Tropentag 2012, Göttingen, Germany
September 19-21, 2012

Conference on International Research on Food Security, Natural Resource Management and Rural Development organised by:
Georg-August Universität Göttingen and University of Kassel-Witzenhausen

Differential Expression of *alp* Gene and Sporulation pattern of *Glomus* with Ri T-DNA Transformed hairy Roots

Anshul,[a] Puri, Shanuja Beri[b] and Alok Adholeya[b]

aTeri University, 10, Institutional Area, Vasant Kunj, New Delhi-110070.

bCentre for Mycorrhizal Research, Bioresources and Biotechnology Division, TERI, Darbari Seth Block, IHC Complex, Lodhi Road, New Delhi-110 003, India.

Introduction

P is taken up by plant roots as phosphate (Pi), which remains as one of the least available nutrient in the soil even after the application of P-fertilizers. For this, plants have evolved a variety of adaptive strategies to improve their Pi-acquisition that includes altered root morphology (Williamson et al. 2001), organic acids exudation, expression of the high-affinity phosphate (Pi) transporter gene (Smith 2002) and release of phosphatases and nucleases so as to solubilize Pi from organic resources. The establishment of symbiosis with arbuscular mycorrhizal (AM) fungi (Hause and Fester 2005) is also an adaptive and evolved strategy for the same. Based on this, the application of mycorrhiza-based biofertilizers is being advocated for reducing the chemical burden in the soil with added benefits (Guar et al. 2000).
Phosphorus transport in the fungus (Pearson and Tinker 1975; Tinker 1975) and its subsequent transfer from the fungal hyphae into the host cell (Cox and Tinker 1976) has been a subject of study for a long time. VAM specific alkaline phosphatase activity has been reported in onions and tobacco (Gianinazzi and Gianinazzi-Pearson 1979; Gianinazzi-Pearson and Gianinazzi 1976) Also, the proportion of the intraradical hyphae showing the enzyme activity is sparse during the first stages of infection but increases sharply as the mycelium proliferates within the roots (Tisserant et al. 1993). Arbuscules are a site of nutrient exchange between the host plant and AM fungi (Cox et al. 1980) formed in the root cortex cells by the penetration of the finely branched hyphae of AM fungi (Bonfante-Fasolo et al. 1986). The host derives phosphorus while the fungus derives the benefits of carbon during this symbiotic association.

alp gene and gene expression
Alkaline Phosphatase gene (*alp*) is AMF specific (Gianinazzi-Pearson et al. 1978; Gianinazzi and Gianinazzi-Pearson 1979) considered to reflect fungal activity within the symbiotic system and not reported in uncolonised roots (Aono et al. 2004) as it is expressed only under symbiotic conditions. ALP enzyme is closely linked to both the mycorrhizal growth stimulation and the arbuscular phase of the infection (Gianinazzi-Pearson and Gianinazzi 1978) and plays a role in the assimilation of phosphorus in the mycorrhizal roots (Gianinazzi-Pearson and Gianinazzi 1978). The symbiotic related genes for the mycorrhizal establishment and association are being studied and the gene pool for the gene expression studies is mainly involved in the nutrient uptake (Balestrini and Lanfranco 2006).

* Corresponding author. Email: aloka@teri.res.in

Material and Methods

Culture establishment
Three root tips for each of the root lines (detailed in table 1) were established in a 90mm Petri plate in M media. After three days, freshly harvested spores of the AMF (~200) were added to the corresponding Petri plates. The plates were sealed and incubated in dark at 25 ± 1 °C for one month. The plates were checked continuously for the absence of contamination and the growth of the symbionts was monitored under a stereomicroscope (Olympus SZ 4045 research microscope, Japan). 3 replicates for each set were subcultured and maintained in jars for *Glomus intraradices* in association with the Ri T-DNA transformed root lines. The cultures were harvested after 10 weeks using citrate buffer (8.25 mM sodium citrate and 1.84 mM citric acid, pH 6.0).

