Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(3): 1740-1747
Received: 06-03-2018
Accepted: 10-04-2018

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Proteomics study during root knot nematode (*Meloidogyne incognita*) infection in tomato (*Solanum lycopersicum* L.)

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Abstract

Tomato (*Solanum lycopersicum* L., 2n=2x=24) is an important vegetable for human consumption because of its enriched nutritional composition that provide the basic body nutritional requirements. Tomato (*S. lycopersicum* L.) is affected by various diseases caused mainly by fungi, bacteria and nematodes. Root-knot nematodes (*Meloidogyne* spp.) found to be very fatal infective agents and cause severe yield loses. The present investigation was undertaken to enhance understanding regarding proteome changes that takes place in the tomato seedlings under root knot biotic stress. Two tomato cultivars AT 3 (Susceptible) and SL 120 (Resistant) grown under sterile and root knot nematode (3000 J_2 stage larvae per plant) inoculated soil were used for proteomics study during root knot nematode (*Meloidogyne incognita*) infection in tomato (*S. lycopersicum* L.). Isozyme analysis for polyphenol oxidase (PPO), peroxidase (POX) and catalse (CAT) revealed one cultivar specific isoform of PPO while two stress induced isoform of POX. The characterization of root proteins by SDS-PAGE had indicated that number of bands had increased in both the cultivars upon transition from control to stress environment and resistant cultivar had shown more number of bands as compare to susceptible cultivar. Protein profiling through 2-D gel electrophoresis had shown total of 506 protein spots out of which 166 proteins were found to be differentially expressed in both the cultivars (susceptible and resistant) under both the conditions (control and diseased). Overall 26 proteins were found to be specifically expressed in resistant cultivar (SL 120) only and absent in susceptible one (AT 3) under both control and diseased conditions, 29 proteins seemed to be differentially expressed under diseased conditions in susceptible cultivar AT 3 and were totally absent in AT 3 (Control). These proteins can be considered as promising candidates for identification markers in the screening of resistant genotype against the root knot nematode.

Keywords: tomato, root-knot nematodes, proteome changes, root proteins

Introduction

Tomato, *Solanum lycopersicum,* is an important vegetable for human use because of its vitamins and minerals content that provide the basic body nutritional requirements (Lorenz and Maynard, 1997) [6]. According to Splittstoesser (1990) [14], it rank 14th among sixteen common vegetables (spinach, lima beans, peas, sweet potato, carrots, cabbage, lettuce, onion, etc) based on total nutritional concentration but ranked first based on the contribution of nutrients to the diet. It is an excellent source of many nutrients and secondary metabolites that are important for human health; mineral matter, vitamins C and E, B-carotene, lycopene, flavonoids, organic acids, phenolics and chlorophyll (Giovanelli and Paradise, 2002) [3]. Tomatoes are widely consumed either raw or after processing and can provide a significant proportion of the total antioxidants in the diet (Martinez-Valvercle *et al.,* 2002) [8]. Tomatoes constitute the predominant source of lycopene and phenols. Nematodes found to be very fatal infective agents and cause severe yield loses in tomato. Root-knot nematodes (*Meloidogyne* spp.) are phytopathogenic obligate endoparasites nematodes that infect many plant species and cause serious damage to agricultural crops per year (Abad *et al.,* 2008) [1]. The annual estimated crop losses due to major plant parasitic nematodes in India have been worked out to be about Rs. 242.1 billion (Jain *et al.,* 2007) [5]. Management of plant parasitic nematodes has always been difficult, and the most successful strategy for many years has been the use of toxic fumigant nematicides, such as the most known methyl bromide which causes deleterious effects on humans and environment (Oka *et al.,* 2000b) [9]. The safe and eco-friendly approach is to use resistant variety. The reprogramming of gene expression during nematode infections is to be accounted towards host adaptation to the invading nematode by developing defence mechanism. As a consequence of restructured gene expression, plant's physiology gets affected and is evident in the form of symptoms. In the last decade proteomics studies have gained increasing importance in plant research. The development of proteomics techniques

allowing increased proteome coverage and quantitative measurements of proteins have been particularly instrumental to characterize proteomes and their modulation during plant development, biotic and abiotic stresses. Till date very limited information is available for changes in protein profile parameter of susceptible and resistant tomato cultivar against the root knot nematode infection. In view of the above reports, the present paper entitled "Proteomics study during Root Knot Nematode *(Meloidogyne incognita)* Infection in Tomato *(Solanum lycopersicum* L.*)"* deals with Proteomic study of roots of resistance and susceptible tomato plants and their differential gene expression.

