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ACID PHOSPHATASE ACTIVITY AND LEAF PHOSPHORUS CONTENT IN TWO WHITE CLOVER (*Trifolium repens* L.) BREEDING LINES

J. Effendy

Jurusan Budidaya Pertanian, Fakultas Pertanian, Universitas Pattimura
Jl. Ir. M. Putuhena, Kampus Poka – Ambon 97233
e-mail: jeffendy@alumni.sfu.ca

ABSTRACT

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Aktivitas ensim asam fosfat (APase) adalah karakter fisiologis yang berhubungan dengan efisiensi penggunaan fosfat (P) dan berbeda secara genetik. Sebagai bagian dari studi tentang karakterisasi kultivar yang berhubungan dengan pengambilan dan penggunaan P dan efisiensi pengaturannya, penelitian ini dilaksanakan untuk mengukur aktivitas enzim APase pada akar dari dua kultivar klover putih [*Trifolium repens* (L.)]. Tujuan penelitian ini adalah (1) mengkarakterisasi enzim APase pada dua kultivar klover putih yang ditumbuhkan pada media cair dengan cukup P dan (2) mengetahui bila ada perbedaan induksi dari aktivitas enzim APase pada dua galur klover putih. Untuk mencapai tujuan tersebut maka pengukuran dilakukan pada parameter-parameter seperti aktivitas enzim APase, kandungan inorganik fosfor (Pi) di daun, total biomass, dan ratio bagian bawah per bagian atas tanaman (R:S ratio). Meskipun tidak berbeda nyata pada aktivitas enzim APase dan kandungan Pi di daun, kultivar (BL) 43 mempunyai aktivitas enzim APase dan kandungan Pi yang lebih besar tapi ini tidak dibarengi dengan berat biomasa yang besar. Sebaliknya dengan BL 45. Korelasi negative didapati antara aktivitas enzim APase dan kandungan Pi pada kedua kultivar. Korelasi positif didapati antara enzim APase dengan semua parameter pertumbuhan untuk BL 43 dan hanya dengan berat basah akar untuk BL 45. Tidak ada korelasi antara kandungan Pi dengan semua parameter pertumbuhan pada kedua kultivar kecuali antara kandungan Pi dengan rasio R:S pada BL 45. Hasil menunjukkan bahwa BL 45 adalah BL diinginkan karena dapat bertumbuh dan berproduksi dengan baik pada tanah-tanah dengan ketersediaan P yang sedang dan ini dapat meningkatkan potensi produksi tanaman klover putih.

Key words: white clover, acid phosphatase, Pi content, breeding lines.

INTRODUCTION

New Zealand soils are phosphorus (P)-deficient, and phosphatic fertilizers are applied primarily to stimulate white clover *(Trifolium repens* L.*)* growth. Application of phosphatic fertilizers to pastures incurs an ongoing cost to farmers, and, during downturns in the economic viability of New Zealand agriculture, these inputs can decline drastically. Selection for increased tolerance of low-P soils and selection for increased efficiency of utilization of P on medium- to high-P soils, where fertilizer is still applied, may result in white clover based pastures requiring lower inputs of P for adequate growth. To identify differences in the uptake and utilization of phosphorus that may have adaptive significance, selections were made in a glasshouse pot trial for differences in P-response (Mackay *et al*., 1990; Caradus *et al*., 1992). P response is the change in dry matter yield with increasing level of P supply such that high P responses are associated with small increases in P supply, the maximum yields being reached at lower P levels than for germplasm with a low P-response. Initially, the P-response of 119 cultivars of white clover was determined over 11 levels of P in pots of soil in a glasshouse (MacKay *et al*., 1990). Large differences were observed among cultivars for the change in response with increasing P, based on fitted response curves. White clover BLs were also identified that exhibited extremes in P-response similar to those observed among cultivars (Caradus *et al*., 1992). In addition, phosphorus is the most expensive nutrient as compared to the other nutrients (Câmara, 2000). The utilization of more adapted and efficient white clover cultivars in phosphorus uptake and utilization would certainly contribute for yield increase. Phosphorus uptake and utilization efficiency requires a vast and well-distributed class of enzymes known as phosphatases (Duff *et al*., 1994). The APase is a hydrolase which promotes the monoester phosphate hydrolysis, transforming organic phosphate in an inorganic form (Breseghelo *et al*., 1992). The APase activity determination is a fast and sensible test for the P status in the plant, and for this reason, Besford (1979a; 1979b) suggested its utilization as a plant P-deficiency indicator. This author observed in tomatoes, in which the unbalance of 15 nutrients was induced, that only P-deficiency caused an increase in leaf APase, hence the suggestion that this enzyme might be used to detect P

