Published with Open Access at Journal BiNET
Vol. 01, Issue 01: 01-15
Journal of Molecular Studies and Medicine Research
Home page: www.journalbinet.com/jmsmr-journal.html

Quantifying the role of arbuscular mycorrhizal colonization and acid phosphatase activity in grass biomass production

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ABSTRACT

Phosphorus (P) is one of the most important plant nutrients but a large portion of soil phosphorus (P) is not available to plants due to sequestration in organic forms. Phosphatase enzymes play an important role in the process, whereby the phosphate molecule is split off from the organic compound and these enzymes are originated from plant roots, mycorrhizal fungi and rhizosphere fungi and bacteria. It is still unclear to what extent these enzymes and arbuscular mycorrhizal symbiosis affect plant biomass. The aim of study is to quantify the role of acid phosphatase activity (APA) and arbuscular mycorrhizal colonization (AMF) in plant biomass and their correlation. A pot experiment of eight different grass species Lolium perenne-cv. Diploid, Lolium perenne-cv. Tetraploid, Lolium multiflorum, Festuca arundinacea, Poa trivialis, Poa pratensis, Phleum pratense, Holcus lanatus grown on a strongly P-fixing soil with two P treatments (with P fertilizer and without P fertilizer) was established. Rhizosphere soil for phosphatase activity, pH and roots for mycorrhizal colonization were collected. AMF colonization significantly differs with P treatments and species had no significant effect, although P treatments and species had no significant effect on acid phosphatase activity and pH. On the other hand, both had highly significant effect on dry weight but the interaction effect between P treatment and species also decline over time. A positive correlation between APA and dry weight in P deficient condition while APA and dry weight positively correlated to pH at fertilization with P. P deficient conditions encourage AMF colonization. The lack of significance differences between species suggests that higher variability of APA may be due to higher pH. Due to same back ground and nutritional status, species and P treatments not any impact on pH although P fertilization had a significant effect on dry weight indicating that plants require P for optimum growth. Species had positive impact on dry weight due to different resource use efficiency. P fertilization effect declines over time due to highly P-fixing capacity of soil. The variation of APA occurs due to pH changes, this positive correlation between dry weight and pH effect can be linked to variability of nutrient content.

Key words: *APA, AMF colonization, dry weight, pH, phosphorus and grass*

Please cite this article as: Sultana, J. & Siddique, M. N. A. (2015). Quantifying the role of arbuscular mycorrhizal colonization and acid phosphatase activity in grass biomass production. *Journal of Molecular Studies and Medicine Research*, 01(01), 01-15.

I. Introduction

Phosphorus (P) is one of the most important plant nutrients that play a vital role in plant metabolism (Bünemann et al., 2011; Frossard et al., 2000) Most of the time plant growth is limited by low

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availability of phosphorus and it's deficiency is a major constraint for plant biomass production globally (Attiwill & Adams, 1993; McGill & Cole, 1981). P is the most immobile and least available plant nutrients due to phosphorus depleted zone which is formed surrounding the roots during P uptake. Plants uptake P as phosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) from soil solution and this uptake depend on mineral phosphates solubility and organic phosphorus mineralization.

Organic phosphorus represents a major portion (sometimes more than 90%) of the total phosphorus and it is protected physically and chemically. This form of phosphorus is not easily available to plant (Hinsinger, 2001) due to slow release of phosphate. Currently agricultural production largely depends on rock phosphate and the reserves of rock phosphate may be depleted within 50-100 years (Cordell et al., 2009). P has been used in agriculture system in a way that resulting in the degradation of terrestrial, fresh water and marine resources (Tilman et al., 2001). Therefore, it is a prerequisite to improve understanding the mineralization of organic phosphorus due to minimizing reliance on mineral fertilizers and subsequent contribution to phosphorus nutrition of plants (Frossard et al., 2000; Jungk et al., 1993; Tarafdar & Claassen, 2003) but our understanding of the process involved in the mineralization of organic phosphorus in nearer to plant roots and the availability phosphate for plant nutrition remains inadequate.

Soil Phosphatase is one type of enzymes which play an important role in the process, whereby the phosphate molecule is split off from the organic compound and lead to assimilate in plants and microorganisms (Jennings, 1995). Actually these enzyme hydrolyse ester-phosphate bonds lead to release of phosphate which are easily available for plant or microorganisms (Ohm et al., 2013; Quiquampoix & Mousain, 2005). These enzymes originated from soil microorganism and plant roots. Although these enzyme released from many organisms (Hass et al., 1992) but mycorrhizal association play a potential role in acquisition of P has been documented in varied environmental conditions for different plants (Jansa et al., 2009) and benefited for plant growth.

The plants benefited from mycorrhizal association due to expansion of the root function. The hyphae of arbuscular mycorrhizal (AM) fungi hyphae can reach to the inaccessible zone of plant roots up to many centimetres compare to root hairs which spread out only few millimetres (Jakobsen et al. 1992; Jansa et al., 2003; 2005). Thereby, plants are able to acquire more nutrients from inaccessible zones of soil. The mycorrhizal association provide a very effective pathway in plant P uptake due to increased P absorption by the hyphae of mycorrhizal plants (Tinker, 1992). The hypha is much thinner to roots then it is accessible to narrow soil pores. It also release compounds which facilitate P discharge from insoluble inorganic and organic P constituents in soil and sometime develop complex fungal structures known as arbuscules and coiled hyphae (Smith & Read, 2010).

