



The presence and role of phytic acid in the alfalfa root and crown
by Mark Robert Campbell

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Agronomy

Montana State University

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Abstract:

Phytic acid, myo-inositol 1,2,3,4,5,6 hexakisphosphate, is the major storage form of phosphorus (P) in seeds, comprising 60 to 90% of total seed P. Phytic acid has also been identified in pollen, vegetative tissues of some aquatic angiosperms, and underground storage structures including roots. No studies have unequivocally demonstrated that phytic acid is indeed present in roots.

Three methods (ferric precipitation, ion-exchange chromatography, and high voltage paper electrophoresis) were used to demonstrate that phytic acid is a P-containing compound within the root and crown tissue of alfalfa (*Medicago sativa* L.). Phytic acid P was found to represent from 5 to 15% of total root and crown P. The effect of varying frequencies and intensity of shoot harvest on root and crown phytic acid P were next studied. More frequent and intense shoot harvests clearly depleted root and crown phytic acid P as compared with less frequent and intense harvest. Phytic acid was also observed to increase substantially in this tissue following hardening off.

These results indicate that phytic acid may be an important P storage compound in alfalfa root and crown that is mobilized to provide P for subsequent shoot growth.

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ABSTRACT

Phytic acid, myo-inositol 1,2,3,4,5,6 hexakisphosphate, is the major storage form of phosphorus (P) in seeds, comprising 60 to 90% of total seed P. Phytic acid has also been identified in pollen, vegetative tissues of some aquatic angiosperms, and underground storage structures including roots. No studies have unequivocally demonstrated that phytic acid is indeed present in roots.

Three methods (ferric precipitation, ion-exchange chromatography, and high voltage paper electrophoresis) were used to demonstrate that phytic acid is a P-containing compound within the root and crown tissue of alfalfa (Medicago sativa L.). Phytic acid P was found to represent from 5 to 15% of total root and crown P. The effect of varying frequencies and intensity of shoot harvest on root and crown phytic acid P were next studied. More frequent and intense shoot harvests clearly depleted root and crown phytic acid P as compared with less frequent and intense harvest. Phytic acid was also observed to increase substantially in this tissue following hardening off.

These results indicate that phytic acid may be an important P storage compound in alfalfa root and crown that is mobilized to provide P for subsequent shoot growth.

INTRODUCTION

Phytic acid (myo-inositol hexakisphosphate) is the major storage form of phosphorus (P) in the plant seed. It is commonly deposited as the mixed "phytate" salts of several physiologically important mineral cations. Upon germination, it serves as a source of P, mineral cations and myo-inositol needed for seedling growth.

Historically, the greatest interest in seed phytic acid has been in its role as an "antinutrient". Mixed phytate salts of mineral cations are often insoluble and are not readily absorbed across the intestinal mucosa of non-ruminants, leading to reduced availability of both P and minerals such as zinc, calcium and iron. Problems associated with high phytate intake may be most severe in populations where cereal and legume grains comprise a major portion of the diet.

The deposition of phytic acid is not limited to seeds alone. Other tissues containing phytic acid include pollen, floral structures, corms, roots, tubers, and other vegetative tissues. Phytic acid is most commonly deposited as discrete inclusions referred to as "globoids". Often the observation of "globoid-like" structures using microscopy is the only evidence provided to indicate the presence of phytic acid. Additional analytical evidence is required to unequivocally

demonstrate the presence of phytic acid.

Shoot P in the perennial forage crop alfalfa (Medicago sativa L.) is important for its nutritional quality and productivity. Phosphorus concentration in this forage has been found to be a limiting component for maximal animal productivity. The alfalfa crown and root tissue serve as a nutrient reserve important for its overwinter survival and subsequent regrowth in the spring. Studies were conducted to determine if phytic acid was present in the alfalfa root and crown tissues and if levels were substantial enough to represent a store of P. The effects of simulated grazing treatments and hardening off on root and crown phytic acid levels were also determined.

REVIEW OF LITERATURE

Seed Phytic Acid

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is the storage form of phosphorus (P) in seeds of nearly all higher plants, and typically represents 60 to 90% of the total seed P (Raboy, 1990). Numerous studies have demonstrated that mature seeds contain a substantial amount of the hexakisphosphate ester of myo-inositol (phytic acid), but at most contain trace levels of the "lower" myo-inositol polyphosphates (myo-inositol bis-, tris-, tetrakis-, and pentakis phosphate, which contain two through five phosphate esters, respectively). These studies used a variety of chromatographic techniques which distinguish between the various phosphate esters of myo-inositol. Recently nuclear magnetic resonance (NMR) methods have also been used to both identify and quantitate phytic acid (Jackson et al., 1982). The "ferric-precipitation method" for quantifying phytic acid can be used for routine analyses, once it is demonstrated that a tissue contains phytic acid and not substantial levels of other myo-inositol phosphates. Since all other myo-inositol polyphosphates are effectively precipitated as ferric salts, this method cannot distinguish among these and alone cannot provide evidence that the tissue contains phytic acid.

Phytic acid is deposited in single-membrane storage microbodies referred to as protein bodies (Lott, 1980). Typically, seed phytic acid is not found uniformly within the proteinaceous matrix of these protein bodies, but rather occurs in discrete electron dense particles known as globoids (Lott, 1986). In seeds, phytic acid is deposited as the mixed "phytate" salt of several physiologically important cations. Energy dispersive X-ray (EDX) analysis has revealed that globoids of both monocots and dicot species predominantly contain potassium and magnesium phytates. Other elements such as calcium, iron, zinc, barium and manganese may also be found in phytate, depending on the tissue region and soil conditions (Lott, 1986). Phytate functions as a source of inorganic phosphorus (P_i), myo-inositol and mineral cations needed for seedling growth (Lott, 1986).

Phytic acid contained in seed-based diets of non-ruminants results in reduced bioavailability of dietary minerals (Graf, 1986). Insoluble phytate salts, formed in the intestinal tract, are not readily absorbed across the intestinal mucosa. Reduced retention of P and mineral cations by phytic acid may lead to mineral deficiencies (Solomons, 1982; Morris, 1986).

Phytic Acid Deposition in Non-Seed Tissues

Phytic acid has been identified in pollen using both chromatographic and NMR methods. Pollen phytic acid is thought to account for its accumulation in soils, especially pine forests (Jackson and Linskens, 1982). Three gymnosperms and 25 angiosperms were assayed for pollen phytic acid. All three gymnosperms were found to contain detectable levels (1.0 to 5.9 mg phytic acid/g dry weight). Angiosperms having mean style lengths greater than 5 mm also contained phytic acid (0.5 to 21.0 mg/g dry weight). Pollen phytate was suggested to function as a source of myo-inositol, a precursor to cell wall polysaccharide synthesis (Jackson et al, 1982).

