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CHANGES ACQUIRED IN PEROXIDASE –A KEY MARKER IN PLANT DEFENSE; UPON ROOT TO ROOT TRANSFER OF *GLOMUS GEOSPORUM* TO *LYCOPERSICON ESCULENTUM* MILL

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ABSTRACT

Plant defense responses are strongly stimulated by AM fungi in host plants by control over defense gene expression during establishment of a successful functional symbiosis. Peroxidase requiring compounds are laid down on cell wall as barrier against microbial incursion thus can be used as an indicator in plant defense responses. In the present study, activity and expression of peroxidase was monitored in *Glomus geosporum* (transferred from roots of *Cyperus rotundus*) colonized and uncolonized two-weeks-old *Lycopersicon esculentum* Mill seedlings for two pathogens *Pseudomonas aeruginosa* and *Fusarium oxysporum* f. sp. *lycopersici* separately. Mycorrhizal colonized plants without pathogen and that infected with *Fusarium oxysporum* f. sp. *lycopersici* showed increased protein and POD activity in comparison to uncolonized plants after 120 hrs. Mycorrhizal colonized plants infected with *Pseudomonas aeruginosa* showed less protein and POD activity than uncolonized plants; while POD activity in these plants increased from 72hrs to 120hrs. Response observed was more promising against soil borne pathogen than air borne pathogen after both 72hrs and 120hrs. Statistical analysis (Unpaired t-test) showed that an increase in total protein content in mycorrhizal than uncolonized plants and decrease in POD activity in mycorrhizal colonized plants infected with *Pseudomonas aeruginosa* than uncolonized plants is insignificant. Except these two sets difference in total protein content and POD activity between colonized and uncolonized plants was statistically significant at both 72hrs and 120hrs.This study concludes that Arbuscular Mycorrhizal Fungi can be transferred from one root to another and it modulates plants defense mechanism by increasing the basal level responses (priming) in the host.

Keywords: *Glomus geopsporum*; Peroxidase; *Pseudomonas aeruginosa; Fusarium oxysporum* f. sp. l*ycopersici*; *Lycopersicon esculentum* Mill.

INTRODUCTION

More than 80% of terrestrial plants form mutualistic, symbiotic associations with Arbuscular Mycorrhizal (AM) fungi (Akiyama *et al*., 2005). Mortan and Benny (1990) classified all the members of AM fungi under class Zygomycota which includes a new order, the Glomales, encompassing six genera comprising of approximately 150 species. Arbuscules – structure unique to AM fungi grow into the root cortex; which grows from intercellular hyphae originated in cortex cells. Arbuscules play role in nutrient cross talk between plants and fungi, transporting mineral nutrients and water from the fungus to the plant and carbohydrates from the plant to fungus

(Strack *et al*., 2003). Apart from the nutritional benefits, root colonization by AM fungi can improve plant resistance/tolerance to biotic as well as abiotic stresses. Plant defense responses are strongly stimulated by AM fungi in host plants indicative of control over defense gene expression during establishment of a successful symbiosis (Garcia-Garrido and Ocampo, 2002). Mycorrhizal plants in comparison to non-mycorrhizal plants suffer less damage as occurrence of disease is decreased or development of pathogen is inhibited. As, AM fungi are established in the roots of host plants, research on mycorrhizal disease interactions has been focused on soil borne pathogens only (Dehne, 1982). Systemic acquired resistance (SAR) is a defense response shown by plants remote to the location from the site of infection or symbiosis (Campos-Soriano *et al*., 2011). So, association of AM fungi with plants may also alter disease resistance in aerial parts like shoots and leaf (Dehne, 1982). In tomato; studies have been done on AM defense response on various soil borne fungi and nematodes (Dehne and Schoenback, 1979; Sikora and Schoenbeck, 1975; Bagyaraj *et al*., 1979; Daft and Okusanya, 1973; Schoenbeck and Spengler, 1978.).