deficiency in these plants (Besford, 1979c). Several reports in the literature indicate that the APase activity is correlated to P-concentrations in tissues of several plant species: *Cucumis sativus* L. (Besford, 1978), *Lycopersicon esculentum* Mill (Besford, 1979a; 1979b), *Triticum aestivum* L. (McLachlan, 1982), *Oryza sativa* L. (Zaini & Mercado, 1985), *Phaseolus vulgaris* L. (Breseghelo *et al*., 1992), *Saccharum officinarum* L. (Silva & Basso, 1993), *Phaseolus vulgaris* L. and *Vigna unguiculata* L. Walp (Fernandez & Ascencio, 1994), *Cajanus cajan* L. and *Gossypium hirsutum* L. (Ascencio, 1994), and *Bactris gasipaes* Kunth (Bovi *et al*., 1998).

It has been suggested that lower root or leaf APase activities would be related to adequate or sufficient P-tissue concentrations, even under low external P, when compared to high APase activity plants under the same conditions (Mc Lachlan, 1980a; 1980b; Silberbush *et al*., 1981; Furlani *et al*., 1984; Helal, 1990; Tadano *et al*., 1993). Such low APase activity plants would be potentially adapted to low-P environments, which might be explained by a lower plant demand for P and a feedback regulation effect on enzyme activity (McLachlan & De Marco, 1982). Therefore, plants adapted to lower external P concentrations would have tissues adequately supplied in P and the lower P demand would inhibit APase activity. Tadano *et al*. (1993) demonstrated differential interspecific genetic variability for root secretion and APase activity for nine plant species, among them, rice, wheat, tomato and lupin, and observed also different magnitudes in root enzyme activity increases in response to P deficiency. McLachlan (1980a; 1980b) found variations in intact root enzyme activity among cultivated wheat species and their wild progenies. The cultivated species presented lower enzyme activities as compared to the wild, suggesting that selection for wheat plants more adapted to low P conditions might have occurred unconsciously. Furlani *et al.* (1984), investigating sorghum BLs, and Helal (1990), working with common beans, observed large differences among tolerant and susceptible cultivars to low external P concentrations, and that the tolerant ones presented lower APase activities. Intraspecific differences in root APase activities were observed among maize hybrids, related also to plant cycle (Kummerová & Buresová, 1990), and among inbred lines (Clark & Brown, 1974). The effects of the P-deficiency degree on the leaf APase activity in this species was also described by Elliott & Läuchli (1986) and Kummerová (1986). To identify differences in intact root APase activity of white clover plants, this research was carried out using two BLs of white clover, correlating the enzyme activity with plant characteristics related to total biomass FW yield and efficiency of P uptake and utilization.

MATERIALS AND METHODS

Plant Materials

Two breeding lines of white clover (*Trifolium repens* L.), BL 43 (accession number C23143: Poor performance in low fertility and high performance in high fertility) and BL 45 (accession number C23145: low performance in low fertility and low performance in high fertility (Table 1) were kindly gifted by Dr. Derek Woodfield, AgResearch Grasslands, Palmerston North, New Zealand.

Table 1. Description of white clover breeding lines evaluated for responsiveness to applied P in a field study in moist hill country. Descriptions include BL number. P-response category from Caradus & Dunn (2000).