Floral structures of Petunia hybrida L. also contain phytic acid and its levels in tissues has been shown to be associated with gametophytic incompatibility genes (Jackson and Kamboj, 1983). Phytic acid was identified in stigmas (0.3 to 0.6 mg phytic acid/g dry weight), upper style (≤ 0.05 mg phytic acid/g dry weight) and lower style (≤ 0.01 mg phytic acid/g dry weight). Among three P. hybrida clones, floral phytic acid accumulation varied according to the incompatibility allele (S) present.

Various studies have identified phytic acid in vegetative tissues. Two aquatic angiosperms of the Lemnaceae family, Wolffiella floridana L. and Lemna gibba L., were found to synthesize phytic acid in their vegetative tissues when grown in an axenic culture including myo-inositol (Roberts and

Loewus, 1968). Phytic acid accumulation was thought to be related to turion (resting frond) formation. Suspension cultures of rice cells (Oryza sativa L.) were found to contain relatively constant amounts of myo-inositol monophosphate while phytic acid levels increased with the length of the culture period (Igaue et al., 1980). Both studies used widely accepted chromatographic methods.

Root phytic acid was first identified in some selected food crops by McCance and Widdowson (1935). Roots of carrot (Daucus carota L.), parsnip (Pastinaca sativa L.), and tubers (modified stems) of jerusalem artichoke (Helianthus tuberosus L.) and the potato (Solanacia tuberosum L.), were found to contain 15.8, 31.4, 25.0 and 23.0% respectively, of their total P in the form of phytic acid. Samotus (1965) found the potato tuber to contain 0.08 to 0.55 mg phytic acid/g. In these studies, phytic acid content was quantified using the ferric-precipitation method. Unequivocal evidence that these tissues solely contained phytic acid and not other myo-inositol phosphate esters was not provided.

Corms and tubers of two granite outcrop inhabiting species Stylidium petiolare Sond. and Philydrella pygmaea (R. Br.) Caruel., are almost fully dehydrated upon entry into summer dormancy and retain the ability to rehydrate and grow after autumn rains. Light microscopy revealed that these corms and tubers contained protein bodies superficially similar to those described for seeds (Pate and Dixon, 1982).

These protein bodies contained globoid-like inclusions rich in P, calcium, magnesium, zinc and, manganese (Dixon et al., 1983).

Inclusions observed in the elongating root tip of Deschampsia caespitosa (L.) Beauv. also are similar to those of phytate containing globoids (Van Steveninck et al., 1987). Electron probe microanalyses have shown that the zinc, potassium, and magnesium contained in these inclusions is in appropriate proportion to P if they were contained as phytate salts. As above, no additional evidence was provided that unequivocally demonstrated the presence of phytic acid or the deposition of minerals as phytate salts. Additionally, vacuolar bodies in the zinc tolerant ecotype of D. caespitosa contained almost three times more zinc than the zinc sensitive ecotype. Globoids appear more frequent in the tolerant ecotypes when exposed to high zinc concentrations. It was suggested that phytate may play a role as a chelator involved in zinc detoxification.

Factors Associated with Phytate Accumulation

Studies of factors affecting phytic acid accumulation have mostly concerned seed phytic acid. Identification of lines differing in phytic acid accumulation may be useful in selecting grain cultivars low in phytic acid. Levels of groat phytic acid among four cultivars of oats (Avena sativa L.) ranked the same over years and locations, suggesting that

phytic acid production is similarly affected by environment (Miller et al., 1980a). Statistically significant differences in groat phytic acid among cultivars were seen, although differences represented only a 0.1% separation between highest and lowest means. In contrast, substantial variation in seed phytic acid content was observed in 163 soybean lines, ranging from 13.9 to 23.0 mg/g dry weight (Raboy and Dickinson, 1984). Batton (1986) found that diploid wheats (Triticum aestivum L.) contained higher seed phytic acid concentrations than tetraploid and hexaploid wheats when grown in nutrient culture under two levels of P. Levels of phytic acid were found to be greater in six varieties of the hexaploid oat than two varieties of diploid oat (A. strigosa and A. strigosa X A. brevis) (Ashton and Williams, 1958). No substantial variation was found within a species.

In seeds, a high positive correlation between phytic acid P and total P has been observed. Lolas and Markakis (1976) observed correlation coefficients (r) between total P and phytic acid P in soybeans (Glycine max L. (Merr.)), oats, barley (Hordeum vulgar L.) and wheat to be 0.98, 0.91, 0.96 and 0.97, respectively. Additional studies with wheat (Miller et al., 1980a), soybeans (Raboy and Dickinson, 1984), and oats (Ashton and Williams, 1958) have confirmed these findings.

Several studies have demonstrated that variation in seed phytic acid closely parallel levels of vegetative P during the period of seed development (Raboy, 1990). Vegetative P in

turn closely parallels levels of nutrient P available to the growing plant. Miller et al. (1980b) found that among seven oat cultivars, groat phytic acid significantly increased with a given increase in available soil P. Similar findings have been observed in other species (Asada et al., 1969; Batten, 1986; Michael et al., 1980; Raboy and Dickinson, 1984).

Several lines of evidence indicate that phytic acid metabolism plays a role in the regulation of cellular P concentration. Early and DeTurk (1944) found that during the cell expansion phase of the developing maize (Zea mays L.) kernel, additional P translocated to the developing kernel was converted to phytic acid while total non-phytic acid P levels remained constant. Similar patterns of total P and phytic acid P accumulation during seed developments were found in wheat (Abernathy et al., 1973; Nahapetian and Bassiri, 1975) rice (Ogawa et al., 1979), soybean (Raboy and Dickinson, 1987) and bengal gram (Verma and Lal, 1966). Variation in phytic acid P usually accounts for nearly all variation in seed total P resulting from differences in nutrient P levels (Raboy, 1990). Dmitrieva and Sobolev (1984) found that during the sixth to eighth day of castor bean (Ricinus communis L.) germination, mobilization of the main stores of phytic acid in the endosperm coincided with phytic acid accumulation in the cotyledons. It was suggested that P_i from phytic acid breakdown in the endosperm resulted in a transiently high P_i concentration in the cotyledons leading to phytic acid

synthesis. Transient cotyledon phytic acid was then mobilized to supply the developing seedling with P. Samotus (1965), suggested a mechanism for regulating organic P in potato, whereby increased deposition of phytic acid lowers levels of P_i .

Alfalfa Root and Crown Tissue, P and Forage Quality

The root and crown of alfalfa contain nutrient stores which are mobilized to support shoot regrowth in its vegetative cycle. High levels of root and crown soluble carbohydrates, starch, NH_4^+ , organic P and P_i have been shown to be associated with harvest tolerance (Chatterton et al., 1977). During the fall hardening period alfalfa undergoes biochemical, biophysical, and morphological changes which enables the root and crown tissue to survive low temperatures. Factors including light, temperature, and soil conditions decrease total water content and increase intracellular sugars and starch in alfalfa root and crown tissue during fall hardening (McKenzie et al., 1988). Although limited data exists on P metabolism and storage in root and crown tissue, much of the P has been found to be combined into organic forms immediately after entry into the root (Rhykerd and Overdahl, 1972).