The phosphatase enzymes which are produced from hyphae play a vital role in the exchange of soil organic P into plant available form. Generally AM plants can acquire more total P than NM plants from the same soil area due to spared out of hyphae in soil (H, 1995). However, current research related to mycorrhizal association in net P uptake and plant biomass provides unclear experimental data. Therefore, the study related to soil microorganisms particularly arbuscular mycorrhizal association with enzyme activity and correlation to plant biomass may be one step forward for understanding the possible complex mechanisms. The association between grass and arbuscular mycorrhizal fungi are ecologically important for plant biomass and net P uptake. In single plant species, different AM fungi do not show same growth response whereas different species respond differently to one single AMF species (Klironomos, 2003). Therefore, I hypothesised that AMF colonization different species of grass. I hypothesized that phosphatase enzyme activity will be different in different species of grass. Optimum level of P is prerequisite for encouraging AMF colonization. if P level exceeds requirement of P may prevent AMF colonization (Grant et al., 2005). Therefore, I hypothesised that AMF colonization will be higher at fertilization without P compare to with P.

The phosphatase enzyme become less important in presence of high concentration of available P compare to low concentration because higher availability inhibits phosphatase synthesis in soil (Persson et al., 2003). It was hypothesised that phosphatase enzyme activity will be higher without P fertilizer compared to P fertilizer due to phosphate inhibits the phosphatase activities of soil. In case of

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dry weight hypothesised that dry weight differ significantly within species and P treatments as the growth response of different species are not same and their response vary with P fertilization. From literature it also known that dry weight increase with P fertilizer, because P nutrition is required for optimum growth (Jouany et al., 2011).

Generally the phosphatase activity can be influenced by numerous factors (Speir & Cowling, 1991). As mineralization of organic phosphorus depends on pH then we expect that acid phosphatase activity also varies with pH and hypothesised that pH also influenced by P treatments and species. The acid phosphatase activity is greater between the pH ranges between 5-6 (McLachlan, 1980). But the mechanism behind the mineralization of organic phosphorus and acid phosphatase activity is very complex and not fully understood. Understanding the possible effect of phosphatase enzyme on P uptake particular attention will be given to role of acid phosphatase activity and AMF colonization on grass biomass. Phosphomonoesters activities are higher compare to phosphodiesterase (Criquet et al., 2007) because P monoesters are also produced from P diesters. Phosphomonoesterase characterized by optimum pH and easily distinguish between acid and alkaline phosphatase. As the soil is acid that's why we choose acid phosphatase enzyme and APA exhibit optima below 6.0 (Vincent et al., 1992).

The aim of this research is to quantify the effect of fertilizer and grass species on AMF colonization and acid phosphatase activity and their correlation to grass biomass production. This research will be contributed to better understanding of the possible role of AMF colonization and acid phosphatase activity (APA) on plant biomass. The main research question is what the effects of P fertilizer species are with regard to AMF colonization, acid phosphatase activity, pH and Dry weight. Therefore, specific hypotheses were P fertilization and species significantly affected AMF colonization, P fertilization and species significantly influenced acid phosphatase activity (APA), P fertilization and species significantly influenced pH, P fertilization and species significantly influenced dry weight and AMF colonization, APA, pH and dry weight strongly correlated.

II. Materials and Methods

Experimental site and soil: The experiment was carried out at the Unifarm greenhouse which is located at Radix, Wageningen University. The soil characteristics included pH 5.6, organic matter 8.5%, total P 823 mg/kg, organic P 439 mg/kg and contains 67% sand, 22% silt and 11% clay. After harvesting soil samples were collected for measuring phosphatase enzyme activity and roots were collected for counting mycorrhizal colonization which was done in the lab of Atlas. For this study samples were collected from a PhD experiment.

Experimental design and management: Two factors were tested namely- fertilization and species of grass .The treatment which was taken into account in this experiment are summarized below in Table 01. The experiment was set up in a "Factorial" block design with three replications.

Table 01. Experimental factors with levels

Factors	Levels
Fertilization	P fertilizer; without P fertilizer
Grass species	8 species

Fertilization: Fertilization was carried out at the start of the pot experiment and after each cut. Two type of fertilizer P fertilizer and without P fertilizer. P fertilizer*:* 120 kg N/ha, 100 kg P_2O_5/ha, 15 kg S/ha, 130 kg K_2O/ha, 70 kg CaO/ha, 15 kg Na_2O/ha + trace elements. Without P fertilizer: 120 kg N/ha, 15 kg S/ha, 130 kg K_2O/ha, 70 kg CaO/ha, 15 kg Na_2O/ha + trace elements.

Grass species: Eight species of grass was used for this experiment. The English name and scientific name of theses grass are given below in Table 02.

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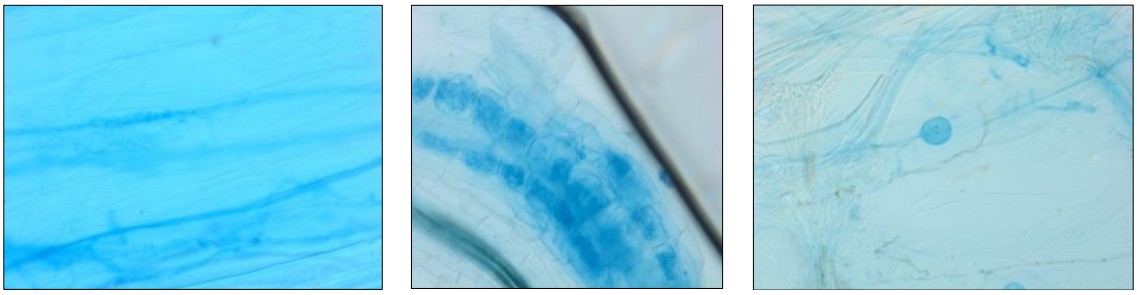
Table 02. Illustrates the name of grass species

English name	Scientific name
English ryegrass (diploid)	*Lolium perenne*-cv. Diploid
English ryegrass (tetraploid)	*Lolium perenne*-cv. Tetraploid
Italian ryegrass	*Lolium multiflorum*
Tall fescue	*Festuca arundinacea*
Rough bluegrass	*Poa trivialis*
Kentucky bluegrass	*Poa pratensis*
Timothy grass	*Phleum pratense*
Velvet grass	*Holcus lanatus*

Laboratory analyses: The laboratory analyses were carried out on the basis of two parameters. These are: enzyme activity and mycorrhizal colonization. All these parameters was analysed according to fertilization and species. The soil was collected from pot experiment and stored in air tight sealed plastic pots for analysing. The enzyme activity was determined using (Tabatabai & Bremner, 1969) method. The roots also collected from that pot experiment.