Materials and Methods

The seeds of tomato cultivars for the present study were procured from the Main Vegetable Research Station; Anand Agricultural University; Anand (Table 1) and the investigation was carried out at Department of Biochemistry in collaboration with Department of Nematology; B. A. College of Agriculture; Anand Agricultural University; Anand; which is situated on 22º- 35' north latitude and 72º- 55' east longitudes and has an elevation of 45 meters above the mean sea level.

Table 1: List of tomato cultivars procured from MVRS

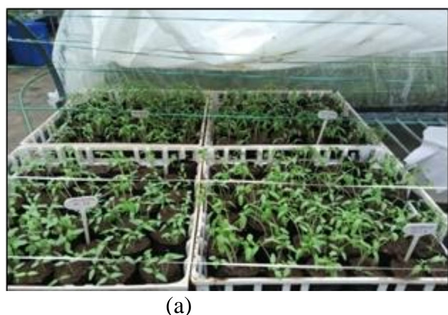
Sr. No.	Tomato Cultivar	Description
1	AT 3	Root knot nematode susceptible
2	SL 120	Root knot nematode resistant

1. Study of root knot index in roots of resistant and susceptible tomato plant (Taylor and Sasser, 1978) [16]

Before planting, tomato seeds were surface-sterilized in 1% v/v hypochlorite solution for 10 minutes and then rinsed in sterile distilled water three times for a total rinse time of 1 hour. Plants were grown under normal and disease condition in earthen pots. Plants were infected at the stage of three true leaves (Figure 1). For the nematode infestation, appropriate inoculum with 3000 J2 stage larvae/ plant was added at the base of each plant in small holes. The design of Experimental prepared was Completely Randomized Design (CRD) in which control and treatments were done in following way:

1. AT 3 Control: Seedlings grown in un-inoculated sterile soil.
2. AT 3 Treated/Inoculated/Stressed: Seedlings grown in soil inoculated with Root knot nematodes (3000 J_2 stage larvae / plant).
3. SL 120 Control: Seedlings grown in un-inoculated sterile soil.
4. SL 120 Treated/Inoculated/Stressed: Seedlings grown in soil inoculated with Root knot nematodes (3000 J_2 stage larvae / plant).

After 45 days of infection, the infected tomato plants of both susceptible (AT 3) and resistant (SL 120) cultivar were carefully removed and the root systems washed free of soil and were used for root knot index study as described by Taylor and Sasser, 1978 [16].



(a)

(b)

Fig 1: Tomato plants grown under normal and disease conditions **(A)** 10 Days after germination **(B)** 45 Days after inoculation

2. Proteomics study

2.1 Total Soluble Protein

The total soluble protein content in roots of tomato plants under control and stressed conditions was analyzed by Lowry *et al.,* 1951 [7].

2.2 Isozyme Analysis Using Native PAGE

Two Hundred milligram roots were homogenized with a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.2) with 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 15 min and supernatant was used for isozymes (PPO, POX and CAT). Native PAGE electrophoresis was conducted on vertical slab gel PAGE unit (BIO-RAD) at 50 mA for 120-130 minutes for different isozyme by following the procedure described by Sadasivam and Manickam, (1992) [12]. The separated bands were visualized under visible light and photographed using BIORAD Gel Documentation system (Bio-Rad Laboratories, USA). Staining of Polyphenol oxidase, Peroxidase and Catalase isozymes was done by the methods explained by Sadashivam and Manickam, 2008, Bhatnagar *et al.,* 2007 [2] and Weydert and Cullen, 2011 [17] respectively. When achromatic bands begin to form on gel, pour off the stain and rinse the gel extensively with ddH_2O.