Phosphorus deficiency in forage is of economic importance as it can be a limiting component for forage and animal productivity in many areas of the United States. Low plant P

in alfalfa may be associated with production problems such as short stand persistency, reduced nitrogen fixation and low seed yield (Griffith, 1978; Pedersen et al., 1972; Rhykerd and Overdahl, 1972). In dairy cattle, low P in forage may result in reduced milk production, depressed appetite and problems in reproduction (Butler and Jones, 1973). Selecting for high P in forage varieties may require a broader understanding of P metabolism throughout the plant.

MATERIALS AND METHODS

Analytical Methods

Phytic Acid Determination in Alfalfa Root and Crown

A modification of the methods of Early and Deturk (1944) was used for the determination of phytic acid. Samples of dry alfalfa root and crown flour (1.0 g) were extracted in 0.4 HCl containing 0.7 M Na_2SO_4 (15 ml) by stirring 18 to 24 hours at room temperature. Samples were centrifuged (10000 g for 15 min.), supernatant decanted into a second tube and centrifuged again (10000 g for 15 min.). Ten ml of the supernatant were placed in a 30 ml Corex glass centrifuge tube and diluted with 10 ml of glass distilled H_2O and 5 ml of 15 mM FeCl_3 in 0.2 N HCl containing 0.35 M Na_2SO_4 . Samples were heated for 1.5 h in a boiling H_2O bath. The ferric phytate precipitate obtained after centrifugation (10000 g for 10 min.) was washed twice with 10 ml of 0.2 M HCl, completely digested on a hot plate (215°C) with 2 ml concentrated H_2SO_4 , H_2O_2 (as needed) and diluted to 12.5 ml with glass distilled H_2O . Phosphorus in the digests was determined colorimetrically (Chen, 1956). Phytic acid P data were expressed on a dry weight basis.

Determination of Total P

Samples of alfalfa root and crown flour (0.2 g) were placed in 50 ml Nessler tubes and digested on a hot plate with 2 ml concentrated H_2SO_4 and H_2O_2 (as needed) to give complete digestion. Samples were diluted to 12.5 ml with glass distilled H_2O , and P determined colorimetrically as before. Total P data were expressed on a dry weight basis.

Ion-Exchange Chromatography

Analysis of alfalfa root and crown myo-inositol phosphates was conducted using a modification of the method of Saio (Saio, 1964). Samples of alfalfa root flour (2.0 g) were extracted in 0.4 M HCl (15 ml) by stirring 18 to 20 hours at room temperature. Following centrifugation, as before, 10 ml of supernatant was diluted 5-fold with glass-distilled water, and passed through a 0.7 X 13 cm column containing 5 ml of AG 1-X8 (Cl^-) resin, pH 5.2. The column was then eluted with a linear gradient of HCl (0.0 to 1.0 N, 1 ml min^{-1} , total 400 ml). Five ml fractions were collected and 2 ml aliquots were dried and digested with 1 ml H_2SO_4 , H_2O_2 (as needed) and diluted with 5.5 ml glass distilled H_2O and 5.5 ml 5 N NaOH. Fraction P was determined colorimetrically (Chen, 1956). Inorganic P was determined directly from selected undigested fractions (Chen, 1956). Elution profiles were compared with those of authentic phytic acid (Sigma) and myo-inositol pentakisphosphate (Calbiochem).

High Voltage Paper Electrophoresis

Crude extracts and portions of selected ion-exchange chromatography peaks were lyophilized, dissolved in glass distilled water, and subjected to paper electrophoresis (0.1 M oxalic acid, pH 1.6; 23 V/cm for 2 hr: Whatman No.1 paper). Standards were phytic acid and a chemical hydrolysate of phytic acid containing a mixture of myo-inositol bisphosphate through myo-inositol pentakisphosphate, prepared as previously described (Desjobert and Peteck, 1956). Phosphorus containing spots were visualized using Hains Isherwood reagent (Bandusrk, 1951) followed by ultraviolet irradiation.

Experiments

Study #1: Phytic Acid Quantification and Confirmation in Alfalfa Root and Crown.

Root and crown tissue from eight alfalfa cultivars (Anstar, Apollo II, Baker, Centurion, Elevation, Ladak-65, Spredor II and Vernal) were collected (October 27, 1988) from a cultivar trial established May 16, 1986 at the Arthur H. Post Experiment Station near Bozeman, MT. These were kindly provided by Dr. Ray Ditterline (Dept. of Plant and Soil Science, MSU). Alfalfa seed was planted at a rate of 11.2 kg ha⁻¹ on a Bozeman silt loam (Agric Pachic Cryoborals) with 515 kg ha⁻¹ P₂O₅. The trial was irrigated as needed to avoid moisture stress. This field study used a randomized design with four blocks. Root and crown samples were collected

following the first hard fall frost (-4.0°C) (October, 27). Plants samples were collected, washed, crowns trimmed to 2.5 cm and roots trimmed to 20 cm. Samples were microwave irradiated 2 minutes (high power), turned and heated for an additional two minutes to inactivate hydrolytic enzymes. Samples were oven dried (50°C for 5 days), a bulk sample representing five or more plants was prepared for each replication and ground in a Thomas-Wiley mill to pass 20 mesh. Phytic acid P and total P were determined as described previously.

Study #2: Effect of Simulated Grazing
Regimes and Hardening Off on Root and
Crown Phytic Acid

Alfalfa root and crown samples from a simulated grazing field study were obtained from Shaun Townsend (Dept. of Plant and Soils, MSU). The study was established on May 1986, and located at the Arthur H. Post Experiment Station. A $2 \times 3 \times 3$ (2 cultivars, 3 harvest frequencies, and 3 clipping intensities) complete factorial randomized complete block design with four replications was used (Agustinho, 1988). Alfalfa was seeded, P_2O_5 applied, and irrigated as described previously. Root and crown tissue from 3 replications were collected following the final seasonal harvest (mid-August) from outside rows of selected plots. Only treatments involving the cultivar Spredor II (fall dormancy 1) were analyzed. Plants were harvested under three clipping intensity treatments (33, 50 and 67% top growth removal as

Table 1. Selected factorial treatments from a simulated grazing study including a graduated treatment.