Pathogen related proteins or defense-related enzymes are keys to study any plant disease resistance mechanism. Changes in expression of isoenzymatic patterns and biochemical properties of some defense-related enzymes such as chitinases, chitosanases and β-1, 3-glucanases have previously been shown during mycorrhizal colonization of tomato roots, with the induction of new isoforms (Pozo *et al*., 2001). Studies have suggested that peroxidases (POD) play a vital role in lignifications (Whetten *et al.*, 1998), suberization (Espelie, 1986), cross-linking of cell wall structural proteins (Fry, 1986), Auxin metabolism (Lagrimini *et al*., 1997), self-defense against pathogens (Chittoor *et al.*, 1997) and senescence (Abeles *et al*., 1988). Blee and Anderson (2000) have remarked from their studies that POD along with catalase play a role in the catabolism of hydrogen peroxide and/or in cross-linking reactions between proteins and polysaccharides in the interface between arbuscules and the plant cell plasma membrane. Since POD can sustain its activity at high temperature and also its activity can be measured conveniently using simple chromogenic reactions, it has been used as a model enzyme in the study of protein structure, enzyme reactions and enzyme functions.

In present study, response of plant defense mechanism of AM fungi when transferred from nut grass root to tomato roots using POD as key enzyme have been studied against Pseudomonas aeruginosa (air borne pathogen) and Fusarium oxysporum f. sp. lycopersici (soil borne pathogen).

MATERIALS AND METHODS

Plant material and chemicals:

Seeds of *Lycopersicon esculentum* Mill were procured from Seedco Company, Jalna, India and were stored at 4° C until used. All the chemicals used were of analytical grade obtained from HiMedia Laboratories, Mumbai, India.

Mycorrhizal and Pathogen material

Glomus geosporum (GenBank accession number: KJ830770) associated with roots of *Cyperus rotundus* (checked by Trypan Blue staining; data not shown) was collected from soil of BRD School of life sciences, Vallabh Vidyanagar, Gujarat, India. *Glomus geosporum* in these roots was established in tomato roots by root to root transfer method (both the roots were kept in vicinity to allow transfer of AM fungus). Tomato roots were stained by Trypan Blue staining method and observed under 40X magnification of compound microscope to confirm the presence of *Glomus geosporum* after 42 hrs (Phillips and Hayman, 1970). *Pseudomonas aeruginosa* (provided by National Chemical Laboratory, National Collection of Industrial Microorganisms, Pune, India) was grown on Nutrient Agar medium at 37° C for 48 hrs. *Fusarium oxysporum* f. sp. *lycopersici* (provided by Microbial Type Culture Collection, Chandigarh, India) was grown on Potato Dextrose Agar medium at 30° C for 10 days.

Growth conditions and Plant harvest

Tomato seeds were grown in autoclaved sand containing 0.5X MS salts (Murashige and Skoog, 1962). Sowing of seeds (10 seeds per cup) was done in medium sized plastic cup at 0.5 inch distance in autoclaved sand. These cups were incubated in dark until the seeds sprouted. Seeds were allowed to grow for 12 days in cups under natural conditions. *Cyperus rotundus* roots colonized with *Glomus geosporum* were kept in vicinity of 12- day- old tomato seedlings for transfer of fungus. *Pseudomonas aeruginosa* (10^6 CFU/ml) and *Fusarium oxysporum* f. sp. *lycopersici* (2.8 x 10^6 conidia/ml) was inoculated in 14 day old tomato plantlets (Shankar *et al*., 2011; Kapoor, 2008). Six different sets: Control plant (C), Plant colonized only with *Glomus geosporum* (C + M), Plant inoculated with *Pseudomonas aeruginosa* (C + Pa), Plant inoculated with *Fusarium oxysporum* f. sp. *lycopersici* (C + Fo), *Glomus geosporum* colonized plants inoculated with *Pseudomonas aeruginosa* (C + M + Pa), *Glomus geosporum* colonized plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici* (C + M + Fo) were used. Analysis was carried out after 72hrs and 120hrs of pathogen inoculation.