2.3 Protein Profile by SDS-PAGE

The total protein from tomato roots (200 mg) was homogenized in 1 mL of protein extraction buffer {Tris- HCl (0.05 M, pH 7.4), 0.02% SDS, 30.03% Urea and 1% β-mercaptoethanol} and kept at room temperature for 24 hours. The homogenates were centrifuged at 13,000 rpm for 15 mins at 4˚C. The clear supernatant mixed with gel loading dye (with SDS) and loaded on the gel and electrophoresis was conducted on vertical slab gel PAGE unit (Bio-Rad) following the methodology as given by Sadasivam and Manickam, 1992 [12]. After the electrophoresis; gels were washed to remove excess of SDS and stained with 0.1% commassie brilliant blue-G250 in a mixture of methanol: acetic acid: distilled water in the ratio 40:10:50. The gels were de-stained by using a mixture of methanol: acetic acid: distilled water in the ratio 40:10:50 without dye. The separated bands were visualized under visible light and photographed using BIORAD Gel Documentation system.

2.4 Two dimensional electrophoresis (2 DE)

Exactly, 500 mg of tomato roots were taken in 2 ml centrifuge tube and 1ml of extraction buffer (7 M Urea, 2 M Thiourea,

50 mM Dithiothritol, 1% β-mercaptoethanol in 0.1 M Tris buffer (pH 7.2) was added and shake well for 6 hrs. Tubes were then centrifuged at 12000 rpm for 15 mins and supernatant was used as protein extract. Ready IPG strips (BIO-RAD) were used for isoelectric focusing. Passive Rehydration method was used for sample application. 40 µl of sample was mixed with 90 µl of rehydration buffer (8M Urea, 2% CHAPS, 50mM DTT, 2% w/v Biolyte (pH- 3-10), BPB trace). This was smeared in rehydration tray and 7 cm IPG strip was overlaid on this with gel side down and kept for 16 hrs for rehydration. Mineral oil was overlaid over strip to prevent sample evaporation. For isoelectric Focusing (IEF), wet small filter paper wicks were kept near poles of focusing tray to collect salts during focusing. Rehydrated strips were kept in focusing tray with positive side of strip to positive side of tray and gel side was kept down. Mineral oil was overlaid and tray was covered and kept in PROTEAN i12 IEF CELL. Following program for focusing was set and allowed to run (Table 2).

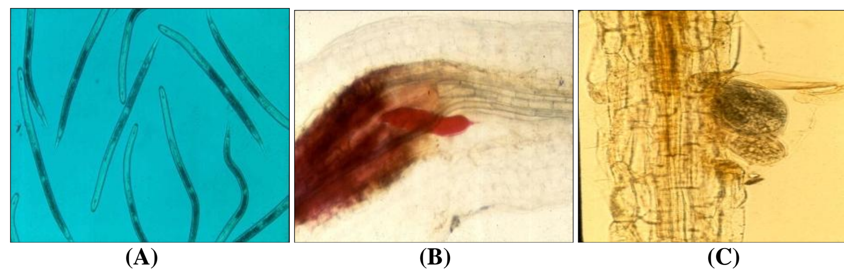
Table 2: Focusing protocol

Step	Voltage	Ramp	µAmp	Value	Unit
1	250	Linear	50	0:45	HH:MM
2	4000	Linear	50	2:30	HH:MM
3	4000	Rapid	50	10,000	Volt
4	50	Hold	50	-	-

After focusing, the strips were transferred in the 10% SDS PAGE gel for second dimensional run. Same protocol was followed as per total protein through SDS PAGE. After electrophoresis the gels were stained with silver staining protocol and analysed by using the software PD Quest Basic version 8.0.1.

Results and Discussion

Uprooted roots were carefully washed with water to remove unwanted soil particles and further analysed by staining as shown in figure 2.



(A) (B) (C)

Fig 2: Microscopic image of Root knot nematode: A) Free living, B) Within host tissue as parasite and C) Reproductive female laying eggs

1. Root Knot Index

Root knot index increased with increase in inoculum level. Therefore the inoculum level was kept constant for both the genotypes under treated condition. Around 2-3 nematode per gram of soil are considered as good population for their pathogenicity. Formation of gall on the roots is the characteristic feature of the root knot disease (Figure 3). Based on the extent of infection prevailed on the root system of the plant; root knot index was given on a scale of 0-5 as described by Taylor and Sasser, 1978 [16] (Table 3). Here index of "0" represents no infection at all and was calculated for AT-3 (Control), SI-120 (Control) and SI- 120 (Treated), While Root Knot index of 2 and 3 was found for AT-3 (Treated) depicting up to 40% and 60% of infected part of roots, respectively.