Root colonization analysis and procedure: Reagents that were used include 50% Ethanol (v/v), 10 % potassium hydroxide solution (KOH) and 2 % hydrochloric acid (HCl). At first the root samples were washed by using tap water to remove the remaining soil and debris, the roots were divided into small pieces (1-2 cm) and stored in 50% ethanol, placed in a 10 % potassium hydroxide solution (KOH) and autoclaved at 121^{0}C for 15 minutes. Then, the root pieces were rinsed thoroughly in tap water to remove the remaining KOH. The root pieces were acidified by soaking in 2 % hydrochloric acid (HCl) for 1 hour, rinsed again thoroughly in tap water and stained overnight in 0.01 % Trypan blue in lactoglycerol (lactic acid: glycerol: de-ionised water, 5:1:1). Then the root pieces were destained for 1-2 hours in 50 % ethanol to remove excess stain and stored roots in acidic glycerol. Finally, slides were prepared from destained root pieces and were observed by a compound microscope (Koske & Gemma, 1989). Each slide viewed randomly 100 times and then counted mycorrhizal colonization (Figure 01) as percentage



(a) (b) (c)

Figure 01. Three types of mycorrhizal colonization (a) hyphae, (b) arbuscules (c) hyphae and vesicle from left to right respectively

Enzyme activity analysis and procedure: Reagents that were used include Modified Universal buffer (MUB), pH 6.5 – preparation method followed from (Skujiņš and Burns 1976), Toluene-Fisher certified reagent, p-Nitrophenyl phosphate (PNP) solution, 0.115M. Dissolve 1.927 g of disodium p-nitrophenyl phosphate tetrahydrate in 50 ml MUB, Calcium chloride, 0.5M. Dissolve 73.5 g of $CaCl_2.2H_2O$ in 1 L demineralised water, Sodium hydroxide, 0.5M. Dissolve 20 g of NaOH in 1 L demineralised water, Standard stock p-nitrophenol solution. Dissolve 1.0 g of p-nitrophenol in 1 L demineralised water and Standard p-nitrophenol solution. Dilute 1 ml of the standard stock p-nitrophenol solution to 100 ml.

For this experiment at first the soil samples were air dried and crushed to pass a 2 mm screen. Then placed 1 g of soil (<2 mm) in a 50-ml Erlenmeyer flask and gradually added 4 ml of MUB, 0.25 ml of Toluene and 1 ml of PNP solution. For mixing the content properly a few seconds swirled the flask and placed it in an incubator for 1 hour at 37^0 C with the stopper. After 1 hour added 1 ml of 0.5M Calcium

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chloride and 4 ml of 0.5M Sodium hydroxide, again swirled the flask for a few seconds and filtered the soil suspension through a Whatman 589/3 filter paper. The absorbance of the filtered soil suspension was measured with a spectrophotometer by/at 405 nm. The colour of this suspension is stable for 24 hours.

The p-nitrophenol content of the filtrate was calculated by reference to a calibration graph of standards containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol. Pipetted 0, 1, 2, 3, 4 and 5 ml standard p-nitrophenol solution in the test tubes and rest volume to 5 ml were filled with mineralized water. Then, added 1 ml of 0.5M $CaCl_2$ and 4 ml of 0.5M NaOH. The test tubes were shaked for few seconds, filtrate the solution through a Whatman 589/3. Finally, I measured the colour intensity. For control, the procedure was same as previous but added 1 ml of PNP solution after addition of 0.5M $CaCl_2$ and 4 ml of 0.5M NaOH.

Dry weight: The data of dry weight were used from a PhD student (Ros Mart) of research group soil quality, Wageningen University. Dry weight data from four harvesting time, these were as follows, harvest 1 was done after 27 days, harvest 2 was done after 48 days, harvest 3 was done after 76 days and Harvest 4 was done after 96 days.

Measurement of pH: Actually, pH is a negative logarithm of the hydrogen ion activity that means pH= -log (H^+). For measuring pH at first placed 5 g of soil sample and 25 ml of 0.01M Calcium Chloride ($CaCl_2$) into sample vial of polyethylene and shacked the suspension 60+10 minutes by a shaker. The mixture leaved for at least 1 hour for settle down. Finally, the pH measured by a pH measurement machine.

Data analysis: Parameters data were collected and entered into excel for advance processing. At first data were verified for normality. Data were analysed using Genstat 14th edition, VSN International (Payne et al., 2008). Two-way ANOVA was carried out to determine the main factors, grass species and P treatment and their interaction. Values varying more than two times the standard deviation to the average were considered as outliers. A tukey post-hoc multiple comparisons were used to test for significance between different treatments. All experimental data was analysed by two-way ANOVA at 5% significance level. The relationships between acid phosphatase activity (APA), AMF colonization (%), pH and dry weight were analysed with correlation coefficient using excel.

III. Results

AMF colonization

Significant difference was identified among P treatments (without P and with P) whereas species had no significant influence on AMF colonization. *P. trivialis*, *H. lanatus* and *L. perenne*-cv. Tetraploid were more resilient towards fertilization without P although colonization with each treatment, *F. arundinacea* tended to have same colonization (Figure 02).

