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IMPACT OF PHYLLOPLANE MICROBE ON THE CELL WALL AND CYTOPLASMIC PROTEIN OF TOMATO LEAVES

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ABSTRACT

An array of microbes colonise surface of leaves where they interact with cells and influence the physiology and biochemistry of the host plant. Inthepresent study, *Aspergillus niger* a dominant phylloplane microfungus, isolated from leaves of field grown tomato plants was inoculated on aseptically raised plants of tomato. The inoculated and non-inoculated leaves were sampled at 0, 24, 48, 72 and 96 hours intervals, and subjected to treatment withformaldehyde (37%) for crosslinking of proteins. Cell wall and cytoplasmic proteins were extracted from crosslinked and non- crosslinked samples of both control and *A. niger* inoculated plants. The proteins were iso-cratically separated on SDS-PAGE. Cell wall proteins were observed to be suppressed or expressed in samples from 24 to 96 hours post inoculation with *A. niger*. After 24, 48 and 96 hours of inoculation, enhanced expression of proteins were observed. However, after72 hours, the protein detected in control sample was not observed in treated sample. Similarly, cytoplasmic proteins were also overexpressed in non-crosslinked treated samples at 72 hours and 96 hours post inoculation. Electrophoretic separation demonstrated crosslinking of proteins in *A.niger* treated samples.

Thus, the results demonstrated that phylloplane microbes are not a static entity on leaf surface rather actively interact with cells and influence their macro-molecular composition. Such interactions could be crucial for the physiology of host tissue.

***Keywords*:** Cross linking; cytoplasmic proteins; cell wall proteins; microfungi; phylloplane

Introduction

Phylloplane, the aerial surface of plant provides a complex terrestrial habitat to a variety of microorganisms including bacteria, filamentous fungi and yeast (Prabakaran *et al*., 2011). The phylloplane microbes interact with host plants through various mechanisms and thus affect its growth and development, crop productivity, abiotic and biotic stress resistance, nutrient uptake and most importantly the host physiology (Windham *et al*., 1986). Phylloplanemicrofungal metabolites have been found to enhance chlorophyll concentration (Mitra *et al*., 2014). Enhancement of carotenoid concentration in rice leaves was observed when sprayed with *T. harzanium* against *B. oryzae* (Abdel-Fattah *et al*., 2007). *Vicia faba* plants treated with *Saccharomyces cerevisiae* had higher carotenoid contents (Elwakil *et al*. 2009). *Trichoderma* spp and *Sebacinales* spp besides being efficient biocontrol agents, also improve the photosynthetic efficiency of plants (Shoresh *et al*., 2010). An increase in RuBisCo activity was also observed in phylloplane microfungal metabolite treated tomato plants (Mitra *et al*., 2014). The protein profiles of *Alternaria alternata* and *Penicillium oxalicum* treated tomato plants had 5 additional proteins (Bashir *et al*., 2016). The influence of pathogenic and non-pathogenic fungal species on plant cell and their responses have been studied for plant protection against biotic and abiotic stress (Muniz and Nombela, 2009). Studies have also revealed that fungal application changes pathogenesis related (PR) protein profiles of plant cells (Khan *et al*., 2012).

In the present study, *Aspergillus niger* was chosen as it was the most dominant phylloplane microfungus on tomato leaves.

Tomato was selected for the study as its cultivation globally occupies maximum acreage as well as it is an important model system for research in plant science. Tomato phylloplane also harbours a large number of microbes which influence its growth and physiology.

Plant cell wall is a dynamic structure that determines the outcome of plant microbe interaction (Bellincampi *et al*., 2014). Pathogens may germinate normally on host surface but are unable to colonise beyond cell wall once they interact with the cell wall impregnated proteins (Sasidharan *et al*., 2011). Plant cell wall proteins (CWPs) are the “lunch bucket workers” which are involved in modification of cell wall components thereby conferring appropriate properties to cell wall (Fry, 2004). CWPs interact with plasma membrane receptors or release signal molecules thereby contributing to signaling (Seifert and Roberts, 2007; Murphy, 2012). CWPs are of various types and their relative abundance is also variable (Albenne *et al*., 2013). The plant cell wall contains integral or peripheral proteins which regulate plant biotic interactions by recognizing microbes, subsequently triggering signalling cascades and thereby controlling physiological