Table 3: Root knot index of tomato genotypes

Tomato Cultivar	Root Knot Index
AT – 3 (Control)	0
AT – (Treated)	2-3
Sl – 120 (Control)	0
Sl – 120 (Treated)	0

(A) (B) (C) (D)

Fig 3: Galling of roots under root knot disease. **A)** AT 3 (Control); **B)** AT 3 (Treated); **C)** SL 120 (Control); **D)** SL 120 (Treated)

2.1 Total soluble protein

The total protein content of roots of both susceptible and resistant tomato cultivars under control and disease conditions ranged from 2.46% - 3.56% (Table 4, Figure 4). Highest total protein content was observed in the susceptible cultivar AT 3 under disease condition (3.56%). There is no any significant change in protein in resistant cultivar during transition from controlled to disease environment while significant rise in protein content was observed in susceptible cultivar AT 3 (Treated) under disease condition (3.56%) as compared to the AT 3 (control) under normal condition (2.46%). The results observed here are in agreement with the results obtained by Shreenivasa *et al.*, 2011 [13].

Table 4: Total Soluble Protein

Samples	Total Protein Content (%)
AT 3 (Control)	2.46
AT 3 (Treated)	3.56
SL 120 (Control)	2.88
SL 120 (Treated)	3.00
S. Em.	0.07
C. D.	0.24
C. V. %	4.19

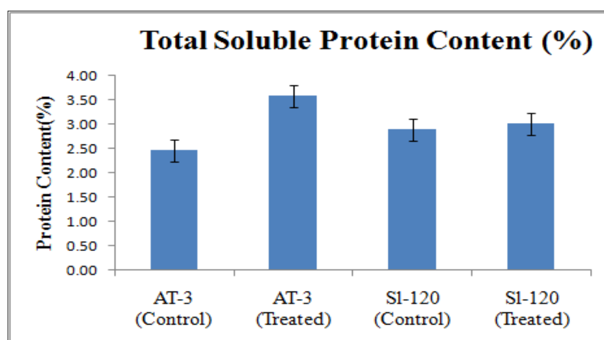


Fig 4: Total protein content of roots of tomato cultivars

2.2 Isozyme Analysis: 1. Polyphenol oxidase

Isozyme pattern for polyphenol oxidase in roots of tomato seedling under stress and control conditions were analyzed as shown in (Plate 1). Root samples of tomato seedling showed presence of eleven bands having Rm value ranging from 0.02 - 0.806 (Table 5). An isoform with Rm value 0.057 was present with light to moderate intensity in the resistant cultivar (SL 120) only under both control and stressed conditions. Another isoform with Rm value 0.088 was found to be present in resistant cultivar only under stressed condition. An isoform having Rm 0.236 was present with light intensity in susceptible cultivar under disease condition only. Electrophoretic banding pattern of PPO activity showed varying intensities of isoforms depending on the resistant and susceptible nature of seedlings. These results are in total agreement with the results reported by Rani *et al.*, (2008) [10].

Table 5: Electrophoretic profile of polyphenol oxidase (PPO) isozymes

Sr. No.	Rm Values	Samples			
		AT 3 (Control)	AT 3 (Treated)	SL 120 (Control)	SL 120 (Treated)
1.	0.020	+	+	+	+
2.	0.057	-	-	+	+
3.	0.088	-	-	-	+
4.	0.130	++	++	+++	+++
5.	0.214	+++	+++	+++	+++
6.	0.236	-	++	-	-
7.	0.369	+++	+++	+++	+++
8.	0.494	+++	+++	+++	+++
9.	0.698	+++	+++	+++	+++
10.	0.748	+	+	+	++
11.	0.806	-	+	+	++

Note: (- Absence of band, + low intensity, ++ moderate intensity, +++ high intensity)