No significant effect was identified on the interaction between P treatments and species (Table 04). The colonization of *L. perenne*-cv. Tetraploid was significantly lower at treatment with P compare to without P.

Acid phosphatase activity (APA)

No significant differences were identified for P treatments (without P and with P) although higher acid phosphatase activity was detected at fertilization without P (Table 05). There was no significant difference between species. The species effect become significant when one outlier was removed (P=0.012). The ANOVA F-value indicates that interaction between P treatment and species had no significant impact on APA. *Lolium* species were more robust towards fertilization without P although *H. lanatus* had the highest acid phosphatase activity at fertilization with P (Figure 03). Acid phosphatase activity of *P. trivialis* showed higher variability of APA at fertilization without P.

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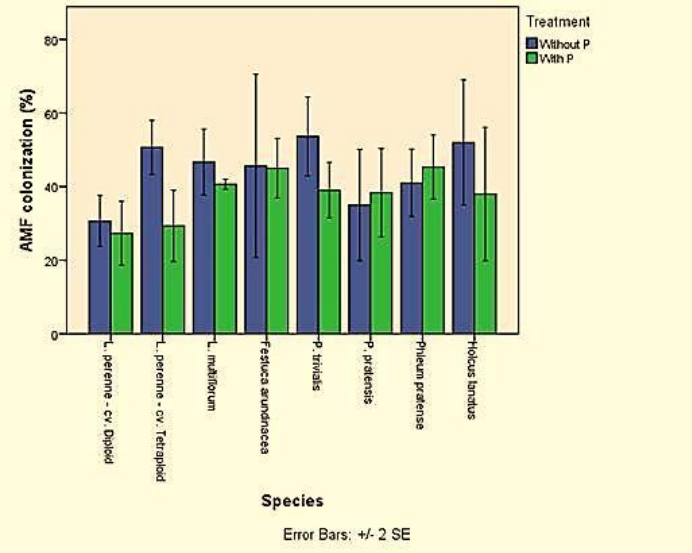

Figure 02. Results of AMF (%) colonization at two P treatment levels. Error bars indicate standard error, n=3

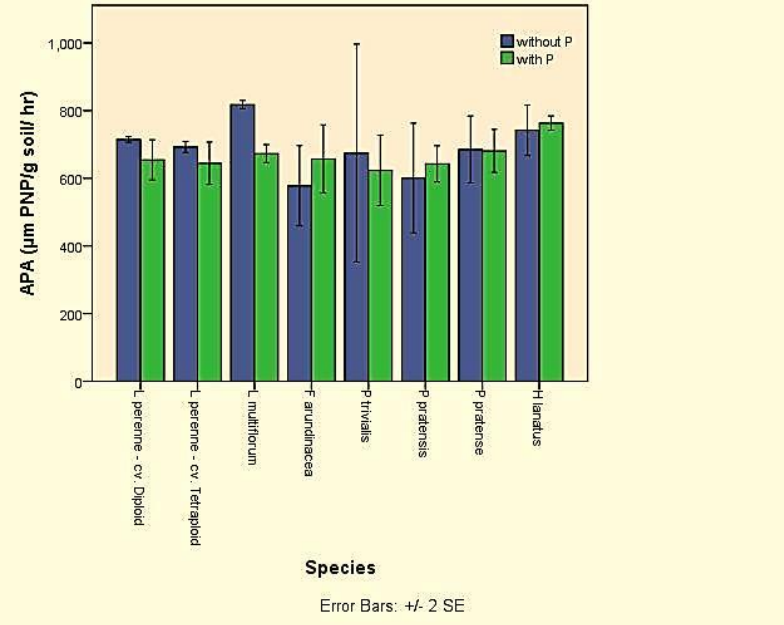

Figure 03. Results of acid phosphatase activity (µm PNP/g soil/hr.) at P treatments level. Error bars indicate standard error, n=3

Soil pH

No significant difference was observed in pH between P treatment (without P and with P) whereas species is slightly related (P=0.06) to pH. At both P treatments, the highest pH was observed in the soil cultivated with *H. lanatus* where the lowest in *P. pratensis*. At both P treatments *P. pratense* showed exactly the same pH. In this experiment the APA calculated by using universal modified buffer (pH 6.5) and observed that *P. pratense* had lower APA value and *H. lanatus* had higer value. Then we considered the lowest (4.4) and highest (5.0) soil pH for calculating the APA of these species. The result explained in Table 03. At pH differences, *H. lanatus* respond 16% more APA where *P. pratensis* only 5% higher APA.

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Table 03. Effect of the acid phosphatase activity and species on pH

pH	Species	APA µm PNP/g soil/hr.
4.4	*P. pratensis*	388
4.4	*H. lanatus*	508
5.0	*P. pratensis*	408
5.0	*H. lanatus*	588

Dry weight (DW)

P fertilization had a significant effect on dry weight (<0.001) of different harvesting time except harvesting time four (Table 04). And species also had significant effect on dry weight. Different grass species grown with P fertilization had a significantly higher dry weight than grown without P fertilization. *L. multiflorum* had highest dry weight in case of fertilization with P in first and second harvest then gradually decrease in third and fourth harvest whereas of *L. perenne* (diploid and tetraploid) had highest dry weight in third harvest. In fourth harvest every species had lower dry weight compare to third harvest. The ANOVA F-value indicated that P treatments had a stronger effect on dry weight of first and second harvest than third and fourth harvest. The interaction between P treatment and species was significant for dry weight of first harvest but no other three harvesting time (Table 04).