outputs inside the cell (Leborgne-Castel and Bouhidel 2014). Cytoplasmic proteins also play an important role in plant microbe interaction. The product of resistance (R) genes are nucleotide binding leucine rich repeat (NLR) proteins which trigger hypersensitive response (HR) upon pathogen recognition. Such proteins are located in the cytoplasm (Wang and Balint-Kurti, 2015). HR effectively restricts the growth and propagation of a large variety of pathogens (Bent and Mackey, 2007). Slootweg *et al*. (2010) demonstrated that *Potato virus* X NLR resistance protein R_X is activated in the cytoplasm to induce resistance.

The present study aimed to understand the impact of *A. niger* on the protein composition of cell wall and cytoplasm are influenced by interaction between the plant cell and phylloplane microbes. Such influences if any would help in understanding the relationship between leaves and the colonizing microbes.

Materials and Methods

Materials

Host Plant: Seeds of tomato (var: Pusa Ruby) were surface sterilised with 0.1% Sodium Hypochlorite solution, aseptically dried and sown in plastic trays (35cmx25cmx6cm; LxWxH) containing sterile soil rite. Plants were grown at 25±1°C and at 70±2% relative humidity with 12- hour (L/D) photoperiod under aseptic conditions. Trays were watered daily with sterilized distilled water and once a week with sterile Hoagland's solution.

Phylloplane micro fungus: *Aspergillus niger* was used for the study.

Methods

Isolation and identification of phylloplane microfungus: *Aspergillus niger* was isolated from abaxial and adaxial leaf surfaces of field grown tomato plants by leaf impression method. The identity of the fungus was confirmed by ITS sequencing (NCBI accession number MK590413).

Preparation of fungal inoculum: Fungal inoculum was prepared by diluting spores in 0.85% saline containing 0.02 µl Tween 20. The spore suspension was adjusted to 10^6 spores/ml through a haemocytometer (Wolk *et al*., 2000).

Treatment and sampling of plants: 8 weeks old seedlings were considered for the experiment to maintain uniformity of age in leaves and divided into two groups of 25 plants each:

Group 1- sprayed with autoclaved distilled water (control) Group 2-inoculated with *Aspergillus niger* spore suspension

Two sets of leaves were sampled at 0, 24, 48, 72 and 96 hours post inoculation from both control and inoculated plants. The first set (non-crosslinked) was stored at -20ºC. The second set (cross linked) was subjected to cross linking and subsequently stored at -20ºC. Three replicates were taken for each experiment.

***In vivo* cross linking of plant tissue using formaldehyde:** *In vivo* cross linking was done according to method followed by Bhuvaneshwari *et al*. (2015) with slight modifications. 1 gram of leaves were taken infalcontubes and washed with autoclaved distilled water twice and then added 10 ml of 1XPBS (Phosphate Buffered Saline, pH-7.4) containing 137 mM NaCl, 2.68 mMKCl, 7.81 mM Na_2HPO_4 and 1.47 mM KH_2PO_4 and vortexed for 10 min. 270 µl of 37% formaldehyde was subsequently added. The tubes were capped with aluminium foil and poked with needle to createholes. The leaves were vacuum infiltrated in a desiccator for 10min. 0.65 ml of 2M glycine was added to stop cross linking and vacuum infiltrated for 5 min. Leaves were taken out carefully and rinsed with ice cold 1X PBS thrice, dried on blotting sheet and stored at -20ºC for further use. Samples were labelled for cytoplasmic protein profiling as follows: Sample 1: Non-crosslinked tomato leaves (control) Sample 2: Non-crosslinked tomato leaves (treated) Sample 3: Crosslinked tomato leaves (control) Sample 4: Crosslinked tomato leaves (treated)