Breeding Line	Accession Number	P-responsive category
43	C23143	Poor performance in low fertility and high performance in high fertility
45	C23145	Low performance in low fertility and low performance in high fertility

Plant Growth Conditions and Growth Measurements

Apical cuttings of each BL, cut to include the first three nodes, were planted in trays of moistened vermiculite and watered with half-strength Hoagland nutrient solution (Hoagland & Arnon, 1950). Plants were maintained in a heated glasshouse (15°C minimum, vented at 25°C, without supplementary illumination), and after two weeks in vermiculite (by which roots had developed), were transferred to 2 L dark colour plastic trays covered with polythene to exclude light. The roots were placed through the hole and submerged in liquid media. After growth in liquid medium for 6 weeks, the plants were sufficient size for use in experiments. Plants from each BL were maintained in half-strength Hoagland nutrient solution (P-containing). At 14 days, a subset of plants of each BL were harvested for use in experiments.

Leaf Phosphate Measurement

At specific time intervals, the first mature leaf from a single stolon from each six plants of each BL maintained in P-containing condition was weight, frozen in liquid nitrogen, ground to a fine powder, and 100 µL of 5.0 M of sulphuric acid (H_2SO_4) added to each sample. Each sample was then vortexed at room temperature for 1 min, 1.0 mL of water was added and after further mixing, the samples were centrifuged at 13,000 x *g* for 5 min. Triplicate (50 µL) aliquots of the supernatant was removed, diluted five-fold with water, and 50 µL of each pippetted into wells of a microtitre plate (A/S Nunc, Rothkilde, Denmark). Fifty µL of a series of P standards, containing 0, 40, 80, 120, 160, and 200 µL KH_2PO_4, were also pipetted in triplicate, into separate wells of the plate. To each sample or standard, 200 µL of the

phosphate assay reagent [1.75% ($w.v^{-1}$) L-ascorbic acid in 13.5% ($v.v^{-1}$) concentrated assay reagent (16 mM ammonium molybdate, 2.25 mM H_2SO_4, 0.15 mM antimony potassium oxide (+) tartrate)], was added, mixed and the absorbance of each well measured at 620 nm using an Anthos HTII plate reader (Anthos Labtec Instruments, Salzburg, Austria). The percentage P per gram FW was calculated using the P standard curve.

Extraction of Cell Wall Protein

Root tissues were excised from plants of both BLs maintained in P-containing media, washed with water, blot dried, weighed and then frozen in liquid nitrogen. The frozen tissue was powdered and extracted with 25 mM 2-mercaptoethanol for 30 min on ice, at a 3:1 extractant:tissue fresh weigh ratio. The slurry was centrifuged at 12 000 x *g* for 10 min at 4 °C, the supernatant removed and designated as the water-soluble whole tissue fraction. The pellet was resuspended with three volumes of 25 mM 2-mercaptoethanol, and after incubation on ice, the slurry was centrifuged as before, the supernatant again removed and discarded and the pellet resuspended with three volumes of 2-mercaptoethanol. This extraction procedure was repeated once more with 2-mercaptoethanol, and then a further 10 times with water. The final water wash extraction was removed from the pellet with two successive centrifugations and the pellet was then extracted with one volume of 1.0 M NaCl at 37 °C for 1 h. This slurry was then resuspended and extracted with one volume of 1.0 M NaCl on ice for 30 min and then one volume of 1.0 M NaCl at 4 °C for 18 h. After centrifugation, the 1.0 M NaCl extracts were pooled, designated the ionically-bound (1 M salt-extractable) cell wall extract, and used in APase assays.

Acid Phosphatase Assay

To measure activity in the root and leaf wall extracts, typically 5 to 20 µL of each fraction was made up to 50 µL with 50 mM sodium citrate buffer (pH 5.8) and 200 µL of substrate [2.5 % (w/v) ρ-nitrophenyl phosphate in 50 mM sodium citrate buffer (pH 5.8) at 20 °C. At specific time intervals, 50 µL was removed, added to 50 µL of 1.0 M NaOH and the absorbance read at 405 nm.