Table 04. Effect of P treatments and species on acid phosphatase activity, arbuscular mycorrhizal colonization and dry weight according to two-way analysis of variance

Parameters*	P treatments (P)		Species (S)		Interaction P×S	
	F- value	P- value	F-value	P-value	F-value	P-value
APA	0.55	0.462	1.64	0.162	0.79	0.6
AMC (%)	4.37	0.045	1.74	0.137	1.1	0.39
pH	0.3	0.59	2.16	0.067	0.44	0.868
DW_1	113.91	<.001	172.64	<.001	5.28	<.001
DW_2	81.52	<.001	124.05	<.001	1.37	0.230
DW_3	16.07	<.001	48.27	<.001	0.81	0.580
DW_3	2.00	0.162	44.88	<.001	0.73	0.651
DW_{1-2}	122.66	<.001	181.01	<.001	2.88	0.010
DW_{1-3}	68.01	<.001	118.74	<.001	0.70	0.673
DW_{1-4}	37.59	<.001	96.04	<.001	0.40	0.897

*(µmPNP/g soil/hr. = micro mole p-nitrophenol phosphate per gram soil per hour)

* APA= Acid Phosphatase Activity (µmPNP /g /hr.); AMC= Arbuscular mycorrhizal colonization (%); pH=soil pH; DW_1 = Dry Weight(g) of first harvest; DW_2 =Dry Weight(g) of second harvest; DW_3 = Dry Weight(g) of third harvest DW_3=Dry Weight(g) of fourth harvest; DW_{1-2} = Dry Weight(g) of first and second harvest; DW_{1-3} = Dry Weight(g) of first, second and third harvest; DW_{1-4} = Dry Weight(g) of first, second, third and fourth harvest.

Correlation between different parameters when fertilization without P

At fertilization without P, a significant positive linear relationship between APA and DW was identified although pH and other 3 parameters (APA, AMC % and DW) did not show any positive relation (Table 06). The relationship between APA and DW was stronger in first harvest but gradually it disappears.

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Table 05. P fertilization effect on APA, AMC (%), DW_1, DW_2, DW_3, DW_4, DW_{1-2}, DW_{1-3} and $DW1_{-4}$

Species		APA	pH	AMC (%)	DW_1	DW_2	DW_3	DW_4	DW_{1-2}	DW_{1-3}	DW_{1-4}
LP(d)	P-	715	4.75	30.7	2.55	5.85	6.88	5.00	8.41 (bc)	15.28	20.28
	P+	654	4.73	27.3	3.47 (b)	6.94	8.03	5.15	10.41 (jk)	18.44	23.60
LP(t)	P-	692	4.79	50.7	2.70	5.89	7.16	5.30	8.59 (bc)	15.74	21.05
	P+	644	4.75	29.3	3.53 (b)	6.87	8.25	5.47	10.40 (jk)	18.65	24.12
LM	P-	818	4.66	46.7	3.99	7.37	7.97	5.12	11.36 (a)	19.33	24.44
	P+	673	4.74	40.7	5.66 (a)	8.33	7.97	4.55	13.99 (i)	21.96	26.51
FR	P-	578	4.69	45.7	1.643	5.11	6.52	5.39	6.76 (d)	13.28	18.67
	P+	657	4.73	45	2.21 (dc)	5.66	7.99	6.33	7.88 (l)	15.87	22.19
PT	P-	674	4.71	53.7	1.10	3.27	2.69	1.32	4.37 (e)	7.06	8.38
	P+	624	4.61	39	1.61 (de)	4.08	3.34	1.79	5.69 (m)	9.03	10.82
PP_1	P-	601	4.64	35	0.80	3.73	3.31	1.50	4.53 (e)	7.83	9.33
	P+	643	4.59	38.3	0.95 (e)	3.92	4.07	1.70	4.87 (m)	8.94	10.64
PP_2	P-	685	4.72	41	1.92	5.71	5.31	3.30	7.63 (cd)	12.93	16.23
	P+	681	4.72	45.3	2.50(c)	6.62	6.16	3.73	9.11(kl)	15.27	19.00
HL	P-	742	4.84	52	3.18	5.97	5.46	3.95	9.15(b)	14.61	18.56
	P+	763	4.81	38	3.78(b)	6.98	5.65	4.14	10.77(j)	16.41	20.55

(Means with the same letter are not significantly different). (µm PNP/g soil/hr. = micro mole p-nitrophenol phosphate per gram soil per hour)

* APA= Acid Phosphatase Activity (µmPNP /g soil/hr.); AMC= Arbuscular mycorrhizal colonization (%); pH=soil pH; DW_1 = Dry Weight(g) of first harvest; DW_2 =Dry Weight(g) of second harvest; DW_3 = Dry Weight(g) of third harvest; DW_4 =Dry Weight(g) of fourth harvest; DW_{1-2} = Dry Weight(g) of first and second harvest; DW_{1-3} = Dry Weight(g) of first, second and third harvest; DW_{1-4} = Dry Weight(g) of first, second, third and fourth harvest; LP (d)= *Lolium perenne*-cv. Diploid; LP (t)= *Lolium perenne*-cv. Tetraploid; LM= *Lolium multiflorum*; FR= *Festuca arundinacea*; PT= *Poa trivialis*; PP_1= *Poa pratensis*; PP_2= *Phleum pratense*; HL= *Holcus lanatus.*

Table 06. Correlation among APA, pH, AMC (%) and DW of different harvesting time without P fertilization