Sporulation Study
The extraradical spore count measurements were carried out by enumerating the number of spores formed outside the root visualized over a stereozoom microscope (Olympus SZ4045 research microscope, Japan). For the intra-radical spore count, the roots were stained with trypan blue (Phillips and Hayman 1970) and the number of the vesicles observed was counted for 100 segments per sample. The intra-radical spore count was enumerated from this by taking into account the total root length of the sample. The sum of the intra-radical spore count and the extra-radical spore count gave the total spore count.

Nitrogen, Phosphorus and Potassium Estimation
Nitrogen was estimated using the Kjeldahl method (Gupta 2004). Phosphorus was analysed spectrophotometrically at 420nm after digesting using Concentrated HNO_3 and 70% $HClO_4$. at 120°C for 30 minutes (Gupta 2004). Systronics flame photometer was used for the potassium estimation (Singh et al. 2007).

RNA extraction and cDNA synthesis
The colonized root tissues were picked with the aid of a stereo microscope (Olympus SZ 16, Japan) and placed in liquid nitrogen in sterile eppendorfs. Using the RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), total RNA extraction was carried out. Thereafter, cDNA was synthesized using the SMART cDNA synthesis kit from Clontech.

Real time expression
The Real time reaction was run using ABI Prism (Applied Biosystems) after RNA and cDNA quantification in a NanoDrop 2000 (Thermo Scientific) spectrophotometer. The cDNA was diluted 1000 times for the study. The reactions were peformed in 20 µl mix volume containing the tested cDNA, 1.75 µl of KAPA SYBR FAST qPCR Master mix (2x), 20 pmol/µl (0.9 µl) of each primer, and sterile water. The reaction consisted of a 10 minute denaturation at 95 °C, followed by 40 amplification cycles (95 °C for 15 s, primer annealing at 54 ˚C for 30 s and extension at 72 °C for 30 s), followed by a melting curve program included at the end of each PCR run according to the thermal profile suggested by the manufacturer (Applied Biosystems (ABI), StepOne™, USA).
Two sets of primers were utilized for the study, the *alp* specific primers for which the alkaline phosphatase gene specific for AMF was selected from the NCBI database (Aono et al. 2004) accession no. AB114298.1 and the primers were designed using the software ABI StepOne™ having the forward sequence GGAATCTGCCAAACACTTAGGTTT and reverse sequence AGAACGTGGCTGGTGTAGCAT; and the 28s G1/G2 primers used as internal positive control (Da Silva et al. 2006) having the sequence
G1: CATGGAGGGTGAGAATCCCG and G2: CCATTACGTCAACATCCTTAACG

Results and Discussion

The application of mycorrhizal biofertilizers to the plants and the benefits bestowed by them has many a times been the subject of research. Based on the limited nutrient available areas, the mycorrhizal benefits have always been seen and reported. This has been attributed to the symbiotic

associations wherein the fungal partner gets benefitted with the carbon supply that it requires which is in line to the nutrients that are transferred to the plants by the fungus.

Sporulation study

In the study, we found that the *Daucus carota* varieties showed a greater intraradical sporulation as compared to *Trifolium.* However, *Daucus carota* var Berlicummer showed the maximum extraradical and hence the total sporulation followed by *Daucus carota* var pusa kesar followed by *Trifolium*

Culture in symbiosis with *Glomus*	Extraradical Sporulation	Intraradical Sporulation	Total Sporulation
Trifolium	31664	13495	45159
Daucus carota var Pusa kesar	65459	24853	90312
Daucus carota var Berlicummer	71079	37031	108110

Table 1: Sporulation pattern in three different Ri T-DNA transformed root hosts in symbiosis with *Glomus intraradices*

Nitrogen, Phosphorus and Potassium Estimation

For the nitrogen, not much difference could be observed across the two carrot varieties, however, for *Trifolium,* lower levels of nitrogen were obtained when in symbiosis. No difference for potassium levels was obtained across the three root hosts and for phosphorus, maximum content was found in *Daucus carota* var Berlicummmer, followed by Daucus carota var Pusa kesar and lowest was in *Trifolium*.