Extraction of cytoplasmic protein from cross linked leaves: One-gram of leaves were crushed in 2ml of cytoplasmicextractionbuffercontaining10mMHEPES(pH-7.1), 40mM KCl, 2 mM $MgCl_2$, 1 mM NaPPi, 1 mM $NaVO_4$, 1mM NaF, 10% glycerine and 1% (w/v) PVP (freshly prepared), 1 mM PMSF and 10 mM DTT at 4ºC. The extract was centrifuged at 10,000×g for 20 min at 4ºC. The supernatant was collected and ultra centrifuged at 1,00,000×g for 1 hour at 4ºC. The supernatant was then concentrated by adding acetone in the ratio 4:6 and incubated overnight at 4ºC and centrifuged at 15,000×g for 40 min at 4ºC. The pellet containing cytoplasmic protein was dissolved in 10mM Tris HCl (pH-6.8) and analysed using SDS-PAGE (Bhuvaneshwari, 2012)

Extraction of cytoplasmic protein from non-cross linked leaves: The homogenization process was carried out as mentioned above. The extract was centrifuged at 500×g for 5 min at 4ºC. The supernatant was collected in new eppendorfs and centrifuged at 15000xg for 20 min at 4ºC. The supernatant thus obtained was collected and stored at -20ºC and used as crude cytoplasmic extract (Bhuvaneshwari, 2012).

Extraction of cell wall: Cell wall was extracted as per the method described by Bhuvaneshwari *et al*. (2015). 1gram of frozen leaf tissue was homogenized in 25 ml of ice-cold acetate buffer (5mM, pH 4.6) containing 0.4 M sucrose, 0.34 % Polyvinyl pyrrolidone (PVP), 5mM Dithiotreitol (DTT) and 1mM Phenyl methyl sulfonyl fluoride (PMSF). The homogenate thus obtained was incubated at 4ºC for 30 minutes with frequent stirring followed by centrifugation at 1000xg for 15 minutes at 4ºC. The supernatant was discarded and the pellet re suspended in 10 ml of 5mM acetate buffer (pH 4.6) containing 0.6 M sucrose and centrifuged at 1000xg for 15 minutes at 4ºC. The supernatant was again discarded and the pelletre suspended in 10 ml of 5mM acetate buffer (pH 4.6) containing 1M sucrose and centrifuged at 1000 x g at 4ºC for 15 minutes at 4º C. Subsequently, the pellet obtained was washed with 10 ml of 5 mM acetate buffer (pH 4.6) and centrifuged at 2000 xg at 4ºC for 20 minutes. This step was repeated thrice. The cell wall pellet thus obtained was stored at 4ºC for further processing.

Extraction of cell wall proteins: The cell wall pellet obtained was resuspended in 2.5 ml of Tris maleate buffer (10 mM, pH 7.3) containing 2 M sodium chloride (NaCl), 1mM Ethylene diaminetetra acetic acid (EDTA) and 2mM Phenyl methyl sulfonyl fluoride (PMSF). The contents were then vortexed for 20 minutes and incubated at 4ºC for 1 hour. The cell wall proteins were obtained twice after centrifugation at 15,000xg at 4ºC for 20 minutes. The

resultant supernatant containing cell wall proteins was precipitated by incubating overnight with 60% ice cold acetone (v/v) at 4ºC. The cell wall protein precipitate was thus obtained by centrifugation at 20,000xg at 4ºC for 1 hour. The final pellet was dissolved in Tris Cl (pH 6.8) and stored at -20ºC for further analysis (Bhuvaneshwari *et al*., 2015).

The protein concentration was quantified using Bradford's assay (Bradford 1976) and proteins were isocratically separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Each sample had equal concentration of protein. The separated proteins were then subjected to silver staining (Gharahdaghi *et al*., 1999).

Results

Cell wall protein profile of tomato leaves treated with *Aspergillus niger*:

The study revealed that phylloplane microfungus, *A. niger* induces the changes in composition of cell wall associated proteins. At 0-hour interval of sampling, post treatment, no change in the protein profile was observed in both control and treated samples (Fig: 1). At 24 hours interval, an additional protein of ~30 kDa was observed in the treated sample only (Fig. 1). After 48 hours of inoculation a ~25 kDa protein was detected in control but not in the treated samples whereas enhanced expression of ~75kDa was observed only intreated samples (Fig. 2). Proteins of ~20kDa and ~25kDa were observed in control samples only at 72 hours post inoculation (Fig. 2). After96 hours there was enhanced expression of ~60 kDa and ~48 kDa in treated samples as compared to controls (Fig. 2). The results thus demonstrate that *A.niger* does influence the expression and association of proteins with cell wall.