Preparation of homogenate:
The extract for POD was prepared using 0.2gm/ml of plantlet. Homogenate was prepared according method given by Summer and Gjessing (1943). Supernatant thus obtained was used as an enzyme source.

Protein determination:
Total protein was estimated quantitatively by absorbance measurements at 550 nm following Lowry's method (1951) using Bovine serum albumin as reference protein.

POD assay and Isoenzyme staining by Agarose Horizontal electrophoresis:
Kinetic studies were performed from crude homogenate to determine optimum pH, optimum temperature, optimum molarity, pH stability, thermal stability and substrate specificity (data not shown). The POD activity in *Lycopersicon esculentum* Mill seedlings was measured using o-dianisidine as substrate (Summer and Gjessing, 1943). The homogenate was prepared as above mentioned method. Optical absorption was recorded on UV-VIS 1800 spectrophotometer (Shimadzu) at 430nm. One unit of enzyme activity is defined as µmoles of enzyme used per µl of substrate per min. Agarose (2%) gel electrophoresis was carried out (Birecka and Garraway, 1975; Hale and Orcutt, 1986) for isoenzyme staining of POD. Gel was stained by modified method of Chanwun *et al*., (2013). Agarose gel was incubated in 0.1 M Acetate buffer (pH 4.6) containing 10% o-dianisidine for 30 min and then transferred to 0.1 M Acetate buffer (pH 4.6) containing 0.03% hydrogen peroxide until brownish red coloured bands appear.

Molecular weight determination:
Molecular weight of POD isoenzymes was determined by AlphaEaseFC software. The standard proteins used were Ovalbumin (43 KDa), Trypsin Soyabean Inhibitor (20.1 KDa) and Lactoglobulin (18.4 KDa). Native electrophoresis was carried out for standard proteins in 10% acrylamide gel and stained with Coomassie Brilliant Blue R-250.

Statistical analysis:
All the experiments were performed in triplicates. Unpaired t-test (Confidence Interval: 95%) was performed using SPSS 22.0.0 to examine statistical difference between protein production and POD activity of Glomus geosporum colonized and uncolonized plants.

RESULTS

Isolation of AM fungus and its establishment via root to root transfer method:
Glomus geosporum was identified from roots of *Cyperus rotundus* and established in tomato roots via root to root transfer method within 42 hrs. Trypan blue staining method showed the presence of arbuscules, vesicles and spores under 40X microscopic observation (Fig. 1).

Total protein and POD assay:
Optimum pH, optimum temperature, optimum ionic strength, pH stability, temperature stability conditions determined for o-dianisidine/H_2O_2 substrate pattern were found to be 6.0, 50° C,

0.1M, 9.0 and 25° C to 50° C respectively(data not shown). After 72 hrs; there was 15.23% more protein in set C+M then set C; 175.9% more protein in set C + M + Fo than in C + Fo; while after 120 hrs there was 224.0% more protein in set C+M then set C and 560% more protein in set C + M + Fo than in C + Fo. While in set C + M + Pa protein was 85.78% less than set C + Pa after 72hrs and 78.53% less after 120 hrs (Table1, Fig. 2). In case of POD; roots colonized with *Glomus geosporum* (C + M) showed 60.34% more POD activity than set C after 72 hrs and after 120hrs it had 101.3% more POD activity. In set C + M + Fo also POD activity was 52.57% more than set C + Fo after 72hrs; while after 120hrs the difference in POD activity increased to 233.3% between both the sets. While in set C + M + Pa there was 33.37% less POD activity than set C + Pa after 72hrs and after 120 hrs C + M + Pa had 11.30% less POD activity than set C + Pa (Table1, Fig. 3).