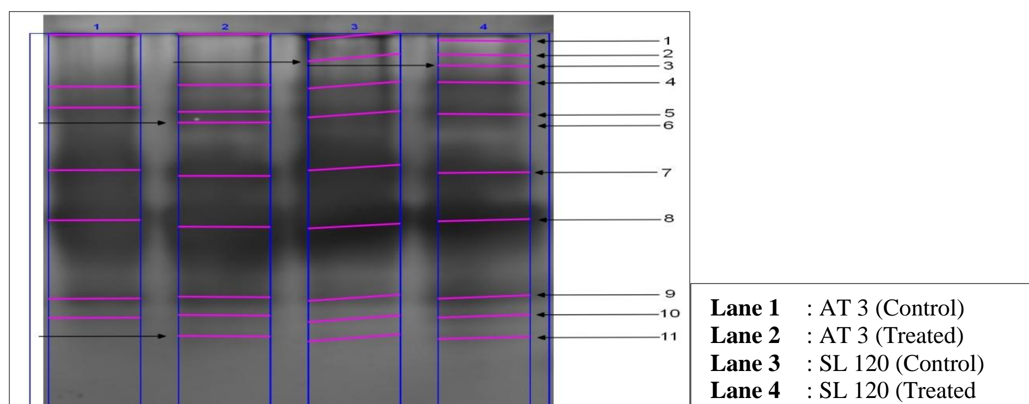


Plate 1: Polyphenol oxidase (PPO) isozyme analysis by Native – PAGE

2. Peroxidase

Isozyme pattern for peroxidase in roots of tomato seedling under stress and control conditions were analyzed as shown in (Plate 2). There was presence of thirteen POX isoforms (Rm 0.034 to 0.794) in roots of tomato seedlings (Table 6). Band 3 with Rm value 0.113 was specifically present in resistant cultivar (SL 120) with light intensity. Most of POX isoforms were similar amongst all the samples. Amongst the thirteen isoforms two isoforms POX 3 and 6 showed differential accumulation of peroxidase between resistant and susceptible cultivar respectively under stressed condition only. These results were in accordance with the results acquired by Rani *et al.,* (2008) [10] and Sreedevi *et al.,* (2013) [15].

Table 6: Electrophoretic profile of peroxidase (POX) isozymes

Sr. No.	Rm Values	Samples			
		AT 3 (Control)	AT 3 (Treated)	SL 120 (Control)	SL 120 (Treated)
1.	0.034	+++	+++	+++	+++
2.	0.072	+	+	++	+
3.	0.113	-	-	-	+
4.	0.159	+	++	++	++
5.	0.214	+++	+++	+++	+++
6.	0.306	-	+	-	-
7.	0.342	+	+	+	+
8.	0.380	+	+	+	+
9.	0.488	+++	+++	+++	+++
10.	0.529	+	+	+	+
11.	0.686	+++	+++	+++	+++
12.	0.732	+	+	+	++
13.	0.794	+	+	+	++

Note: (- Absence of band, + low intensity, ++ moderate intensity, +++ high intensity)

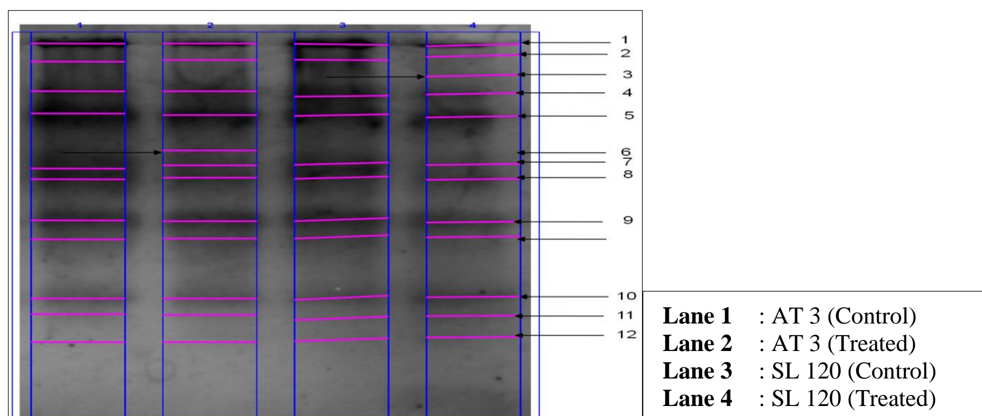


Plate 2: Peroxidase (POX) isozyme analysis by Native – PAGE

3. Catalase

Isozyme pattern for catalase in roots of tomato seedling under stress and control conditions were analyzed as shown in (Plate 3). Catalase gels (10% gels) had only one major band that rarely saturates getting larger with increasing catalase activity and another minor lighter band was also found as well. These results are in total harmony with the results found by Weydert and Cullen, (2010) [17].