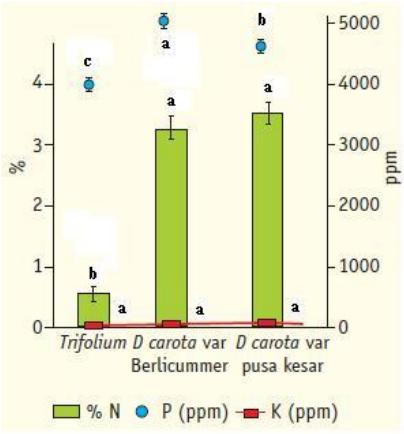

Figure 1: Nutrient estimation across three Ri T-DNA transformed root hosts in symbiosis with *Glomus intraradices*

Real Time Expression Study

The *alp* expression was found to be host dependent with the maximum expression in *Daucus carota* var Pusa kesar followed by *Trifolium,* followed by *Daucus carota* var Berlicummer.

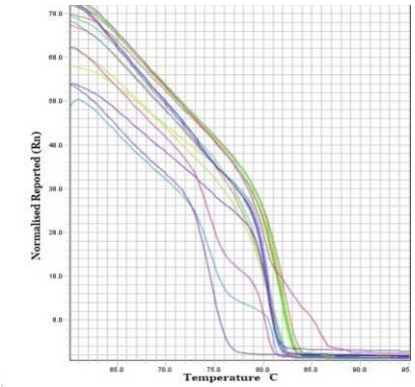

Figure 2a: Melting curve for the real time expression study

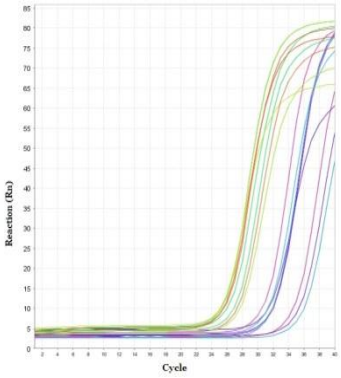

Figure 2b: Amplification plot obtained for the real time expression study

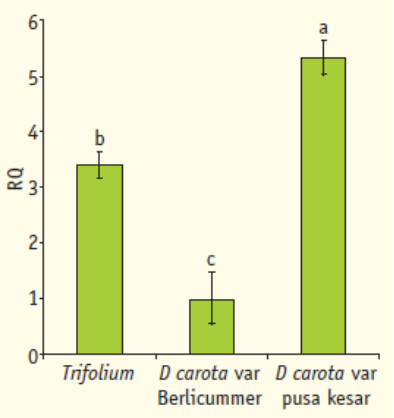

Figure 3: *alp* expression profile in three Ri T-DNA transformed hairy roots in symbiosis with *Glomus intraradices*.

Graphs were created using Microsoft ® Excel 2003. The computations were carried out at $p < 0.01$, using the Duncan Multiple Range Test. All data from real-time RT-PCR analyses were statistically compared between treatments at each time point using one-way ANOVA

The correlation studies conducted for the sporulation, phosphorus content and gene expression studies in pairs Gianinazzi-Pearson and Gianinazzi (1978) also reported a close correlation between the mycorrhiza-specific phosphatase activity and development of both the infection and the host plant. The high correlation value obtained in this study points that the results are almost linear for the gene expression result and the P present in the colonized roots at a time frame.

	Expression	P present	P present	Sporulation	Expression	Sporulation
Trifolium	3.4	4653.27	4653.27	45159.4	3.4	45159.4
D carota var Berlicummer	1	3969.85	3969.85	90312.2	1	90312.2
D carota var pusa kesar	5.3	5124.89	5124.89	108110	5.3	108110
correlation r	**0.9993**		**0.1715**		**0.2092**	

Table 2: Correlation values for the gene expression and P concentration present

The alp activity similar to mycorrhizal activity (Gianinazzi and Gianinazzi-Pearson1979) in the vacuoles has been reported in *Saccharomyces cerevisiae* (Bauer and Sigarlakie 1975) and *Candida albicans* (De Nollin et al. 1975). As the alp activity increases with the maturation of arbuscular hyphae (Gianinazzi and Gianinazzi-Pearson 1979), the total activity within the mycorrhizal root should depend on the number of active arbuscules present within the infection. Hence, a correlation of the intraradical colonization can be an indicative of the alp activity and vice versa.