Cytoplasmic protein profile of tomato leaves inoculated with *Aspergillus niger*:

In the present study, cytoplasmic protein-protein interaction was studied using chemical cross linker. Non-cross linked and cross linked proteins of both control and treated samples were compared at different time intervals i.e. 0, 24, 48, 72- and 96-hours postin oculation. At 0-hour and 24 hours four proteins were observed innon-crosslinked fungus treated and control samples (Fig. 3 and Fig. 4 respectively). *A. niger* inoculation led to an enhanced expression of 5 proteins after 48 hoursinnon-cross linked samples (Fig. 5). After 72 hours of inoculation, a total of 12 proteins were observed in non-cross linked control and treated samples with an over expressed protein band of ~32k Dain treated samples (Fig. 6). However, proteins of ~85kD a and ~50kD a were over expressed in non-cross linked control as compared to treated samples (Fig. 6). A poorly expressed protein (95kDa) was observed in non-cross linked control samples but was not detected in microbe inoculated non-cross linked samples (Fig. 7). A ~50 kDa protein was over expressed in *A. niger* treated non-cross linked samples at 96 hours. Other proteins of ~42kDa, ~37kDa and ~30 kDa were also seen to be prominently expressed in non-cross linked treated plants as compared to its control.

The 0-hour cross linked samples from treated and control plants had 4 protein bands of ~66 kDa, ~60 kDa, ~52 kDa and ~50 kDa each (Fig. 3) whereas after 24 hours, the treated samples had 3 additional proteins of ~75kDa, ~45kDa and ~40kDa (Fig. 4). After 48 and 72 hours of inoculation both control and treated cross linked samples had a similar protein profile (Fig:5 and Fig: 6 respectively). A prominent band of ~53kD a was observed in cross linked treated sample which was very insignificant in cross linked control samples (Fig. 5). At 96 hours post inoculation, a protein of ~95 kDa which was recorded in cross linked control but not from cross linked treated sample. However, proteins of ~85kDa, ~70kDa, ~60kDa, ~55kDa had enhanced expression in cross linked treated samples as compared to its control (Fig. 7).

Discussion

A cell or tissue is basically dependent upon its protein profile for its physiological function (Ponten *et al*., 2009). Previous studies have demonstrated that application of both pathogenic and non- pathogenic fungal species have induced expression of proteins, Q1M1W6 and Q9M1W6 in tomato where Q1M1W6 plays a significant role in guaiacyl lignin biosynthesis and Q9M1W6 in biosynthesis of jasmonic and Indole acetic acid contents (Bashir *et al.*, 2016). Studies have revealed that in addition to structural roles, plant cell walls play an important role in connecting extrac ellular and intracellular environments thereby sensing and transducing signals and activating cellular responses (Pogorelko *et al*., 2013) to environmental change and pathogen attack (Azizetal, 2004). Similarly, the present study has shown that *A. niger* on interaction with tomato leaves inducesthe express ion of cell wall proteins. It either suppresses the existing protein as seen in 48 hours interval or enhances the protein as seen in 96 hours of inoculation. The most active defense weapons of the plant cells are the proteins and thus the antifungal resistance in plants depends upon the protein profile modifications of plant cells (Zhu *et al*., 2010). Studies have shown that application of fungi could change the pathogenesis related (PR) protein profile of host (Khan *et al*., 2012). *A. niger*, in the present study has also shown alteration in the cell wall protein profile of tomato. GtLTP1 protein from *Prosopis juliflora* and GtLTP2 protein from *Vitis vinifera* were strongly expressed in the roots and stems while there was improved tolerance in tobacco plants due to over expression of GtLTP1 (Kiba *et al*., 2012). The expressed or suppressed cell wall proteins in our study will probably have some functionality related to the physiology of the host. As proteins perform structural, regulatory and enzymatic functions in a biological system, the profile of plant cell wall proteins during host-microbe interaction can play important role in establishing the outcome of plant microbe interaction.