Treatment	Clipping interval	Clipping Intensity
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FACTORIAL

	days	% removal
* 1	8	33
2	8	50
* 3	8	67
4	16	33
* 5	16	50
6	16	67
* 7	32	33
8	32	50
* 9	32	67

* Graduated:

	Clipping interval	Clipping intensity
	days	% removal
	8	33
	8	33
	16	33
	16	50
	32	50
Fall Management		67

* Selected treatments included for analysis.

measured by plant height), and three clipping frequencies (8, 16, and 32 day clipping intervals) (Table 1). Of nine factorial treatments, five representing high, low and intermediate harvest severities were selected for all analyses. A graduated (GRAD) treatment was also included with an early season harvest at 8 d frequency with 33% forage removal. Harvest intensity as well as cutting interval were increased with progression of growing season (Table 1). A control plot (hay) was harvested at 10% bloom to a 10 cm stubble height. Management treatments were not imposed the establishment year. Five plant samples per replication were collected in 1988, and processed as before. The flour was bulked, and stored in a desiccator until analysis. For 1989, 10 samples were collected per replication and processed as before, with the exception of grinding with a Thomas-Wily Mill, (Model 4, Arthur H. Thomas Co., Philadelphia, PA) and reground through a Cyclone Sample Mill (UD Corporation, Boulder, CO) using a 0.5 mm size screen. Phytic acid P (ferric-phytate precipitation) and total P were determined using methods previously described.

Alfalfa root and crown tissue from the same study was collected on a second date (September, 16) in 1989 following a hard frost (-4.0° C, September, 11). Ten plants per replication were collected and tissue processed as before. Phytic acid and total were P determined as described previously.

In an additional study, the root and crown tissue of five cultivars (Ladak-65, Arrow, Vernal, Garst-636 and Thor) was obtained from two intra-state variety trials located at the Arthur H. Post Experiment Station established by Dr. Ray Ditterline. The trials were planted in 1989 (location 1) and 1988 (location 2) in a randomized block design with four replications. Ten plants were collected from each replication 2 days prior to the final seasonal harvest (August 3), approximately 3 weeks following (August 23), and after a hard frost (-4°C) (October 8). Plants were trimmed and processed. Total P and phytic acid P were analyzed as previously described. Treatment effects were determined using a split plot test over date.

RESULTS AND DISCUSSION

Phytic Acid Quantification and
Confirmation in Alfalfa Root and Crown

Samples of alfalfa root and crown tissue of the eight cultivars contained phytic acid P, as determined by the ferric-phytate precipitation method (Table 2). Means ranged from 0.22 to 0.29 mg phytic acid P per g dry tissue. Mean root and crown total P ranged between 1.91 to 2.54 mg per g dry tissue. Phytic acid P as a percent of total P ranged from 10.5 to 14.1%. Phytic acid P was found to be positively correlated with total P ($r = 0.41$; $P = 0.045$). Although statistically significant differences in traits measured were not observed between cultivars a trend for higher levels of phytic acid P, total P and phytic acid P as percent of total P was seen with cultivars of higher dormancy rating (3 and 4) as compared with cultivars of lower dormancy rating (1 and 2).

Three cultivars (Vernal, Spredor II, Ladak-65), representing the range of phytic acid P levels observed using the ferric precipitation method, were selected for further chromatographic analyses. Ion-exchange chromatography identified a compound (fractions 50 to 65) with an elution profile similar to authentic phytic acid present in the extracts of each cultivar (Figure 1). A second peak

Table 2. Mean^a phytic acid P, total P and phytic acid P as the percent of total P in alfalfa root and crown tissue as determined using the ferric precipitation method.

Cultivar	Dormancy Rating	Phytic Acid P	Total P	Phytic acid P as the percent of Total P
		---mg/g dry tissue---		%
ANSTAR	4	0.29	2.03	14.1
APOLLO II	4	0.29	2.28	13.0
ELEVATION	3	0.29	2.17	13.4
CENTURION	3	0.29	2.54	11.5
VERNAL	2	0.22	1.91	11.2
BAKER	2	0.27	2.13	12.8
LADAK-65	1-2	0.23	2.19	10.5
SPREDOR II	1	0.25	2.21	11.2
Mean		0.23	2.14	11.0
LSD (0.05)		0.08	0.41	4.1
CV%		16.3	3.9	18.4

^a Data represents the mean of three replications.

occurring at approximately the 5th to 11th tubes represented inorganic P (P_i) and was confirmed by direct colorimetric determination of P_i from selected column fractions. Elution profiles from each cultivar indicated no substantial levels of other partially phosphorylated inositol polyphosphate intermediates (myo-inositol bisphosphate to myo-inositol pentakisphosphate) in the root and crown extracts. Ion-exchange chromatography fractions representing phytic acid peaks from the three cultivars were pooled and concentrated (Figure 1). High voltage paper electrophoresis of each pooled sample, as well as crude extracts yielded a single spot co-migrating with standard phytic acid (Figure 2). No lower myo-inositol polyphosphates were observed.

Table 3. Comparison of root and crown phytic acid P quantified by ion-exchange chromatography verses the ferric precipitation method.

Cultivar	<u>Phytic Acid P</u>		Percent Recovery
	Column Chromatography	Ferric Precipitation	
	---mg. phytic acid P/g tissue----		%
Vernal	0.15	0.18	83.3
Spredor II	0.16	0.18	88.8
Ladak-65	0.08	0.10	<u>80.0</u>
Mean			84.3