Statistical Analysis

Breeding lines were evaluated regarding APase activity, leaf Pi content, shoot FW, root FW, total biomass (BM), and R:S ratio. Results were submitted to statistical analyses using the SAS – System for Windows 6.11 (SAS Inc., 1996) program, through the GLM procedure. Analysis of variance of the treatment degrees of freedom were performed, allowing comparisons between both BLs. When test F was significant, the Duncan's test ($\alpha = 0.05$) for multiple mean comparisons was applied to identify differences between BLs. Correlations between APase activity and total biomass production to the other growth parameters were calculated using SAS, through the procedure CORR, calculating the Pearson's correlation coefficients within each BL.

RESULTS AND DISCUSSION

Although not statistically significant, the acid phosphatase activity of both BLs varied from 1.7348 to 1.9979. BL 43 presented a higher APase activity when compared to BL 45. No significant difference was observed in leaf Pi content between two BLs (Table 2). Although the substrate P concentration was considered medium level, P content in the leaf varied from 0.0097906 to 0.0104714 with BL 43 presented the higher and BL 45 presented the lower leaf Pi content (Table 2).

Differences in the values for root APase activity do not always evidence plant differences regarding P uptake and utilization efficiency (Furlani & Machado, 2002). Studies with genetic markers in bean genotype, one efficient and another inefficient proved that the gene locus for root APase was not associated to the loci for P uptake and utilization efficiency (Yan *et al.*, 2001). High root enzymatic activity, however, can result in plant stress signals for the need of higher external P supplies for plant development (Furlani & Machado, 2002).

Table 2. Intact root APase activity and leaf Pi content in two white clover breeding lines. Average of six replications, two plants per pot

Breeding Lines	APase activity Cell wall fraction (min^{-1} g^{-1} FW)	Pi content (% P g^{-1} FW)
43	1.9979a [a]	0.0104714 a
45	1.7348 a	0.0097906 a
Analyses of Variance	Breeding lines	
Means	1.866362	0.010131
F value[b]	2.04ns	2.58ns
C.V. (%)	48.34761	40.98204

[a] Means in the same column with the same letters are not significantly different by Duncan Multiple Range Test ($p<0.05$). All values are means of six replicates.

[b] *, **, *** differences significant at <0.05. <0.01, <0.001

There are two prevailing hypothesis about the role of acid phosphatases in plants and its relationship with plant nutritional P status, as follows: (1) plants adapted to low P conditions (efficient in P uptake and utilization) would present high leaf or root APase activity as a sign of the ability in hydrolyzing and remobilizing P, by root secretion and or leaf synthesis, making P more available to the plant, from soil or other older plants parts (Lee, 1988; Lefebvre *et al.*, 1990; Barret-Lennard *et al.*, 1993); or (2) plants adapted to low P conditions (efficient in P uptake and utilization) would present a lower P demand and consequently, a lower leaf or root APase activity under stress P conditions as compared to the non-adapted or higher P demanding plants. APase activity would then be a chemical indicator of the plant P deficiency severity degree, and the more the plant is stressed in relation to P, the higher the APase activity and the less adapted the plant would be (McLachlan 1980a; 1980b; Silberbush *et al.*, 1981; Furlani *et al.*, 1984; Elliot & Lauchli, 1986; Helal, 1990; Tadano *et al.*, 1993).

The first hypothesis anticipates a direct role of acid phosphatases on plant P uptake and use efficiency mechanisms, and the second assumes an indirect relationship, that is, an indication of a lower or higher P demand by the plants. It is impossible to conclude on any of the two above mentioned hypotheses, what might be credited to the fact of working with open-pollinated, high variability breeding lines and to the fact of working with very young plants, which might have been the case of the subpopulations, the results of which could not be confirmed by the correlation coefficients.