	APA	pH	AMC (%)	DW_1	DW_2	DW_3	DW_4	DW_{1-2}	DW_{1-3}	DW_{1-4}
APA	1									
pH	0.27	1								
AMC (%)	0.21	0.39	1							
DW_1	0.87**	0.37	0.21	1						
DW_2	0.72*	0.25	0.00	0.93	1					
DW_3	0.48	0.17	-0.06	0.81*	0.91**	1				
DW_4	0.31	0.29	0.00	0.70(*)	0.80*	0.96***	1			
DW_{1-2}	0.80*	0.31	0.10	0.98***	0.98***	0.88**	0.77*	1		
DW_{1-3}	0.68	0.26	0.03	0.93***	0.98***	0.96***	0.88**	0.98***	1	
DW_{1-4}	0.58	0.27	0.02	0.89**	0.95***	0.98***	0.94***	0.94***	0.99***	1

[(*) =5-10% level, *=5% level, **= 1% level,*** = less than 1% level of significance] (µm PNP/g soil/hr. = micro mole p-nitrophenol per gram soil per hour)

* APA= Acid Phosphatase Activity (µm/g soil/hr.); AMC= Arbuscular mycorrhizal colonization (%); pH=soil pH; DW_1 = Dry Weight(g) of first harvest; DW_2 =Dry Weight(g) of second harvest; DW_3 = Dry Weight(g) of third harvest; DW_4 =Dry Weight(g) of fourth harvest; DW_{1-2} = Dry Weight(g) of first and second harvest; DW_{1-3} = Dry Weight(g) of first, second and third harvest; DW_{1-4} = Dry Weight(g) of first, second, third and fourth harvest.

If the dry weight is plotted against acid phosphatase activity, the result basically displayed a significant and positive correlation between dry weight and acid phosphatase activity. (R^2=0.64) The higher acid phosphatase activity exhibited higher dry weight (Figure 04).

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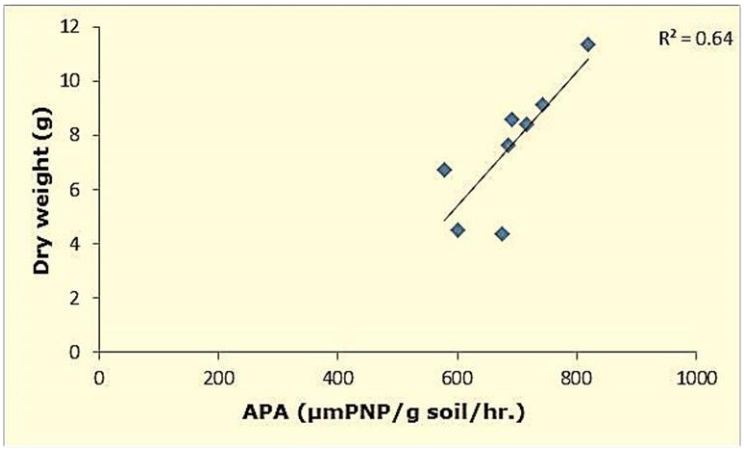

Figure 04. Correlation of acid phosphatase activity with dry weight (g)

Correlation between different parameters when fertilization with P

The results show the correlations among different parameters. The values of different parameters are in Table 07. There was a significant positive relationship between pH and acid phosphatase activity but no relationship was found between mycorrhizal colonization and pH (Table 07). Positive relationship also found between pH and dry weight and it vary with different harvesting time. No correlation was found between acid phosphatase activity and dry weight of different harvesting time.

Table 07. Correlation among APA, pH, % of MC and DW of different harvesting time with P fertilization

	APA	pH	AMC (%)	DW_1	DW_2	DW_3	DW_4	DW_{1-2}	DW_{1-3}	DW_{1-4}
APA	1									
pH	0.71*	1								
AMC (%)	0.17	0.11	1							
DW_1	0.42	0.73*	-0.20	1						
DW_2	0.48	0.84**	-0.16	0.94***	1					
DW_3	0.07	0.70(*)	-0.27	0.66	0.77*	1				
DW_4	0.18	0.77*	-0.16	0.52	0.66	0.94***	1			
DW_{1-2}	0.46	0.80*	-0.19	0.98***	0.99***	0.73*	0.60	1		
DW_{1-3}	0.33	0.82*	-0.23	0.92***	0.97***	0.90**	0.79*	0.96***	1	
DW_{1-4}	0.30	0.84**	-0.22	0.85**	0.92**	0.95***	0.88**	0.90**	0.99***	1

[(*) =5-10% level, *=5% level, **= 1% level and *** = less than 1% level of significance] (µm PNP/g soil/hr. = micro mole p-nitrophenol phosphate per gram soil per hour)

* APA= Acid Phosphatase Activity (µm/g soil/hr.); MC= Mycorrhizal colonization (%); pH=soil pH; DW_1 = Dry Weight(g) of first harvest; DW_2 =Dry Weight(g) of second harvest; DW_3 = Dry Weight(g) of third harvest; DW_4 =Dry Weight(g) of fourth harvest; DW_{1-2} = Dry Weight(g) of first and second harvest; DW_{1-3} = Dry Weight(g) of first, second and third harvest; DW_{1-4} = Dry Weight(g) of first, second, third and fourth harvest

If the pH value is plotted in x-axis and acid phosphatase activity in y-axis, the result discovered a positive correlation between pH value and acid phosphatase activity (R^2=0.51). Therefore, pH changes can be explained by variation of acid phosphatase activities (Figure 05).