Comparing *Glomus geosporum* colonized plants inoculated with *Pseudomonas aeruginosa* and that with *Fusarium oxysporum* f. sp. *lycopersici*; later had 32.84 times more protein production than former after 72hrs and that after 120hrs it was 2.98 times more; similarly POD activity was 48.15 times more after 72hrs and 56.43 times more after 120hrs.

Statistical analysis:

Unpaired t-test (Table 3) shows that total protein mean values between sets C and C + M after 72hrs was statistically insignificant (P value= 0.0645) and POD activity mean values between sets C + Pa and C + M + Pa after 120 hrs is statistically insignificant (P value= 0.1145).

Molecular Weight determination:

Molecular weight was determined using AlphaEase FC software. In total 6 bands were observed on 3rd day out of which 4 were acidic

Table 1. Total protein and POD Increase [↑]/Decrease [↓] in Mycorrhizal colonized plants than -colonized plants

Set name	Protein Percentage Increase/Decrease (%) after 72hrs	Protein Percentage Increase/Decrease (%) after 120 hrs	POD Percentage Increase/Decrease (%) after 72hrs	POD Percentage Increase/Decrease (%) after 120hrs
C + M	15.23[↑]	224.0[↓]	60.34[↑]	101.3[↑]
C + M + Fo	175.9[↑]	560.0[↑]	52.57[↑]	233.3[↑]
C + M + Pa	85.78[↓]	78.53[↓]	33.37[↓]	11.30[↓]

Table 2. Unpaired t-test analysis result after 72 hrs of mycorrhizal association

Set name	Total protein		Set name	POD Activity	
	Mean	P-value		Mean	P-value
C	0.3327±0.018[a]	0.0654	C	1011.7±36.7[a]	0.0004
C + M	0.3834±0.009[a]		C + M	1254.65±7.06[b]	
C + Fo	0.067±0.0072[b]	0.0001	C + Fo	2087.33±34.74[c]	0.0001
C + M + Fo	1.675±0.0274[c]		C + M + Fo	3184.75±52.29[d]	
C + Pa	0.3588±0.0237[d]	0.0001	C + Pa	99.26±2.106[e]	0.0008
C + M + Pa	0.051±0.0006[e]		C + M + Pa	66.13±5.83[f]	

(Means with same alphabet are statistically not significant)

C= Control, C + M= *Glomus geosporum* colonized plants, C + Fo= Plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici*, C + M + Fo= *Glomus geosporum* colonized plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici*, C + Pa = Plants inoculated with *Pseudomonas aeruginosa*, C + M + Pa= *Glomus geosporum* colonized plants inoculated with *Pseudomonas aeruginosa.*

(anionic) (64.22 KDa, 26.64 KDa , 15.63 KDa and 43.47 KDa) and 1 was basic (cationic) band (31.69 KDa); while on 5th day only 4 acidic bands were seen no basic bands were observed. 4th number of acidic band (43.47 KDa) was seen only in C+M+ Fo and 5th (31.69 Kda) number of basic band (31.69 KDa) was seen in C + Pa and C+ M+ Fo both. While on 5th day all four acidic bands were seen in all six sets (Table 4, Fig. 4, 5).