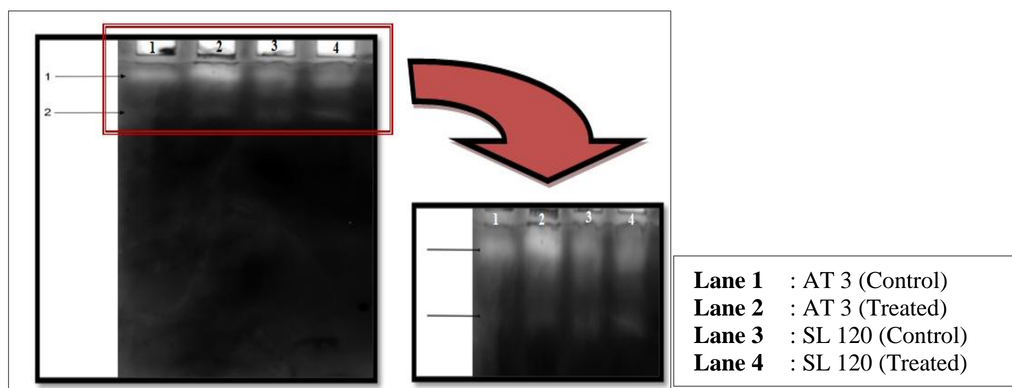


Plate 3: Catalase (CAT) isozyme analysis by Native – PAGE

2.3 Protein Profile by SDS-PAGE

The total soluble root proteins were fractioned into 23 bands, which showed heterogeneity among both the genotypes under both the conditions (Plate 4). The maximum numbers of bands (22) were observed in resistant cultivar SL 120 under root knot nematode biotic stress condition followed by SL

120 (Un-inoculated) and AT 3 (Inoculated) showing 19 bands each and at last AT 3 (Un-inoculated) with 17 bands (Table 7). The susceptible genotype AT 3 had 17 and 19 bands under control and disease conditions respectively whereas the resistant genotype SL 120 had 19 and 22 bands respectively. The resistant cultivar was differentiated from susceptible cultivar by the presence of four unique bands with Rm values 0.042, 0.29, 0.79 and 0.85. First three were light intensity band found to be present only in resistant cultivar (SL 120) and absent in susceptible cultivar (AT 3) whereas the forth one with moderate intensity had Rm value 0.85 was found to be present only in the susceptible cultivar (AT 3). Moreover one stress specific band with Rm value 0.18 was found to be present only under root knot biotic stress and absent under control condition in both the cultivars.