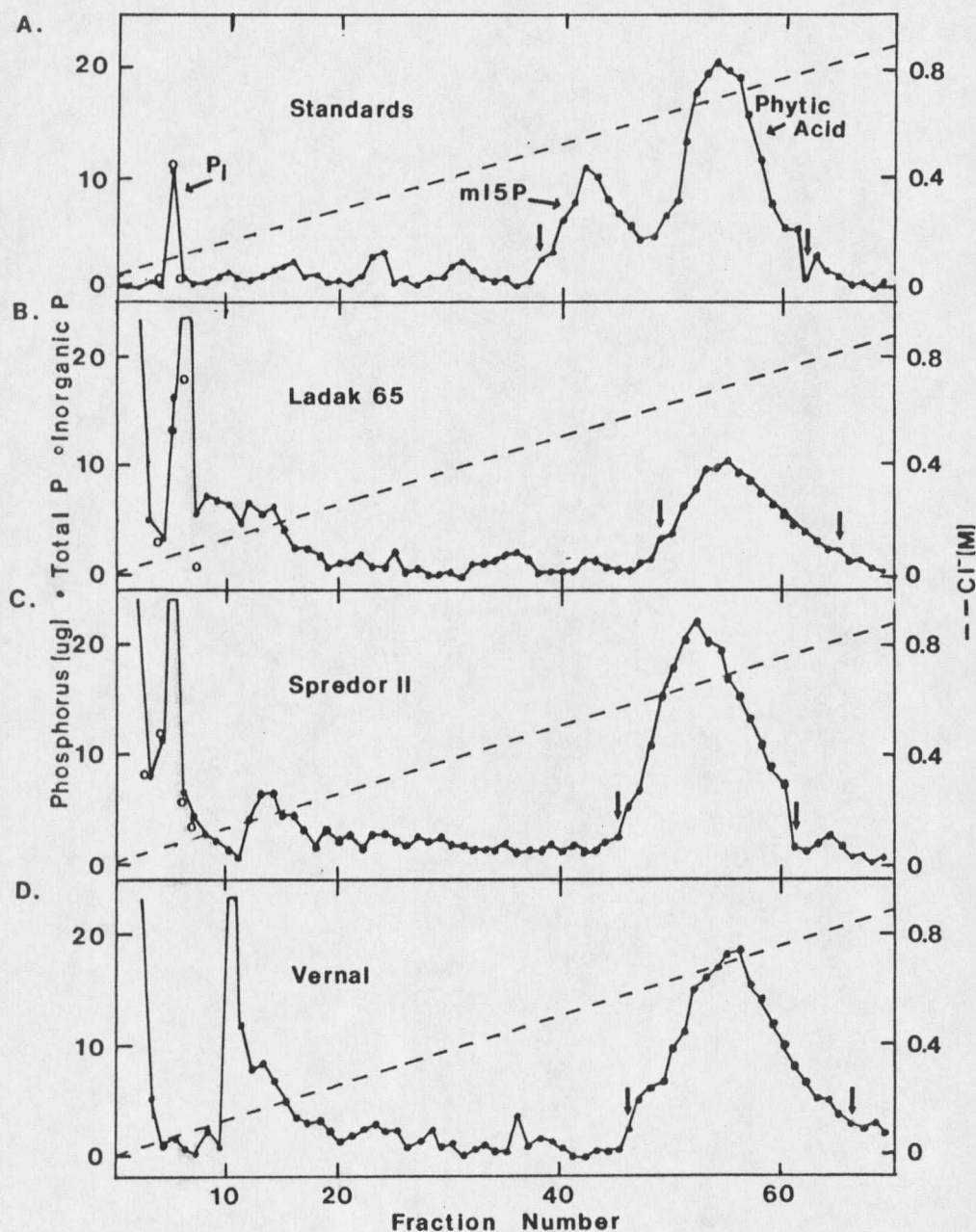


Figure 1. Ion-exchange chromatography of alfalfa root and crown extracts. (A) Standards: P_i (inorganic P); mI5P (myo-inositol pentakisphosphate); phytic acid. (B, C, D) Chromatography of root and crown extracts representing the indicated cultivars. Fractions within the arrows were pooled for subsequent analysis.

Figure 2. High-voltage paper electrophoresis analysis of alfalfa root and crown extracts. Lanes 1 through 3: Concentrated pooled fractions from ion-exchange chromatography of root and crown extracts representing the alfalfa cultivars Vernal (Lane 1), Spredor II (Lane 2), and Ladak-65 (Lane 3). Lane 4: Standards P2 through P5 (myo-inositol bis- through pentakisphosphate) and P6 (phytic acid). Lane 5: Pooled ion-exchange chromatography fractions comprising myo-inositol pentakisphosphate and phytic acid. Lanes 6 through 8: Concentrated crude extracts of root and crown tissue representing the cultivars Vernal (Lane 6), Spredor II (Lane 7) and Ladak-65 (Lane 8).

To confirm the ferric precipitation method as a means of quantifying alfalfa root and crown phytic acid P, P was calculated from selected fractions (See Figure 1) representing phytic acid peaks. These values were compared to phytic acid P values determined from the ferric-phytate precipitate (Table 3). Phytic acid P quantified by ion-exchange chromatography recovered from 80.0 to 88.8% of phytic acid P determined by the ferric precipitation method for the three alfalfa cultivars.

This study provides initial evidence that phytic acid P represents a substantial fraction, approximately 10 to 15%, of alfalfa root and crown total P. Chromatographic analyses provided both qualitative and quantitative confirmation of this observation. Phytic acid P, as determined using ion-exchange chromatography, closely paralleled values obtained using the ferric-phytate method. No other myo-inositol polyphosphates were detected at this level of sensitivity. This indicates that differences in P from the ferric precipitation method are primarily due to differences in phytic acid. The ferric precipitation method is therefore valid for the determination of alfalfa root and crown phytic acid P.

The fraction of alfalfa root and crown total P represented by phytic acid P (approximately 10%) is less than that typically observed for seeds (60 to 90%). However, it is similar to that observed in other underground vegetative

tissues, such as the thickened tap root of carrot and parsnip (McCance and Widdowson, 1935). The correlation between phytic acid P and total P ($r = 0.41$) in the alfalfa root and crown, while statistically significant ($P < 0.05$), is also reduced compared to that typically observed in seeds ($r > 0.90$) (Lolas and Markakis, 1976; Miller, 1980; Raboy, 1984). A lower r value in this tissue may simply reflect that in alfalfa roots phytic acid P represents a much smaller proportion of total P than in seeds.

Most likely, the primary role for phytic in the alfalfa root and crown is as a P storage product. Van Steveninck et al. (1987) suggested an additional function for phytic acid as a compound involved in detoxifying zinc within the elongating root tip of Deschampsia caespitosa. Their study represents the only previous report of the presence and role of phytic acid during root growth. Their evidence for both the presence and role of phytic acid in the elongating root tip of D. caespitosa was not unequivocal. Clearly, additional studies are required to determine both the presence and role of phytic acid in annual and perennial roots. While the primary role may be as a P storage product, an important secondary role may be as a chelator of minerals.

Phosphorus level in the alfalfa shoot is an important factor in its nutritional quality and productivity. Since root phytic acid represents a significant component of alfalfa root and crown total P, studies designed to alter alfalfa

shoot P levels should take this into account.

Effects of Simulated Grazing and Hardening
Off on Root and Crown Phytic Acid P

Two major events in the vegetative cycle of cultivated alfalfa are shoot harvest and hardening off. Each is followed by shoot regrowth which utilize root and crown storage reserves. Phytic acid P was previously found to represents 10 to 15% of alfalfa root and crown total P, and the ferric-precipitation method proved reliable for determining phytic acid P in this tissue. This method was therefore used to determine the effects of these two events on phytic acid levels in the root and crown of alfalfa.

The alfalfa cultivar Spredor II was subjected to seven different harvest regimes in 1988 (Table 4). In August, root and crown samples were collected during the final seasonal harvest, prior to hardening off, and phytic acid P and total P were determined (Table 4). Those treatments representing the most frequent and intense shoot harvests (Treatment 1 and 2) clearly had depleted root and crown phytic acid P as compared with treatments representing the least frequent and intense harvests (Treatments 4 and 5). A similar trend was observed for root and crown total P. The most frequent and intense harvesting regimes also reduced the percent of total P represented by phytic acid when compared with the least intense and frequent harvests. The hay treatment (treatment

Table 4. Mean total P, phytic acid P, and phytic acid P as the percent of total P content in alfalfa root and crown tissue from a simulated grazing study in 1988^a.