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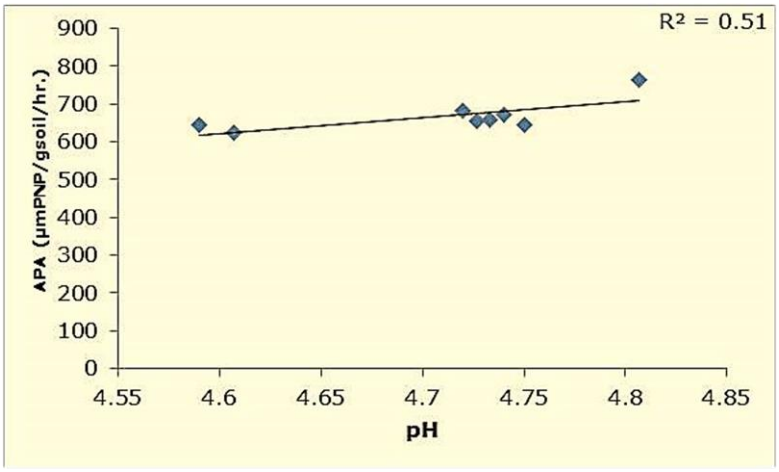

Figure 05. Correlation of pH with acid phosphatase activity

When analysing soil pH and dry weight, a strong positive relationship was found between them (R^2=0.63). Therefore, soil pH differences can be described by changing the dry weight of grass (Figure 06).

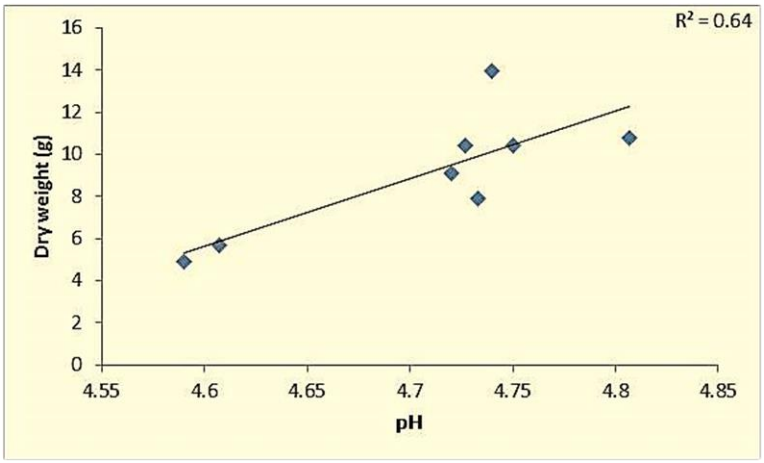

Figure 06. Correlation between pH and dry weight (g) at fertilization with P

IV. Discussion

AMF colonization

P treatments changed AMF colonization to different extents. Different grass species had higher AMF colonization in case of fertilization without P. The possible mechanisms behind the observations will be explained below.

Effect of species or cultivar on AMF colonization: We hypothesised that AMF colonization varies significantly with in grass species due to varying levels of AMF colonization and reliance on AMF. In contrast to expectation, no significant relation between species and AMF colonization was found. The literatures related to AMF colonization represent that colonization varies in different species (Klironomos, 2003) due to different genetic character. However, the study result does not support that. Therefore our results not confirmed hypothesis because colonization rate not differ significantly.

Effect of P treatments on AMF colonization: P deficient condition encourages arbuscular mycorrhizal colonization. This supports one part of first hypothesis that fertilization without P had significant effect on AMF colonization, as seen in previous studies (Grant et al., 2005; Kahiluoto et al., 2012; Ryan & Graham, 2002). This was possibly due to high nutrient availability which decreases plant

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dependency on AMF. Actually AMF colonization may be favourable only for the situation where P fertilizer is detrimental.

Effect of soil P levels on AMF colonization: AMF colonization has a tendency to decline with increasing background soil P level. As our soil was highly p fixing then may be P fertilization increase background level of P. Gradually resulting higher plant P status and decrease AMF colonization, as seen in other observations Kahiluoto et al., (2001) where higher mycorrhizal association at low P content compare to intermediate and mycorrhizal association might decrease plant growth at high soil P level. Basically, availability of P in the soil solution affects plant available P and AMF colonization.

Acid phosphatase activity

Effect of species on acid phosphatase activity: No significant differences were identified which reject our second hypothesis. However wide ranges in phosphatase enzyme activity were obtained with in different grass species due to different rooting system. Different species have not same capacity to discharge the enzyme. One species *Poa trivialis* showed higher variability of APA although the factors driving changes in higher variability cannot be identified. This was possibly may be due to pH changes resulting from changing of organic matter or any other biotic and abiotic environment.

Effect of P treatments on acid phosphatase activity: We hypothesized that fertilization with P would decrease acid phosphatase activity due to increase levels of available P which inhibits acid phosphatase activity and it is supported by a number of studies (Allison & Vitousek, 2005; Makoi & Ndakidemi, 2008). In contrast to anticipation, no significant result was outcome between P treatments in any grass species. This may be partly due to high P protection character of the soils (Turrión et al., 2000). Other possible reason chemical immobilization through adsorption, precipitation reactions and P applied as fertilizer had been immobilized as microbial biomass. The *Lolium* species were more resilient towards fertilization without P and *L. multiflorum* had the highest acid phosphatase activity (Figure 03). It may be due to P deficient plants had greater activity than sufficient P level (Gilbert et al., 1999; Šarapatka et al., 2004) On the other hand, *H. lanatus* had the highest acid phosphatase activity when fertilization with P and other two species namely *P. pratensis* and *P. pratense* respond positively to fertilization with P which could be resulted by the root system or poorer fertilization.

Effect of P treatments and species on pH

At soil pH either species or P treatments had no significant effect. This result cannot be explained in proper way because one species had higher pH and one had lower where one had exactly same pH in both P treatments. There is no perfect pattern for both P treatments. As the soil have same back ground and nutritional status. Therefore species and P treatments not influence so much about changing the pH.

