; Ca^{2+}-phytate (mixed phosphate)	83·6; 115	1·4
Ca^{2+}-phytate	230	1·4
NaH_2PO_4; Inositol	167·2; 41·8	1·4
NaH_2PO_4; Inositol; $CaNO_3.4H_2O$	167·2; 41·8; 127·4	1·4

Plants were harvested for dry wt measurements after 50 days, prior to seed development and at the end of the arbuscular stage. Before weighing, leaves and roots were dried at 55 °C for 48 h. Differences among treatments were compared using analysis of variance with least significant difference (Zar, 1974).

Other plants harvested at the same time were analyzed for chlorophyll content, and inorganic phosphate and organic phosphate concentrations. Chlorophylls *a* and *b* were measured spectrophotometrically (Ross, 1974). For phosphate determinations, the tissue was thoroughly homogenized at 4 °C in 50 mM Tricine buffer (pH 7·5); an aliquot was removed for perchloric acid digestion and the remaining homogenate centrifuged to remove cellular debris. Inorganic phosphate was assayed spectrophotometrically using unhydrolyzed extract (McCaman, Smith and Cook, 1965) and total phosphate was estimated following perchloric acid digestion (Dittmer and Wells, 1969). Organic phosphate was calculated by taking the difference between total and inorganic phosphate. Concentration differences among treatments were compared using the Mann–Whitney U-test for nonparametric data (Zar, 1974).

Root surface acid phosphatase activity was assayed using 30-day-old plants (the time of maximal arbuscule development) growth in the phytate medium with 1·4 or 0·14 mM phosphate. Plants were removed from the soft agar substrate, rinsed in twice-distilled water, and the roots placed in test tubes with 4 ml of 25 mM *p*-nitrophenyl phosphate in 0·1 M sodium acetate buffer, pH 5·5. After the tubes had shaken for 15 min, the roots were removed and 9 ml of 0·2 M K_2HPO_4 added to each tube to stop the reaction. The preparatory procedure for determination of alkaline phosphatase activity was similar except that a 10 mM *p*-nitrophenyl phosphate solution was prepared with a Tris buffer (pH 8·2) and the roots shaken in the solution for 30 min. In both analyses, freed nitrophenol was measured spectrophotometrically at 410 nm (Gould, Coleman and Rubink, 1979). Rates of activity expressed as nM *p*-nitrophenyl phosphate hydrolyzed s^{-1} were compared between mycorrhizal and non-mycorrhizal plants using Student's *t*-test.

Localization of surface acid phosphatase activity was determined as described by Gould, Anderson and Coleman (pers. comm.). The agar medium with intact control or VA mycorrhizal plants was carefully removed from the plate, placed upside down in a large Petri dish, and flooded with 50 ml of a solution containing 50 mM α-napthyl phosphate, paraosanilin HCl, and $NaNO_2$ in 0·2 M sodium acetate buffer, pH 6·0. Acid phosphatase activity was observed as areas of dark red colour.

Results

Phosphate source, calcium and inositol all significantly affected establishment of VA mycorrhizae (Table 2). After 50 days, 75 % infection was noted in plants grown in the phytate medium; about 20 % infection occurred with sodium phosphate and mixed phosphate and no infection developed in the plants from the sodium phosphate with added inositol and inositol plus calcium media.

Both medium constitution and mycorrhizal infection influenced growth of *B. gracilis* (Table 3). Plants grown in the sodium phosphate medium had the lowest dry wt of the non-mycorrhizal treatments. The addition of inositol plus calcium to the sodium phosphate medium resulted in an increase in plant dry wt equal to that found in the phytate medium. VA mycorrhizal infection significantly increased root dry wt in the phytate treatment and leaf dry wt in the sodium phosphate treatment. No significant dry wt change was apparent following infection in the

Table 2. *Effect of phosphate source on VA mycorrhizal development in 50-day-old seedlings* (±*s.e.*)

Medium	Infection frequency (% of 1 mm root segments)
NaH_2PO_4	19±3b*
NaH_2PO_4 + Ca^{2+}-phytate	22±3b
Ca^{2+}-phytate	75±2a
NaH_2PO_4 + inositol	0±0c
NaH_2PO_4 + Ca^{2+} + inositol	0±0c

* Different letters denote significant differences (L.S.D. 0·05).