Protein-protein interaction can be studied through proteomic analysis of cellular components which may occur due to reorientation of cellular signaling events (Fukao, 2012). Cross linking of proteins by chemical cross linkers are known to be the conventional method for understanding protein-protein interaction (Klockenbusch and Kast, 2010). Results thus indicate that presence of higher molecular weight proteins in cross linked samples as compared to its respective non-cross linked samples possibly arises from cross linking of low molecular weight proteins observed in non-cross linked samples. Formaldehyde prevents the interacting proteins from separating during processing of samples for protein extraction. The results possibly indicate that the cross linker binds two or more interacting proteins which probably underwent interaction on induction due to interacting with tomato leaves. The results therefore possibly point towards the establishment of protein-protein interaction in cytoplasm which may lead to establishment of signaling

cascade, leading to induction of gene expression. Genetic, molecular and proteomic analysis have led to identification and characterization of CWPs (Borderies *et al*., 2003). Studies demonstrated that susceptible varieties of tomato inoculated with Ralstonia solanacearumshowed increased expression of endochitinase (PR-3) and PR-5 family proteins while resistant variety showed up regulation of nucleoside diphosphate kinase (NDPK) and subtilase (Dahal *et al*., 2010). The enhanced concentration of some proteins could be a result of enhanced expression of genes due to signaling cascade induced by *A. niger*. A few dominant phylloplane colonisers viz. *Aspergillus niger*, *Alternaria alternate*, *Chaetomium globosum* and *Trichoderma viride* were found to induce Systemic Acquired Resistance (SAR) inplants (Mitra *et al*., 2013). It was reported that strains of *Aspergillus* spp. showed biocontrol property against specific strains of *Fusarium* spp. by inhibition of mycelial growth as well as improving development with highest shoot (SDW) and root (RDW) dry weight values (Hamdi *et al*., 2018). Similarly, in the present work *Aspergillus niger* has the tendency to alter the cytoplasmic and cell wall proteins thus promoting physiological effect in tomato plants. Phylloplane microbial communities are known to produce antimicrobial compounds and thus indirectly protect the plants from pathogens. *In vitro* studies have demonstrated that the toxin produced by *P. syringae pv. syringae* 22d inhibited the growth of the near isogenic foliar pathogen *P. syringaepv. glycinea* (Braun *et al*., 2010). The present study helps to understand that the phylloplane colonizing microfungi may regulate the protein profile of cell wall and cytoplasm and thereby affect the plant'sphysiology.

Conclusion

The current study focussed on the impact of plant and phylloplane microbe interaction. The investigation evidenced the alteration of protein profile in the host cell wall and cytoplasm. This signifies that alteration in the expression of protein may lead to signaling molecules thus influencing the physiology of the host. The identification of expressed proteins shall further support to understand their functional role in the host physiology.

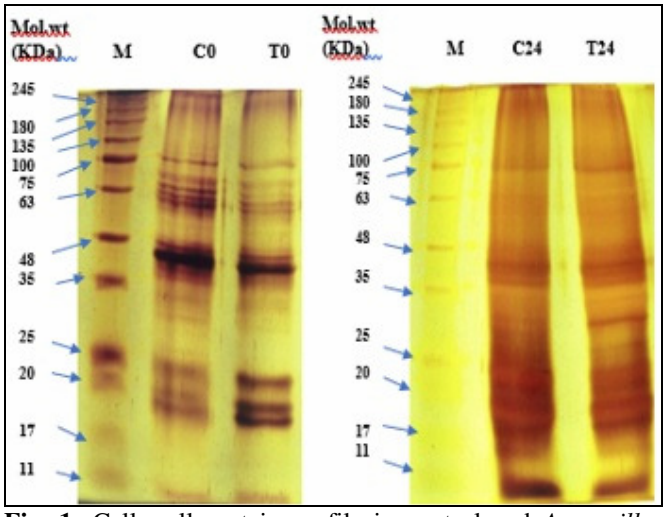

Fig. 1: Cell wall protein profile in control and *Aspergillus niger* treated tomato leaves (sampling at 0- and 24-hours post inoculation). M= protein marker, C= control, T=Treated.