<u>Treatment</u>		<u>Phosphorus</u>		Phytic Acid P as the percent of total P
No.	Description ^b	Phytic Acid	Total	
		-----mg/g tissue-----		%
1	8d/67%	0.25 A ^c	2.20 A	10.6 AB
2	8d/33%	0.26 AB	2.52 AB	10.4 A
3	16d/50%	0.32 ABC	2.62 AB	12.2 ABC
4	32d/67%	0.39 C	2.79 B	14.2 C
5	32d/33%	0.36 BC	2.85 B	12.7 ABC
6	--HAY--	0.32 ABC	2.71 AB	11.7 ABC
7	GRADUATED	0.36 BC	2.79 B	13.0 BC
MEAN		0.31	2.56	12.2
LSD (0.05)		0.10	0.53	2.51
CV %		27.5	14.2	19.13

^a See appendix (Table 12) for analysis of variance.

^b Selected treatments from the simulated grazing study.

^c Means within a column followed by the same letter are not significantly different ($P < 0.05$) using LSD.

6) appeared to be intermediate in its levels of phytic acid P, total P and percent phytic acid P. The graduated treatment (treatment 7) had relatively high levels of phytic acid P, total P and phytic acid P as a percent of total P.

In 1989, root and crown mean phytic acid P and total P were determined in August following the final seasonal harvest and in September following a hard frost (Table 5). These two sample dates permitted determination of both harvest frequency and intensity effects on root and crown mean phytic acid P as well as the effects of hardening off. For August samples, the greatest mean phytic acid P value occurred with the least frequent and intense shoot harvest (treatment 5). Mean total P also was highest in this treatment as well as phytic acid P when expressed as a percent of total P (Figure 3). Similar effects of the harvest regimes were observed for samples collected in September. Again, treatments represented by a low harvest frequency and intensity (treatments 4 and 5) maintained both greater mean phytic acid P, total P and phytic acid P as a percent of total P, as compared with treatments representing more frequent and intense harvest, confirming the initial observation. From the two collection dates no trends were observed for either the hay or graduated treatments.

A comparison of root and crown mean phytic acid P between August and September revealed that with the exception of treatment 1, substantial increases in phytic acid P were observed following hardening off. Increases among treatments

Table 5. Mean total P, phytic acid P, and phytic acid P as the percent of total P content in alfalfa root and crown tissue from a simulated grazing study in 1989^a.

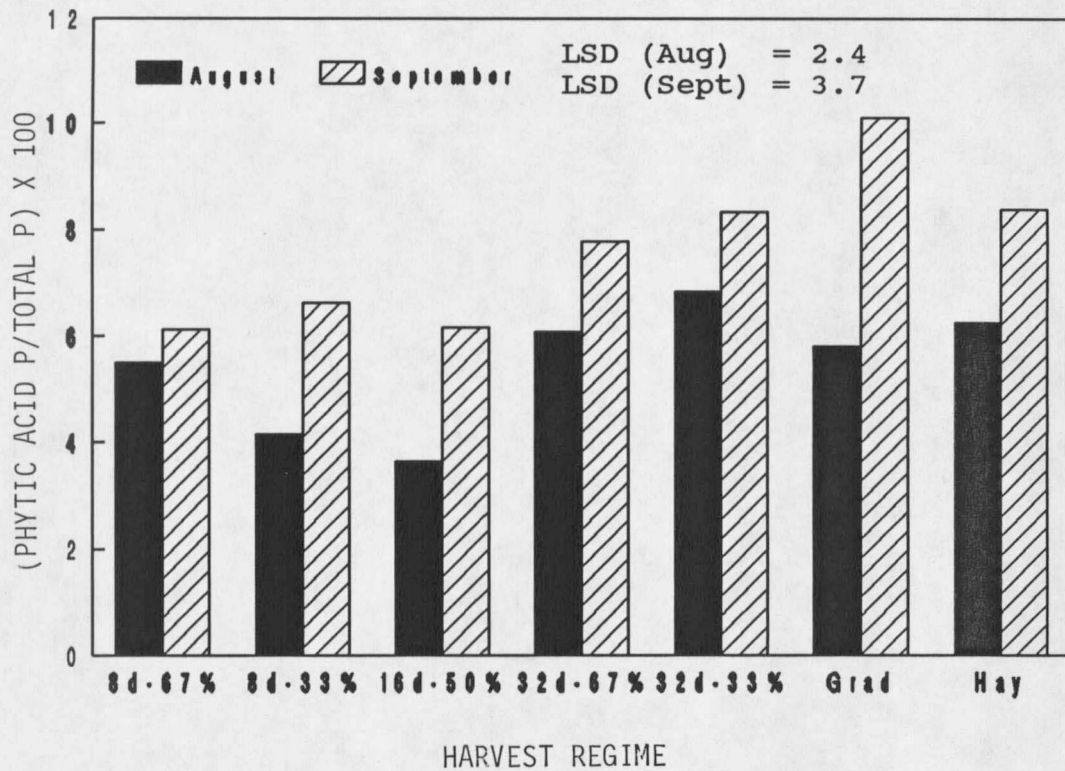
<u>Treatment</u>		<u>Phytic Acid</u>		<u>Total P</u>	
No.	Description ^b	August	September	August	September
-----mg/g tissue-----					
1	8d/67%	0.11 ABC ^c	0.11 A	1.97 AB	1.75 A
2	8d/33%	0.07 A	0.12 AB	1.66 A	1.87 A
3	16d/50%	0.08 AB	0.11 A	1.83 A	1.89 A
4	32d/67%	0.11 ABC	0.15 ABC	1.88 AB	1.94 AB
5	32d/33	0.15 C	0.19 BC	2.23 B	2.26 C
6	--HAY--	0.13 BC	0.17 ABC	2.00 AB	2.11 BC
7	GRADUATED	0.10 ABC	0.22 C	1.73 A	2.14 C
Mean		0.12	0.17	1.88	1.98
LSD (0.05)		0.05	0.06	0.36	0.09
CV %		27.5	36.3	14.2	8.9

^a See appendix (Table 13) for analysis of variance.

^b Selected treatments from the simulated grazing study.

^c Means within a column followed by the same letter are not significantly different ($P < 0.05$) using LSD.

Figure 3. Phytic acid P as the percent of total P in August and September root and crown tissue collected from the 1989 simulated grazing study.



were seen for mean total P, again with the exception of treatment 1 where mean total P decreased. The inability of treatment 1 to replenish root and crown phytic acid and total P may be related to the severity of shoot harvest during the growing season. Additionally, for each treatment phytic acid P, when expressed as a percent of total P, increased following a period of hardening off (Figure 3).

A second study was conducted to test the observation that

root and crown phytic acid P levels increase in response to hardening off. In the previous study only one cultivar (Spredor II) was examined. In the following study, root and crown phytic acid P was examined in five cultivars. With few exceptions, root and crown phytic acid P levels were observed to increase following hardening off in both locations, although differences between date means for location 1 were not statistically significant (Tables 6 and 7). For location 2, phytic acid P increased nearly 75% when the overall means for the first harvest are compared with the overall means following a hard killing frost. In contrast, no clear trends were observed for total root and crown P (Tables 8 and 9). However, the percent of total P represented by phytic acid P clearly increased from August to October (Tables 10 and 11).