Fig-1. Trypan blue stained tomato root

(*A – Vesicle, B – Arbuscules, C- Appresoria, D- Spore)*

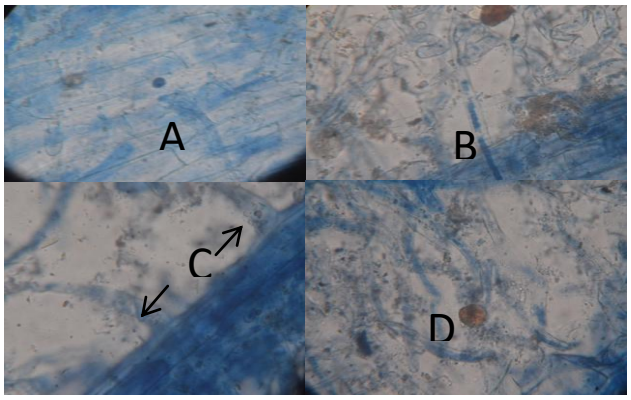
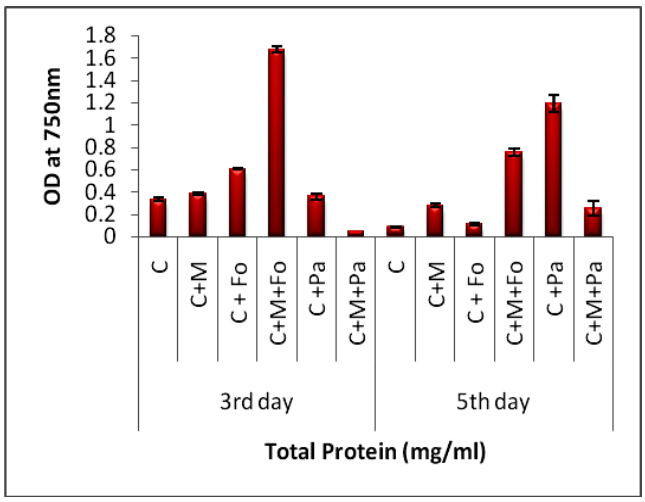


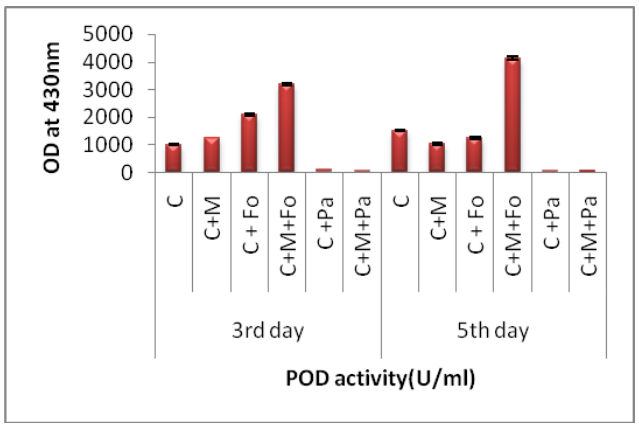
Fig-2. Total protein analysis for all the sets after 72hrs and 120hrs



DISCUSSION

Trypan Blue stained roots showed presence of Arbuscules, Appresorium, Vesicles and Spores within 42 hours of transfer; which suggests that AM fungus can be established from one root to another root by itself; known as Common Mycorrhizal Networks (CMNs). Barto *et al*., (2012) postulated that Network Enhanced Bioactive Zone (NEBaZ) model in CMNs increase the bioactive zones of chemicals by serving as connecting links between plants below ground. It was further stated that chemical transport via CMNs allows systemic defense across plant populations and directed allelochemical delivery to targeted plants.

Fig-3. POD molecular weight determination for all the sets after 72hrs



[C= Control, C + M= Glomus geosporum colonized plants, C + Fo= Plants inoculated with Fusarium oxysporum f. sp. lycopersici, C + M + Fo= Glomus geosporum colonized plants inoculated with Fusarium oxysporum f. sp. lycopersici, C + Pa = Plants inoculated with Pseudomonas aeruginosa, C + M + Pa= Glomus geosporum colonized plants inoculated with Pseudomonas aeruginosa].