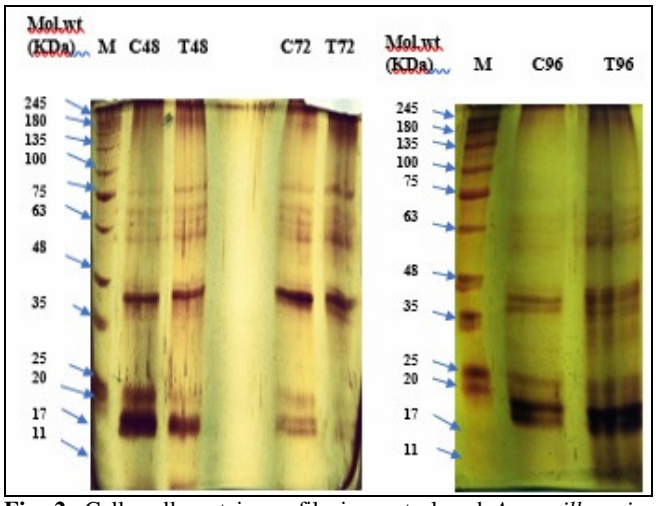

Fig. 2: Cell wall protein profile in control and *Aspergillus niger* treated tomato leaves (sampling at 48,72- and 96-hours post inoculation). M= protein marker, C= control, T=treated

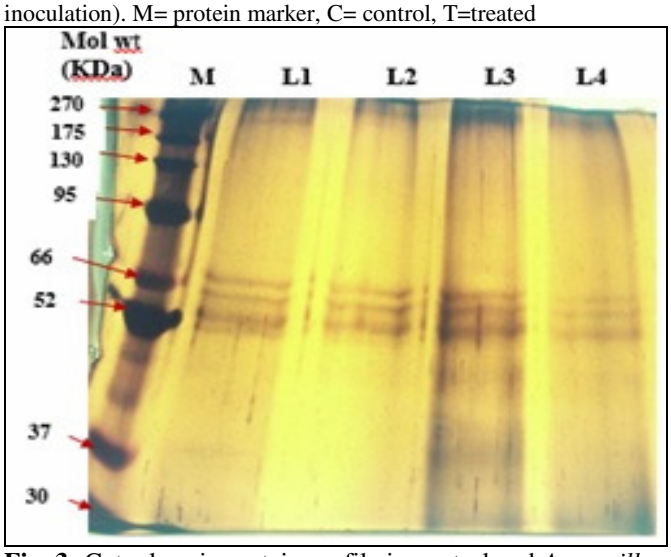

Fig. 3: Cytoplasmic protein profile in control and *Aspergillus niger* treated tomato leaves with and without crosslinking (Sampling at 0hr). M- Protein marker, L1- non crosslinked control, L2- non-crosslinked treated, L3- crosslinked control, L4- crosslinkedtreated.

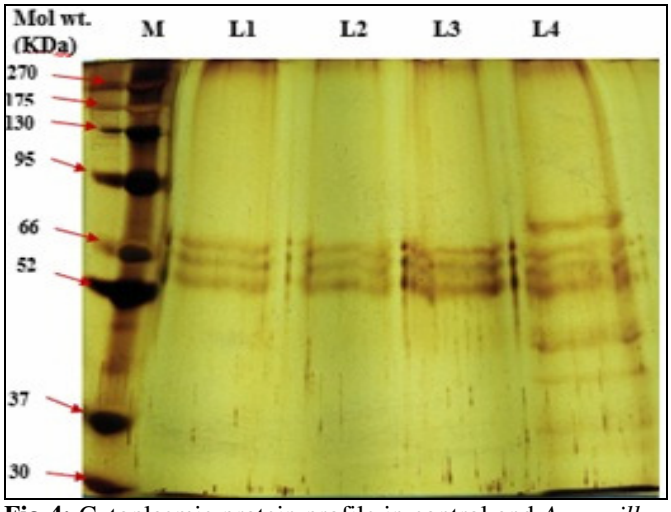

Fig 4: Cytoplasmic protein profile in control and *Aspergillus niger* treated tomato leaves with and without crosslinking (Sampling at 24 hrs). M- Protein marker, L1- noncrosslinked control, L2- non-crosslinked treated, L3- crosslinked control, L4- crosslinkedtreated.