Statistically significant differences between cultivar means were also observed but no clear relationship between fall dormancy and mean root and crown phytic acid P could be established (Table 6 and 7). In both locations Ladak-65 (fall dormancy 1-2) maintained lowest phytic acid P levels. Arrow (fall dormancy 3) consistently had the greatest phytic acid P levels, while Thor (fall dormancy 4) maintained less relative to other cultivars. For total root and crown P, no trends among cultivars were observed (Tables 8 and 9). A similar pattern seen previously with phytic acid P among cultivars was also observed upon examination of the percent of total P represented by phytic acid P (Tables 10 and 11).

Table 6. Root and crown phytic acid P among five cultivars and three collection dates at location one.^a

Cultivar	DR ^b	Phytic Acid P			Cultivar Mean
		Aug 3	Aug 23	Oct 8	
		-----mg/g tissue-----			
Thor	4	0.09	0.11	0.17	0.12 a ^c
Arrow	3	0.17	0.15	0.17	0.17 b
Garst-636	2	0.09	0.13	0.19	0.14 ab
Vernal	2	0.09	0.13	0.17	0.13 ab
Ladak-65	1-2	0.08	0.10	0.14	0.11 a
Date mean		0.11 A	0.12 A	0.17 A	

Cultivar mean LSD (0.05) = 0.035; Date Mean LSD (0.05) = 0.070
CV% = 33.2%

^a See mean squares for treatment effects (Table 14).

^b Dormancy Rating.

^c Means between date (upper case) and cultivar (lower case) followed by the same letter are not significantly different ($P < 0.05$) by LSD.

Table 7. Root and crown phytic acid P among five cultivars and three collection dates at location two.^a

Cultivar	DR ^b	Phytic Acid P			Cultivar
		Aug 3	Aug 23	Oct 8	Mean
		-----mg/g tissue-----			
Thor	4	0.12	0.13	0.22	0.16 ab ^c
Arrow	3	0.19	0.19	0.22	0.20 c
Garst-636	2	0.19	0.21	0.22	0.20 c
Vernal	2	0.18	0.20	0.21	0.20 bc
Ladak-65	1-2	0.10	0.11	0.18	0.13 a
Date mean		0.15 A	0.17 AB	0.21 B	

Cultivar Mean LSD (0.05) = 0.042; Date Mean LSD (0.05) = 0.045
CV% = 36.6%

^a See appendix for analysis of variance (Table 14).

^b Dormancy Rating.

^c Means between date (upper case) and cultivar (lower case) followed by the same letter are not significantly different ($P < 0.05$) by LSD.

Table 8. Root and crown total P among five cultivars and three collection dates at location one.^a

Cultivar	DR ^b	Total P			Cultivar
		Aug 3	Aug 23	Oct 8	Means
-----mg/g tissue-----					
Thor	4	3.00	2.79	2.61	2.80 c ^c
Arrow	3	2.69	2.64	2.39	2.57 abc
Garst-636	2	2.66	2.84	2.53	2.68 bc
Vernal	2	2.75	2.50	2.36	2.54 ab
Ladak-65	1-2	2.72	2.39	2.38	2.35 a
Date mean		2.67 B	2.63 B	2.45 A	

Cultivar Mean LSD (0.05) = 0.239; Date Mean LSD (0.05) = 0.160
CV% = 11.8%

^a See appendix for analysis of variance (Table 15).

^b Dormancy Rating.

^c Means between date (upper case) and cultivar (lower case) followed by the same letter are not significantly different ($P < 0.05$) by LSD.

Table 9. Root and crown total P among five cultivars and three collection dates at location two.^a

Cultivar	DR ^b	Total P			Cultivar
		Aug 3	Aug 23	Oct 8	Mean
-----mg/g tissue-----					
Thor	4	2.46	2.54	2.79	2.59 ab ^c
Arrow	3	2.67	2.76	2.75	2.73 b
Garst-636	2	2.40	2.71	2.39	2.50 a
Vernal	2	2.61	2.70	2.69	2.67 ab
Ladak-65	1-2	2.26	2.86	2.83	2.65 ab
Date mean		2.48 A	2.71 A	2.69 A	

Cultivar Mean LSD (0.05) = 0.210; Date Mean LSD (0.05) = 0.449
CV% = 12.6%

^a See appendix for analysis of variance (Table 15).

^b Dormancy Rating.

^c Means between date (upper case) and cultivar (lower case) followed by the same letter are not significantly different ($P < 0.05$) by LSD.

Table 10. Root and crown phytic acid P as the percent of total P among five cultivars and three collection dates at location one.^a

		Phytic Acid P as the Percent of Total P			
Cultivar	DR ^b	Aug 3	Aug 23	Oct 8	Cultivar Mean
		-----%			
Thor	4	3.1	3.8	7.4	4.7 a ^c
Arrow	3	6.4	5.8	7.4	6.5 b
Garst-636	2	3.4	4.4	6.8	4.9 a
Vernal	2	3.3	5.4	7.0	5.2 ab
Ladak-65	1-2	3.9	4.0	5.6	4.5 a
Date mean		4.0 A	4.7 AB	6.8 B	

Cultivar Mean LSD (0.05) = 0.014; Date Mean LSD (0.05) = 0.026
CV% = 35.3%

^a See appendix for analysis of variance (Table 16).

^b Dormancy Rating.

^c Means between date (upper case) and cultivar (lower case) followed by the same letter are not significantly different ($P < 0.05$) by LSD.

Table 11. Root and crown phytic acid P as the percent of total P among five cultivars and three collection dates at location two.^a

		Phytic Acid P as the Percent of Total P				
Cultivar	DR ^b	Aug 3	Aug 23	Oct 8	Cultivar Mean	
-----%-----						
Thor	4	4.9	5.3	7.5	5.9	ab ^c
Arrow	3	5.9	6.9	9.3	7.4	bc
Garst-636	2	7.6	7.8	8.7	8.0	c
Vernal	2	6.5	7.4	7.9	7.3	bc
Ladak-65	1-2	4.6	3.9	6.5	5.0	a
Date Mean		5.9 A	6.3 AB	8.0 B		

Cultivar Mean LSD (0.05) = 0.015; Date Mean LSD (0.05) = 0.019
CV% = 27.5%

^a See appendix for analysis of variance (Table 16).

^b Dormancy Rating

^c Means between date (upper case) and cultivar (lower case) followed by the same letter are not significantly different ($P < 0.05$) by LSD.

Ladak-65 had the lowest amount of phytic acid P as the percent of total P while Arrow maintained relatively greater values at both locations. Thor, the least dormant of the cultivars, had a smaller percent of total root and crown P as phytic acid relative to most other cultivars.

Previous studies have demonstrated that phytic acid serves as the major P storage compound in seeds and pollen. It accumulates during the development of this tissue and is broken down during germination to supply the growing seedling and pollen tube with P_i and myo-inositol. The first study demonstrated that alfalfa root and crown contained phytic acid P. The following studies indicate that phytic acid P levels fluctuate in response to P supply and demand during the alfalfa vegetative cycle.