This corroborates with the present study where POD response expressed by Mycorrhiza inoculated plants is an indication of defense response against both soil borne and air borne pathogens. In our study, total protein after 72hrs (0.3384mg/ml) was more than colonized plants inoculated with either of the pathogen. Similar results have been observed in mycorrhizal tobacco roots; which was observed to coincide with appresorium formation and fungal penetration into the root (Blilou *et al*., 2000). Similarly in our study also an increase in POD activity of mycorrhizal colonized plants soil

Figure-4 & 5. POD molecular weight determination for all the sets after 72 & 120hrs

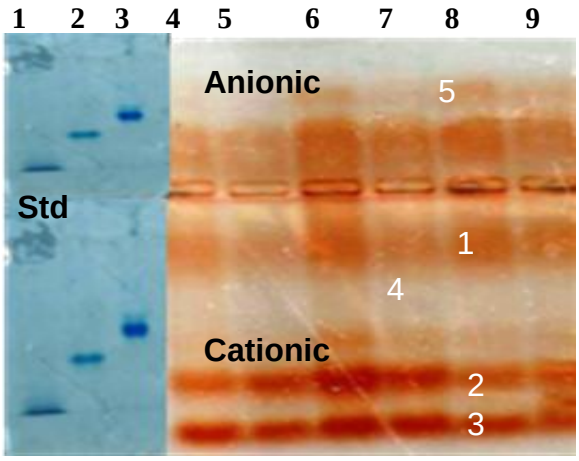
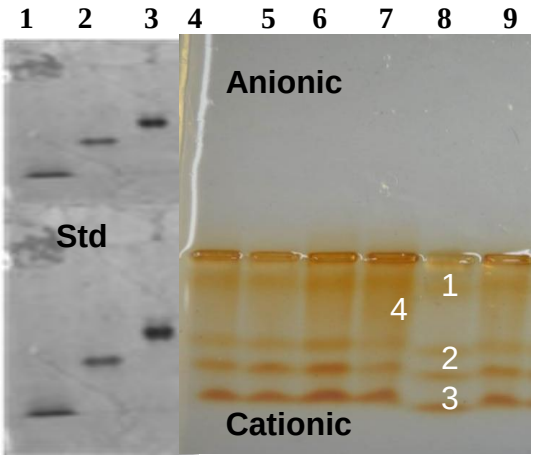


Fig-4. After 72 hrs



Fog-5. After 120 hrs

(Lane 1: Lactoglobulin-18.4 Kda, Lane 2-Ovalbumin-43 Kda, Lane 3- Trypsin Soyabean Inhibitor-20.1 Kda, Lane 4- C, Lane 5 - C+ M+ Pa , Lane 6 -C+M+ Fo, Lane 7 C+M, Lane 8 - C + Pa, Lane 9 - C+ Fo)

Table 3. Unpaired t-test analysis result after 120 hrs of mycorrhizal association

Set name	Total protein		Set name	POD Activity	
	Mean	P-value		Mean	P-value
C	0.0861±0.0055[a]	0.0003	C	1512.2±7.399[a]	0.0001
C + M	0.279±0.015[b]		C + M	1045.33±36.02[b]	
C + Fo	0.115±0.01[c]	0.0001	C + Fo	1237.33±38.31[c]	0.0001
C + M + Fo	0.759±0.033[d]		C + M + Fo	4124.41±58.77[d]	
C + Pa	1.189±0.078[e]	0.0001	C + Pa	64.76±3.313[e]	0.1145
C + M + Pa	0.254±0.065[f]		C + M + Pa	73.09±6.36[e]	

(Means with same alphabet are statistically not significant)

C= Control, C + M= Glomus geosporum colonized plants, C + Fo= Plants inoculated with Fusarium oxysporum f. sp. lycopersici, C + M + Fo= Glomus geosporum colonized plants inoculated with Fusarium oxysporum f. sp. lycopersici, C + Pa = Plants inoculated with Pseudomonas aeruginosa, C + M + Pa= Glomus geosporum colonized plants inoculated with Pseudomonas aeruginosa.

could be attributed to appresorium formation in tomato roots (Fig.1).