Clark & Brown (1974) observed differences for intact root APase activity between two maize inbred lines, using young plants, and the higher activity inbred line presented also the higher P content, that is, a direct relationship, indicating a better plant P nutritional status. In the present study, the breeding line having the higher P content had the APase activity, and the breeding line with the lower P content had a lower APase activity.

There was a clear differentiation for leaf Pi content. A lower or a higher leaf Pi content was inversely related to total biomass FW including root FW and shoot FW (Tables 2 and 3), BL 43 presented the lower biomass FW and the higher leaf Pi content, whereas BL 45 had the higher biomass FW and the lower leaf Pi content, evidencing a nutrient dilution which occurred as a consequence of BL 45 having a faster plant growth (Tables 2 and 3).

Linear correlation coefficients (r) were calculated between intact root APase activity and Pi content to all growth parameter characteristics (Table 4). For BL 43, there were positive correlations between APase activity with all growth parameters, while for BL 45 a positive correlation was found only between APase activity and root FW (Table 4). Further, for both BL 43 and BL 45, there were no correlations between Pi content and all growth parameters except for Pi content and R:S ratio in BL 45. In addition, for the existing correlations, the variables were not always the same, or even, sometimes directly or inversely correlated. For instance, APase activity presented negative correlation ($P<0.001$) with leaf Pi content ($r=-0.232$) in BL 43 and ($r=-0.345$) in BL 45 (Table 4) indicating that a decrease in the leaf Pi content induced increase in the enzyme activity in both breeding lines. On the other hand, BL 45 had a positive correlation between APase activity and root FW but has a negative correlation between Pi content and R:S ratio (Table 4). For BL 43, no correlations were found between Pi content and all growth parameters (Table 4), indicating an inconsistent behavior between both breeding lines with relation to APase activity and P-efficiency characters.

White clover breeding line with a higher biomass production, lower APase activity and leaf tissue P content is more desirable than breeding line with a lower biomass production but has higher APase activity and leaf Pi content. A higher biomass production observed in BL 45 showed that this BL has the ability to hydrolyzing P from organic forms to inorganic P, and remobilizing it to the apical growing (Duff *et al.*, 1994; Furlani & Machado, 2002).

Table 3. Total biomass FW of plant parts (root, shoot, and whole plant) and R:S ratio of white clover breeding lines. Average of six replications, 2 plants per pot

Breeding lines	Total biomass FW (g/plant)			R:S ratio FW
	Shoot FW (g/plant)	Root FW (g/plant)	Total FW (g/plant)	
43	6.951b [a]	2.4111b	8.818b	0.28084a
45	10.487a	1.8662a	12.898a	0.24442b
Analyses of Variance	Breeding lines			
Means	8.718972	2.138651	10.85762	0.262634
F value [b]	11.54***	4.90*	10.31***	6.12*
C.V (%)	58.47600	56.36950	57.33149	27.46696

[a] Means in the same column with the same letters are not significantly different by Duncan Multiple Range Test ($p<0.05$). All values are means of six replicates.

[b] *, **, *** differences significant at <0.05. <0.01, <0.001

Positive and negative correlations have been demonstrated between leaf or root enzyme activity and P content in plants (Besford, 1978; 1979; 1980; McLachlan & DeMarco, 1982; Dracup *et al*., 1984; Elliot & Lauchli, 1986; Fernandez & Ascencio, 1994); or between APase activity and tolerance or susceptibility to P deficiency (McLachlan 1980b; Silberbush *et al*., 1981; Furlani *et al*., 1984; Helal, 1990; Tadano *et al*., 1993). Nevertheless, the comparison of results is difficult because of the lack of systematization and standardization of methods, and criteria for the plant APase activity determination, in such way that, data of the literature are inconsistent or contradictory in relation to different species and different laboratory techniques.