Table 3. *Dry wts of 50-day-old VA mycorrhizal (M) and non-mycorrhizal (NM) plants grown in the medium treatments*(±*s.e.*)

Medium	Mycorrhizal treatment	Dry wts (mg)		
		Roots	Leaves	Total
NaH_2PO_4	NM	3·7±0·6c	9·8±1·3c	13·6±1·9c
	M	4·2±0·5c	14·8±1·7bc*	19·4±2·2bc*
NaH_2PO_4 + Ca^{2+}-phytate	NM	3·9±0·6c	15·4±2·2bc	19·3±2·7bc
	M	4·8±1·2bc	14·1±4·1bc	18·8±5·3bc
Ca^{2+}-phytate	NM	5·7±0·7bc	22·9±2·6a	28·6±3·1a
	M	7·9±1·0a*	21·4±2·4a	29·3±3·2a
NaH_2PO_4 + inositol	NM	4·9±0·9bc	17·8±2·7ab	22·8±3·5ab
NaH_2PO_4 + Ca^{2+} + inositol	NM	6·3±0·8ab	22·6±2·5a	28·7±3·3a

Table 4. *Organic and inorganic phosphate concentrations in leaves of 50-day-old VA mycorrhizal (M) and non-mycorrhizal (NM) plants*

Medium	Mycorrhizal treatment	Fresh wt (mg)	Phosphate concentration		Phosphate content per plant	
			Total (mmol kg^{-1})	Organic (% of total)	Total (μg)	Organic (μg)
		(1)	(2)	(3)	(4)	(5)
NaH_2PO_4	NM	36·3	42·2	26	146	38
	M	56·1	93·2*	57*	497*	283*
NaH_2PO_4	NM	57·2	61·1	43	332	143
+ Ca^{2+}-phytate	M	56·2	91·9*	75*	491*	368*
Ca^{2+}-phytate	NM	84·9	6·5	38	52	20
	M	80·8	9·8*	61*	75*	46*

* Denotes significant difference between NM and M within a medium at a confidence level $\geq$ 0·95.

Table 5. *Organic and inorganic phosphate concentrations in roots of 50-day-old VA mycorrhizal (M) and non-mycorrhizal (NM) plants*

Medium	Mycorrhizal treatment	Fresh wt (mg)	Phosphate concentrations: Total (mmol kg^{-1} tissue)	Phosphate concentrations: Organic (% of total)	Phosphate content per plant: Total (μg)	Phosphate content per plant: Organic (μg)
NaH_2PO_4	NM	93·6	7·06	64·0	62·8	40·2
	M	105·1	6·15	59·7	61·4	36·7
$NaH_2PO_4 + Ca^{2+}$-phytate	NM	97·2	13·04	78·7	120·4	94·8
	M	127·0	9·83	74·6	118·6	88·5
Na^{2+}-phytate	NM	142·0	1·12	67·0	15·1	10·1
	M	197·8	1·69*	64·0	31·8*	20·3*

* Denotes significant difference between NM and M within a medium at a confidence level ≥ 0·95.

Table 6. *Chlorophyll concentrations of leaves from 50-day-old VA mycorrhizal (M) and non-mycorrhizal (NM) plants*

Medium	Mycorrhizal treatment	Chlorophyll concentrations (mg kg^{-1} fresh wt) *a*	*b*	total	*a/b* ratio
NaH_2PO_4	NM	1070	350	1420	3·1
	M	1640*	570*	2210*	2·9
$NaH_2PO_4 + Ca^{2+}$-phytate	NM	1166	394	1560	3·0
	M	3340*	1090*	4430*	3·1
Na^{2+}-phytate	NM	136	63	199	2·2
	M	262*	119*	381*	2·2

* Denotes significant difference between NM and M within a medium at a confidence level ≥ 0·95.

Table 7. *Root surface phosphatase activity of 30-day-old VA mycorrhizal (M) and non-mycorrhizal (NM) plants grown in the Ca^{2+}-phytate*

			p-Nitrophenyl phosphate (PNP) activity (nM PNP hydrolyzed s^{-1})			
			Activity per plant		Activity kg^{-1} dry wt	
Mycorrhizal treatment	Phosphate concentration in medium (mM)	Mean root dry wt (mg)	Acid	Alkaline	Acid	Alkaline
NM	0·14	2·6	0·297	—	$1·14 \times 10^5$	—
M	0·14	4·4	0·389*	—	$8·84 \times 10^4$	—
NM	1·40	2·4	0·261	—	$1·09 \times 10^5$	—
M	1·40	5·4	0·422	—	$7·81 \times 10^4$	—
NM	1·40	3·6	—	0·02	—	$6·11 \times 10^3$
M	1·40	4·9	—	0·06*	—	$1·22 \times 10^4$*

* Denotes significant differences between NM and M at a confidence level ≥ 0·95.

mixed phosphate medium. Root branching appeared to increase with infection, especially in the phytate treatment.

Phosphate uptake and utilization were influenced significantly by phosphate source and by mycorrhizal infection. Total phosphate concentration in leaves was increased 121, 50 and 51% with mycorrhizal infection in the sodium phosphate, mixed phosphate, and phytate media, respectively (Table 4, column 2). The concentration of organic phosphate increased 384, 162 and 142% respectively (column 3). In contrast, the only mycorrhiza-stimulated increase in root phosphate occurred in plants from the phytate medium (Table 5). Organic phosphate contents of roots with and without infection were not significantly different. Both the leaves and the roots of plants grown in the inorganic phosphate media had higher total phosphate levels than did leaves of plants grown in the medium with phytate only (Tables 4 and 5).