Events like activation of plasma membrane-bound enzymes, the activation of kinases, phosphatases, phospholipases, and the production of signal molecules, including active oxygen species has been found in the plant interaction with AMF which results in the transcriptional activation of defence-related genes. This result correlates with our results in which POD activity increases in mycorrhiza colonized plants inoculated with soil borne pathogen. An oxidative burst could be detected at sites of hyphal tips of *Glomus intraradices* (García-Garrido and Ocampo, 2002). Blilou and his co-workers (2000) recount increase in catalase and ascorbate peroxidase with their antioxidant role against any active oxygen molecules generated during initial stage of

fungus penetration. Song *et al.,* (2010) also showed that there was increase in POD activity in *Alternaria solani* infected tomato plants upon CMNs formed by donor plant containing AM fungus. In this study also POD activity increases in colonized plants inoculated with *Fusarium oxysporum* f. sp. *Lycopersici* after 120 hrs.

Jung and his co-workers (2012) showed that functional symbiosis between plant and fungus during mycorrhiza establishment leads to modulation of plant defense responses. As a result of this infection, a mild, but operative activation of the plant immune response seemsto occur, locally as well as systemically. This activation leads to a primed state of the plant that allows a more efficient activation of defense mechanisms in response to attack by potential enemies. This hypothesis also provides basis for elevated POD activity showed against *Pseudomonas aeruginosa* in present study, though it is an air borne pathogen. POD activity in colonized plants inoculated with *Pseudomonas aeruginosa* after 72 hrs was less than every other set but this activity increased after 120 hrs in colonized plants while decreased in uncolonized plants. This result may be attributed to air borne nature of pathogen; as Mycorrhiza associates with roots and pathogen is air borne it may show delayed response than soil borne pathogen. But increase in POD activity shows that it though the response is delayed it shows defense response against air borne pathogen too.

If the study had been carried out further for more number of hours POD activity might have increased in this case too. Rodríguez *et al.,* (2001) showed presence of 4 bands only on polyacrylamide gel when tomato roots were inoculated with *Glomus clarum or Glomus fasciculatum*. However, in present study, agarose horizontal electrophoresis was carried out for the same association to observe changes in both cationic and anionic bands. In total, four cationic bands and one anionic band was observed. Basic (cationic) bands were only observed on 3rd day of analysis in plant associated with only AM fungus and that with *Pseudomonas aeruginosa*; while intensity of 4th band was highest in plant associated with mycorrhiza and *Fusarium oxysporum* f. sp. *lycopersici*. Similarly; Santos *et al*., (2001) also showed presence of one common band in all mycorrhiza (*Gigaspora albida, Scutellospora heterogama, Glomus clarum*) colonized plant which was absent in uncolonized plant. Rodríguez *et al.,* (2001) also showed difference in isoenzymes expression upon mycorrhizal inoculation.

CONCLUSION

Present study envisages that AM fungi can be established from one root to another via network formation. Presence of AM fungi in host plant enhances defense mechanism in plants by priming defense related genes against both soil borne and air borne pathogens; and POD is one of the earliest expressing enzymes.

Table 4. Molecular weight data obtained from software AlphaEaseFC

MARKERS ---------			
Band	**Position**	**Mol. Wt.(KDa)**	**Rf value(cm)**
Trypsin Soyabean Inhibitor	323	18.40	0.880
Lactoglobulin	244	20.10	0.665
Ovalbumin	199	43.00	0.542
POD bands			
Band	Position	Mol. Wt.(KDa)	Rf value(cm)
1	104	64.22	0.283
2	246	26.64	0.670
3	330	15.83	0.899
4	167	43.47	0.455
5	268	31.69	0.594

[Band numbers are written according to that marked in Fig. 4]

Also, differential band pattern of POD is observed during pathogen attack in AM fungus associated plants. The expression of one additional basic POD band in AM fungus associated plants can be correlated with the capacity of cells to produce alkaloids (Limam *et al.*, 1998), one of the distinguished features of defense in plants. So, POD can be used as one of the early marker to spot the presence of mycorrhiza in plants and change in its expression pattern can be an indicative of Mycorrhiza induced resistance against pathogen attack.

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