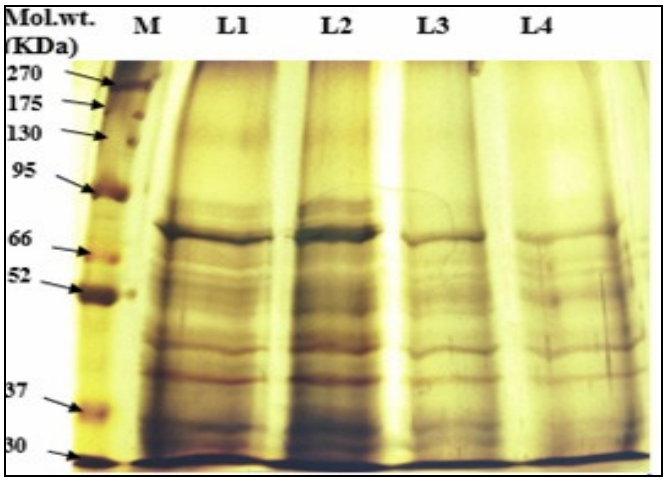

Fig. 5: Cytoplasmic protein profile in control and *Aspergillus niger* treated tomato leaves with and without crosslinking (Sampling at 48 hrs). M- Protein marker, L1- noncrosslinked control, L2- non-crosslinked treated, L3- crosslinked control, L4- crosslinkedtreated.

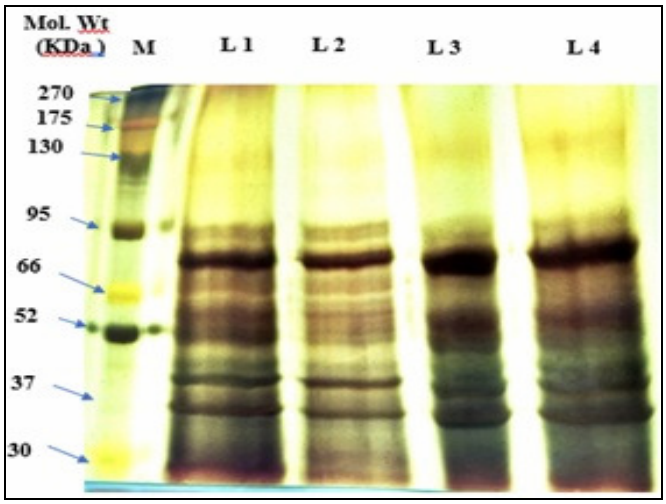

Fig. 6: Cytoplasmic protein profile in control and *Aspergillus niger* treated tomato leaves with and without crosslinking (Sampling at 72 hrs). M- Protein marker, L1- non crosslinked control, L2- non-crosslinked treated, L3- crosslinked control, L4- crosslinkedtreated.

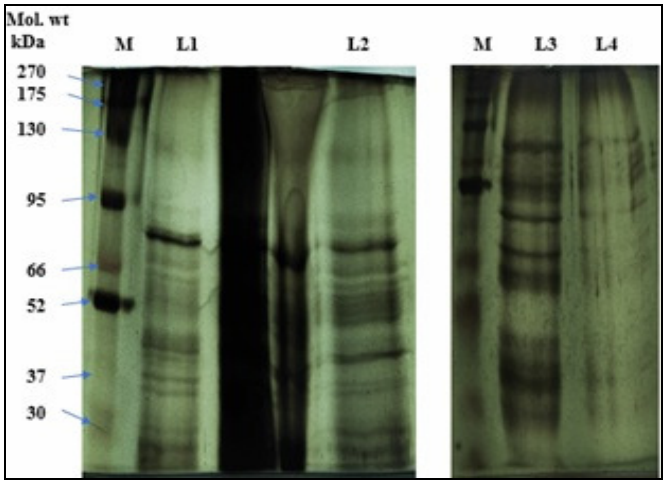

Fig. 7: Cytoplasmic protein profile in control and *Aspergillus niger* treated tomato leaves with and without crosslinking (Sampling at 96 hrs). M- Protein marker, L1- noncrosslinked control, L2- crosslinked treated, L3- non-crosslinked treated, L4- crosslinkedcontrol.

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