Chlorophyll concentrations increased significantly with infection, although the *a/b* ratios remained constant (Table 6). The sodium phosphate and mixed phosphate grown plants also had significantly higher chlorophyll concentrations and *a/b* ratios than did plants from the phytate medium.

Phosphatase activity at root surfaces was altered with mycorrhizal infection. Although acid phosphatase activity increased significantly per plant in mycorrhizal as compared with non-mycorrhizal plants, the increase was not proportional to the root biomass increase (Table 7). Microscopic observations of the stained medium indicated that acid phosphatase activity in mycorrhizal plants was localized only along the roots and not along fungal hyphae. External alkaline phosphatase activity was not significant in non-mycorrhizal plants but was significantly increased with infection (Table 7) when expressed either as quantity per plant or as quantity per unit of root dry wt.

DISCUSSION

The influence of calcium–phytate (Ca^{2+}–inositol hexaphosphate) components on the establishment of VA mycorrhizae and on the growth of *B. gracilis* suggests that a complex relationship existed between substrate, fungus and plant. Mycorrhizal infection was higher in the phytate medium than in the presence of inorganic phosphates, possibly a response to the phytate (Mosse and Phillips, 1971) or the low phosphate concentrations in the plants (Sanders, 1975; Menge *et al.*, 1978). Addition of inositol and calcium to the sodium phosphate prevented infection by mycorrhizal fungi and increased biomass suggesting that these components may limit plant growth. Inositol is required by some monocots in tissue culture (Kaul and Sabharwal, 1975) and Rhodes and Gerdemann (1979) observed that the hyphae of mycorrhizal fungi could transport calcium into the plant.

Mycorrhizal plants had greater phosphate concentrations than did non-mycorrhizal plants. The increased uptake may have resulted from: (1) increased absorbing area contributed by hyphae (Hattingh, Gray and Gerdemann, 1973; Pearson and Tinker, 1975); (2) greater affinity of sites transporting phosphate (Cress, Throneberry and Lindsey, 1979); (3) an enhanced phosphate sink; and (4) increased external phosphatase activity. Mycorrhizal fungus hyphae can transport ^{32}P across distances of up to 7 cm (Rhodes and Gerdemann, 1975). Cress *et al.* (1979) demonstrated that the V_{max} for $H_2PO_4^-$ uptake was similar in mycorrhizal and nonmycorrhizal tomato roots but that the K_m was significantly lower in the former to suggest that the number of absorbing sites was the same but that the phosphate binding affinity of mycorrhizae was greater.

Conversion of phosphate from inorganic to organic forms in the leaves always increased with mycorrhizal infection suggesting greater photophosphorylation (Simonis and Urbach, 1973). The higher chlorophyll levels observed in mycorrhizal plants with no change in a/b ratio may indicate a greater number of photosynthetic units (Alberte, Fiscus and Naylor, 1975). Recently, Allen *et al.* (unpublished data) demonstrated increased photosynthesis in *B. gracilis* with infection. Increased inorganic to organic phosphate conversion rates could reduce the inorganic phosphate gradient from roots to leaves, possibly increasing the phosphate sink and enhancing uptake (Bieleski, 1973).

In the phytate medium, $1{\cdot}15 \times 10^{-5}$ M inorganic phophate was present (Allen *et al.*, 1979). Therefore, about 0·11 mg of inorganic phosphate was available in each dish. However, approximately 0·34 and 0·53 mg of phosphate were present in non-mycorrhizal and mycorrhizal plant tissue per dish, respectively, indicating that phosphate from the phytate molecule was utilized by both mycorrhizal and non-mycorrhizal *B. gracilis*. External acid phosphatase activity was restricted to the root surface. External alkaline phosphatase activity was associated with the mycorrhiza. Thus, mycorrhizal *B. gracilis* utilized more phytate and exhibited phosphatase activity over a wider pH range than did non-mycorrhizal plants.

Although this study and other published data indicate that plants utilize organic phosphates (Martin, 1973; Halstead and McKercher, 1975), their significance in phosphorus nutrition remains controversial (Bartlett and Lewis, 1975; Pearson and Read, 1975; Tinker, 1975). However, in a recent simulation model of phosphorus cycling in semi-arid grasslands (Cole *et al.* 1977), the maximum mineralization rates of organic phosphate by saprophytic microbes could not account for total phosphorus movement into plants. In regions where low precipitation could limit phosphate diffusion (Nye and Tinker, 1977), the utilization of organic phosphates and an enhanced phosphate sink caused by higher photosynthetic rates accompanying mycorrhizal infection could significantly benefit the plant symbiont.

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The method for localizing surface acid phosphatase is now in press as:

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