Conclusions and Outlook (250 words)

In this study we obtained an increased AMF development in terms of colonization with high proportions of alkaline phosphatase active mycelia / arbuscules within the roots which was host specific. From the correlation studies of the sporulation, phosphorus concentration and gene expression, it can be inferred that the alp gene expression leads to an increase in the phosphorus content in the colonised roots. However, an increase in these does not lead to an increase in the sporulation of the fungus, which we assume is dependent on the carbon levels.

References

Aono T, Maldonado-Mendoza IE, Dewbre GR, Harrison MJ and Saito M, 2004, Expression of alkaline phosphatase genes in arbuscular mycorrhizas, New Phytologist, 162: 525-534.

Balestrini R and Lanfranco L, 2006, Fungal and Plant gene expression in arbuscular mycorrhizal symbiosis, mycorrhiza, 16:509-524.

Bauer H and Sigarlakie E, 1975, Localization of alkaline phosphatase in Saccharomyces cerevisiae by means of ultrathin frozen sections. Journal of ultrastructural Research, 50, 208

Bonfante-Fasolo P, Vian B, and Testa B, 1986, Ultrastructural localisation of chitin in the cell wall of a fungal spore. Biol. Cell. 57:265-270

Cox G, Morgan KJ, Sanders F, Nockolds C, Tinker PB, 1980, Translocation and trnafer of nutrients in vesicular – arbuscular mycorrhizas. 3. Polyphosphate granules and phosphorus translocation. New Phytol 84: 649-659.

Cox G and Tinker PB, 1976, Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. I. The arbuscule and phosphorus transfer: a quantitative ultrastructural study. New Phytologist, 77, 371.

De Nollin S, Thone F and Borgers M, 1975, Enzyme cytochemistry of Candida albicans. Journal of Histochemistry and Cytochemistry, 23: 758.

Guar A, Guar A, Adholeya A, 2000, Growth and flowering in Petunia hybrid, *Callistephus chinensis* and *Impatiens balsamina* inoculated with mixed AM inocula or chemical fertilizers in a soil of low P fertility. Sci Hortic 84: 151-162

Gianinazzi S and Gianinazzi-Pearson V, 1979, Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. III. Ultrastructural localization of acisd and alkaline phosphatase in onion roots infected by Glomus mosseae (Nicol & Gerd.), New Phytologist, 82: 127-132.

Gianinazzi-Pearson V and Gianinazzi S, 1976, Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. I. Effect of mycorrhiza formation and phosphorus nutrition on soluble phosphatase activities in onion roots, Physiologie Végétale, 14: 833

Gianinazzi-Pearson V and Gianinazzi S, 1978, Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza II. Soluble alkaline phosphatase specific to mycorrhizal infection in onion roots, Physiological Plant Pathology 12: 45-48

Gupta PK 2004. Soil, Plant, Water and Fertilizer Analysis. Agrobios, India.

Hause B and Fester T 2005, Molecular and cell biology of arbuscular mycorrhizal symbiosis, Planta 221: 184-196

Pearson V and Tinker PBH, 1975, Measurement of phosphorus fluxes in the external hyphae of endomycorrhizas. In: Endomycorrhizas, Sanders FE, Mosse B and Tinker PB (eds), p277, Academic Press, London, New York

Phillips JM and Hayman DS, 1970, Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society, 55, 158-160.

Smith FW, 2002. The phosphate uptake mechanism. Plant Soil 245:105114.

Tinker PBH, 1975, Effects of vesicular-arbuscular mycorrhizas on higher plants, In: Symbiosis; Symposium Society Experimental Biology, 29, 325.

Tisserant B, Gianinazzi-Pearson V, Gianinazzi S, Gollotte A, 1993, In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol Res, 97: 245-250

Williamson LC, Ribrioux SP, Fitter AH and Leyser HM, 2001, Phosphate availability regulates root system architecture in *Arabidopsis*. Plant Physiol. 126:875-82