Effect of P treatments and species on dry matter

P fertilization had a significant effect on dry weight of grass and this is supported Jouany et al. (2011) observation where yield increase significantly by P supply under not limited N condition. This may be explained as all plants required P for optimum growth and metabolism. The dry weight differs significantly among different grass species. The *Lolium* species and *H. lanatus* had higher dry weight compare to other species. On the other hand *F. arundinacea* had medium dry weight and *Poa species* had lower dry weight. It may be due to different species have different resource use efficiency. As for example *Lolium perenne* have adaptive power to nutrient rich environment where *Festuca* have better adaptation power to low fertility (Jouany et al., 2011) and it has resource conservation strategies. The species with conservation strategies have chemical composition that result delay decomposition rate and nutrient availability take more time (Fortunel et al., 2009). This explanation matches for *F. arundinacea,* because this species had highest dry weight of 4th harvest compare to other species. It was observed that P fertilization effect declines over time this may be due to soil character. Because, the soil is highly P-fixing. At early stage of phosphorus application, it is more available to plant but gradually it is fixed by soil and less available to plant. For this reason, may be dry weight of 4th harvest

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is low compare to 3rd harvest and P fertilization effect declines over time and that's why interaction effect between P treatment and grasses species also decline over time.

Correlation between different parameters

Several correlations were found correspond with results of others. In our experiment, we didn't found any correlation between AMF colonization and acid phosphatase activity of soils in both P fertilizations (Table 06 and 07). It may be due to acid phosphatase originated by organisms other than AMF. These enzymes also originated from plants (Juma & Tabatabai, 1998; Tadano et al., 1993). The study reveals a positive correlation between acid phosphatase activity and dry weight of grass without P fertilization (Figure 04). It may be explained that P deficient condition enhances APA which directly correlated to dry weight. This correlation is in good agreement with the results of (Raposo et al., 2004) observation where they found linear correlation between acid phosphatase activity and shoot biomass in soybean but several studies showed that acid phosphatase negatively correlated to dry matter under P deficiency condition (de Loudes Breseghelo et al., 1992; Furlani et al., 2002). We actually did not found any observation related to correlation between acid phosphatase activity and dry weight among different grass species. However, no correlation found between pH and AMF colonization. There was no relation between dry weight and AMF colonization. Although positive relation between AMF colonization and plant biomass also observed by (Tawaraya et al., 2007). The study confirmed a positive correlation between acid phosphatase activity and soil pH with P fertilization (Figure 05). Whereas others found the negative correlation between acid phosphatase activity and soil pH (Gehlen & Schröder, 1990; Šarapatka et al., 2004). Actually the variation of APA occurs due to pH changes and greatest acid phosphatase activity in acidic range between 5 to 6 (McLachlan, 1980). With positive relation between APA and pH, the result also showed same relationship between dry weight and pH (Figure 05). This may be explained by variation in total nutrient content (due to pH change) related to differences in dry weight (Finn et al., 1993).

V. Conclusion

AMF colonization significantly differs with P treatments; especially P deficient condition encourages AMF colonization although species had no significant effect on AMF. In case of APA and pH either P treatments or species had no significant effect. The lack of significance differences between species suggests that higher variability of APA may be due to higher pH. Due to same back ground and nutritional status, species and P treatments not any impact on pH. P treatments significantly increase dry weight but the impression disappears over time due to highly P-fixing capacity of soil. Species had positive impact on dry weight due to different resource use efficiency. Acid phosphatase activity correlated positively to dry matter under P deficiency condition. P deficient condition enhances APA which may be directly correlated to dry weight. APA and dry weight positively correlated to pH at fertilization with P. The variation of dry weight can be linked to variability of nutrient content due to pH changes.

VI. Implications and recommendations

P fertilization effects on mycorrhizal colonization but not in the acid phosphatase activity .Time period is too short to observe the exact effect of P fertilization on acid phosphatase activity and suggest that more replication would be included. There were only two P treatments. Therefore, more p treatments rather than without or with P would be suitable for further research. There was no correlation between dry weight and arbuscular mycorrhizal colonization. The mechanisms of AMF colonization in phosphorus uptake and plant biomass is still required to understand the contribution of these associations in the below ground environment. Therefore, it could be suggested that considering other organisms related to phosphatase activity to broaden the understanding the possible role of microorganisms in P uptake and plant biomass. Although plant P uptake parameters were not included due to budget and time also a factor. The soil is highly P fixing acid soil and understanding the possible role of phosphatase in organic phosphorus mineralization required to include different type of soil. It was expected that top soil (0-10 cm) have positive effect on colonization compare to subsoil but it was not found any rhizosphere effect on mycorrhizal colonization. It may be due to same background

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status between two depths of soil. Generally acid phosphatase decrease with soil but we calculated only APA of top soil due to time shortage. Consequently, not only the top soil but also the sub soil would be included for future study. This study was as pot experiment and then, it is obligatory to practice this experiment on field condition representative to complex natural situations. Overall results advocate a possible role of AMF and APA in plant biomass production and sustainable use of fertilizer. Because effective P management practices whether through efficient fertilizer use or encourage of mycorrhizal associations is important for minimizing adverse effects of environment and also for economic use of P fertilizer. Finally, improved knowledge on complex interaction among microorganisms, plant and soil phosphatase may help in improving P uptake and plant biomass production.

VII. Acknowledgements

Authors were indebted to supervisor Thom Kuyper, who always supported and inspired by enthusiasm, valuable and constructive comments in order to improve this research. Second supervisor Ros Mart always helped and supported during the study. Many thanks to Jaap Nelemans, Willeke van Tintelen and Thamas Salanki for their assistance in the lab and microscopic works. Also thanks to Cor Langaveld, who was study advisor and helped and supported always to do study in time in Wageningen. Gratitude to all the people that helped in different phases of the study, without their contribution this study might be impossible. Special thanks to Renee, Edvaldo, Jingju, Gregory, Anupol. Deepest thanks drive to whole Soil Quality group for their friendliness and delightful knowledge and perception.

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