Increases in shoot harvest frequency and intensity tend to deplete root and crown phytic acid P while levels clearly increase following hardening off. Chatterton (1980) observed that in the root and crown tissue of alfalfa, total P and P_i levels were associated with tolerance to harvest intensity. Here, evidence is presented which indicate that phytic acid is an important component of alfalfa root and crown tissue. Related work by Townsend (1990) and Agostinho (1988) observed other factors which respond similarly to severity of harvest and hardening off in this study. Increases in root and crown total nonstructural carbohydrates (TNC) levels, root diameters and forage yield were associated with infrequent and less

intense harvests. Similarly, during hardening off of the alfalfa plant, P mobilized from other plant tissues or from pools within the root and crown tissue may be stored as phytic acid presumably to be mobilized in response to subsequent regrowth the following season. Increases in root and crown TNC were also observed following a period of hardening off in this study.

This study also revealed differences among cultivars. Ladak-65, a relatively dormant cultivar, consistently was found to have the lowest observed levels phytic acid P as well as phytic acid P as a percent of total P. However, Thor, a relatively non-dormant cultivar also tended to have low levels of phytic acid P and phytic acid as a percent of total P. In the first study, a clearer trend indicating a relationship between dormancy rating and phytic acid P was observed. The physiological basis, if any, between root and crown phytic acid and dormancy rating is not understood at present. More detailed studies need to be conducted to determine whether a relationship in fact exists.

Phosphorus concentration in alfalfa has been identified as an important component for its nutritional quality (Butler, 1973) and productivity (Griffith, 1978; Pedersen, 1972; Rhykerd, 1972). Root and crown phytic acid deposition may be involved in the plants ability to regulate both root and shoot P concentrations. In addition, root and crown phytic acid may also function as a store of myo-inositol, mineral cations and

P as in seeds. Clearly, future studies of P metabolism and distribution in alfalfa need to address the role of root and crown phytic acid P.

SUMMARY

Phytic acid is the major storage form of P in seeds, pollen and several other plant structures. The purpose of this study was to determine whether phytic acid was present in the root and crown tissue of alfalfa. Phytic acid was found to represent approximately 10% of root and crown total P.

Ion-exchange chromatography and high voltage paper electrophoresis were used to confirm the presence of phytic acid in root and crown tissue. Ion-exchange chromatography also confirmed the reliability of the ferric precipitation method for routine quantification of phytic acid P in the tissue.

Root and crown samples were selected from a simulated grazing study to evaluate levels of phytic acid P, total P and percent total P represented by phytic acid. Phytic acid P as well as the other traits were found to be maintained at high levels when cutting was infrequent and forage removal was less intense. Comparisons were also made between plant samples collected following the final seasonal harvest and a hard freeze. In nearly every case phytic acid as well as phytic acid P when expressed as a percent of total P increased after a period of hardening off.

Additionally, differences in root and crown phytic acid were seen among cultivars. Several cultivars maintained

either high or low levels of phytic acid P and phytic acid P as a percent of total P. A relationship between these traits with cultivar dormancy rating was inconclusive.

Phosphorus is an important component in the nutritional quality of alfalfa. Since it appears that phytic acid is an important P component of alfalfa root and crown total P, future studies need to explore possible relationships with forage P content.

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APPENDIX

Table 12. Mean Squares (MS) and Degrees of Freedom (DF) for phytic acid P, total P and percent total P as phytic acid P for 1988 harvest treatments.

<u>Phytic acid P</u>		
Source	DF	MS
Blocks	2	0.0010905
Treatments	6	0.0085206
Residual	12	0.0032349

<u>Total P</u>		
Source	DF	MS
Blocks	2	0.03921
Treatments	6	0.15113
Residual	12	0.08947

<u>Percent Total P as Phytic Acid</u>		
Source	DF	MS
Blocks	2	0.0150
Treatments	6	5.3708
Residual	12	1.9950

Table 13. Mean Squares (MS) and Degrees of Freedom (DF) for phytic acid P, total P and percent total P as phytic acid P for August and September samples of 1989 harvest treatments.

<u>Phytic acid P</u>			
Source	DF	August	September
		-----MS-----	
Blocks	2	0.00080476	0.0036000
Treatments	6	0.00207620	0.0050635 *
Residual	12	0.00078810	0.0014278

<u>Total P</u>			
Source	DF	August	September
		-----MS-----	
Blocks	2	0.015490	0.010305
Treatments	6	0.108770	0.096941 **
Residual	12	0.041007	0.012544

<u>Percent Total P as Phytic Acid</u>			
Source	DF	August	September
		-----MS-----	
Blocks	2	8.3407	3.3313
Treatments	6	6.2940	4.0245
Residual	12	4.3668	1.7558

*,** Significant at $P = 0.05$ and $P = 0.01$ respectively.

Table 14. Mean Squares (MS) and Degrees of Freedom (DF) for phytic acid P as affected by date of root and crown collection and cultivar at location one and two.

<u>Location 1</u>		
Source	DF	MS
Blocks	3	0.00159
Date	2	0.19259
Error A	6	0.00809
Cultivar	4	0.00589 *
Date X Cultivar	8	0.00146
Error B	36	0.00183

<u>Location 2</u>		
Source	DF	MS
Block	3	0.00651
Date	2	0.01455 *
Error A	6	0.00267
Cultivar	4	0.01240 **
Date X Cultivar	8	0.00147
Error B	36	0.00263

*,** Significant at $P = 0.05$ and $P = 0.01$ respectively.

Table 15. Mean Squares (MS) and Degrees of Freedom (DF) for total P as affected by date of root and crown collection and cultivar at location one and two.

<u>Location 1</u>		
Source	DF	MS
Blocks	3	0.09753
Date	2	0.25457 *
Error A	6	0.04251
Cultivar	4	0.34074 **
Date X Cultivar	8	0.06235
Error B	36	0.08312

<u>Location 2</u>		
Source	DF	MS
Block	3	0.28832
Date	2	0.34000
Error A	6	0.33673
Cultivar	4	0.08837
Date X Cultivar	8	0.08837
Error B	36	0.06427

*,** Significant at $P = 0.05$ and $P = 0.01$ respectively.

Table 16. Mean Squares (MS) and Degrees of Freedom (DF) for phytic acid P as the percent of total P as affected by date of root and crown collection and cultivar at location one and two.

<u>Location 1</u>		
Source	DF	MS
Blocks	3	0.00021
Date	2	0.25457
Error A	6	0.00115
Cultivar	4	0.34074 *
Date X Cultivar	8	0.06235
Error B	36	0.08312

<u>Location 2</u>		
Source	DF	MS
Block	3	0.00056
Date	2	0.00244
Error A	6	0.00057
Cultivar	4	0.00180
Date X Cultivar	8	0.00015
Error B	36	0.00035

*,** Significant at $P = 0.05$ and $P = 0.01$ respectively.

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