Elliot & Lauchli (1986) observed inverse relationship between plant P deficiency degree and leaf APase activity only for cases of severe P-deficiency. The enzyme activity was not useful to detect moderate to light P deficiency degree was also determined for maize plants, as a function of the leaf position in the plant (Kummerová, 1986). Variations derived from genetic differences were also observed by Kummerová & Buresová (1990), when working with one early and one late maize hybrids, in the form of inverse relation between root dry matter yield and APase activity. The early hybrid presented lower root dry matter yield and higher enzyme activity whereas the inverse was observed for the late one, high yielding and low enzyme activity. For other plant species, the results are also quite inconsistent, and inverse relationships between APase activity and root P concentrations or contents have been found for sorghum (Furlani *et al*., 1984), white clover (Dracup *et al*., 1984) and common beans (Helal, 1990) between leaf and root APase activity and P deficiency in wheat plants (McLachlan & De Marco, 1982). On the other hand, Ascencio (1994) and Fernandez & Ascencio (1994) did not observed any relationship between leaf or root APase activity and P deficiency in bean, cowpea, pigeon pea, and cotton plants.

Consequently, such results are of difficult interpretation and comparison because APase activity data are derived from plants growing under different external P concentrations, or from different plant parts and/or from different breeding lines, the origin of which might influence the gene expression of the APase activity under a specific external P availability. Besides, the root ability to secrete APases can only be evaluated when intact roots are use for the APase activity determination, and the secreted root APase activity may or my not be related to the plant P nutritional status, which is more easily demonstrated when leaves are also analyzed. Another complicating factor to be considered is that the gene control of APase synthesis and activation seems to be independent from the one that confers to the root the ability of enzyme secretion, which is usually activated under low P levels (Fukuda *et al*., 2001). Fukuda *et al*. (2001), Wasaki *et al*. (2001), and other researchers have investigated the possibility of gene transfer of the root character responsible for APase secretion, in order to obtain plants more efficient in making self P available. However, the root secreted APase activity gene expression should be related to a significant root P-uptake efficiency expression, in order to obtain an effective gain in P-uptake and utilization efficiency.

Table 4. Simple linear correlation coefficient (r) estimates between root APase activity and white clover breeding line P-efficiency characters.

Breeding lines	Variable	APase activity	Pi content
43	Pi content	-0.232***	
	Root FW	0.220***	0.035ns
		P= 0.002	P-Value = 0.629
	Shoot FW	0.167*	0.018ns
		P= 0.020	P-Value = 0.803
	Biomass FW	0.180*	0.022ns
		P= 0.012	P-Value = 0.763
	R:S ratio	0.158*	0.045ns
		P= 0.028	P-Value = 0.539
45	Pi content	-0.345***	
		P= 0.000	
	Root FW	0.144*	-0.101ns
		P= 0.046	P-Value = 0.164
	Shoot FW	0.111ns	-0.037ns
		P= 0.124	P-Value = 0.609
	Biomass FW	0.119ns	-0.049ns
		P= 0.101	P-Value = 0.496
	R:S ratio	0.017ns	-0.153*
		P= 0.819	P-Value = 0.034

*, **, ***, differences significant at <0.05, <0.01 and <0.001 levels respectively; (ns = not significant).

From the actual knowledge on the high variability existing within white clover breeding lines as to their root ability in APase synthesis and secretion, and also on the independent gene control of such processes, it is not recommended using APase activity as a physiological marker for evaluation of plants in relation to P uptake and utilization efficiency under low P conditions. Results of this work are evidence that differentiated white clover plant mechanisms are involved in the soil P acquisition and in the internal P-remobilization, and being the need for further biochemical and molecular studies to characterize the genes responsible for these mechanism controls.

CONCLUSION

1. BLs 43 and 45 have negative correlations between APase activity and leaf Pi content.
2. No statistically significant were found in both breeding lines for APase activity and leaf Pi content, however, a higher total biomass yield in BL 45 indicates the existence of different mechanisms involving P mobilization in the soil and internal plant P remobilization.
3. The results suggest that APase may not be a major mechanism for scavenging or acquiring P. Other factors such as root architecture, secretion of low molecular weight organic acids, and microbial interactions will also affect on P acquisition capacity. Examination of APase response in low-P soil will provide more real information on the role of APase in acquiring P under P-limited field conditions.

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