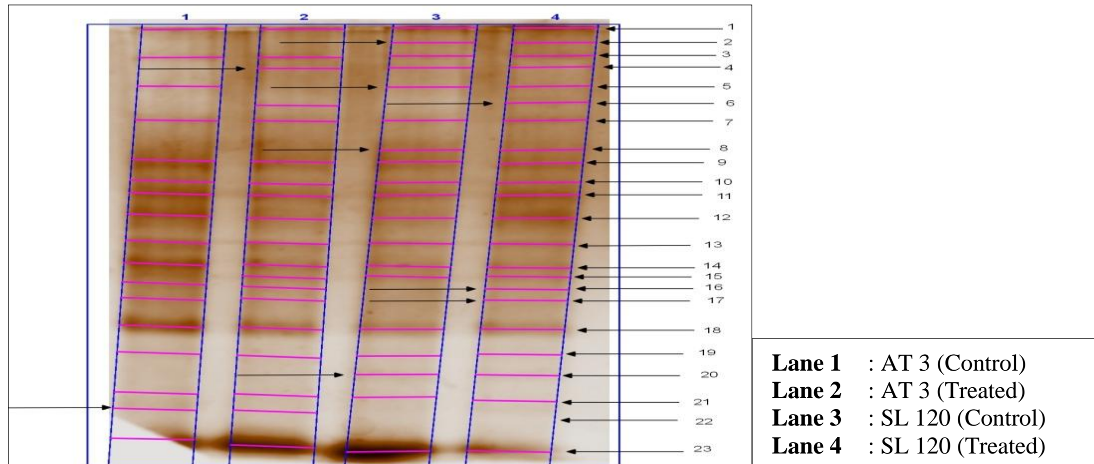


Plate 4: Protein profile by SDS – PAGE

Table 7: Electrophoretic profile of total soluble proteins by SDS – PAGE

Sr. No.	Rm Values	Samples			
		AT 3 (Control)	AT 3 (Treated)	SL 120 (Control)	SL 120 (Treated)
1.	0.012	++	++	++	+++
2.	0.042	-	-	+	+
3.	0.075	+	+	+	++
4.	0.097	-	+	+	+
5.	0.141	+	-	+	+
6.	0.178	-	+	-	+
7.	0.218	++	++	++	+++
8.	0.285	-	-	++	++
9.	0.315	+++	+++	+++	+++
10.	0.358	+	+	+	+
11.	0.387	+++	+++	+++	+++
12.	0.439	+++	++	++	+++
13.	0.497	++	++	++	++
14.	0.551	+++	+++	++	+++
15.	0.571	-	+	+	+
16.	0.600	+	+	-	+
17.	0.625	+	+	-	+
18.	0.690	+++	+++	+++	+++
19.	0.747	+	+	+	+
20.	0.794	-	-	+	+
21.	0.854	+	+	+	+
22.	0.871	+	+	-	-
23.	0.966	+++	+++	+++	+++

Note: (-Absence of band, + low intensity, ++ moderate intensity, +++ high intensity)

2.4 Proteomic Study Through 2-DE

Each sample had shown many differentially expressed protein spots which were quantified by their relative intensity through the PDQuest software, Bio-Rad (Version 8.0.1). Total of 506 protein spots were found out of which 166 proteins were found to be differentially expressed in both the cultivars (susceptible and resistant) under both the conditions (control and disease). Out of which AT 3 (Control) had shown a total of 111 spots where as AT 3 (Treated) under disease environment had yield 109 spots. At the same time SL 120 (Control) had shown 153 spots while SL 120 (Treated) under root knot nematode biotic stress had revealed the presence of 133 spots (Plate 5-8). Among 506 proteins, 62 and 70 proteins were up-regulated by root knot nematode biotic stress, under disease environment compared to control in AT 3 and SL 120 respectively. While down-regulated proteins were 78 and 91 in root knot nematode biotic stress, under disease environment compared to control in AT 3 and SL 120 respectively.

Overall 26 proteins were found to be specifically expressed in resistant cultivar (SL 120) only and absent in susceptible one (AT 3) under both control and disease conditions. Out of

which spot with SSP no. 7801 had shown highest up regulation during transition from control to stress condition in resistant cultivar SL 120. There were two particular spots with their SSP no. 2502 and 8501 were found to be expressed only during control condition and were suppressed totally under stress condition in SL 120. These could be considered as a stress responsive proteins. The remaining 24 spots could be considered as promising candidates for identification markers in the screening of resistant genotype against the root knot nematode via marker assisted selection in the breeding programme for the development of the root knot resistant variety.

Similarly only 5 proteins were specifically expressed in susceptible cultivar (AT 3) only and absent totally in resistant one (SL 120) under both control and disease conditions. Out of which two particular spots with their SSP no. 6507 and 8102 were found to be expressed only during disease condition and were absent totally under control condition in AT 3. There were 29 proteins seem to be differentially expressed under disease condition in susceptible cultivar AT 3, which were totally absent in AT 3 (Control). These resulting protein spots were in total harmony with the findings of Gong *et al.,* (2014) [4] who analyzed stress responsive proteins in roots of tomato using iTRAQ. The same result was also found by Rodriguez-Celma *et al.,* (2010) [11] on his work on tomato root response to low (10µM) and high (100µM) cadmium concentration at the root proteome level. The resulting proteomics expression analysis could be used as promising candidates for the further investigation regarding better understanding of plant responses to specific stress.

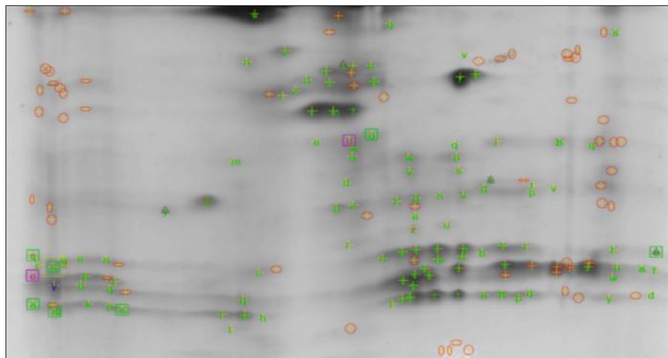


Plate 5: Protein profile by 2 – D electrophoresis for AT – 3 (Control)

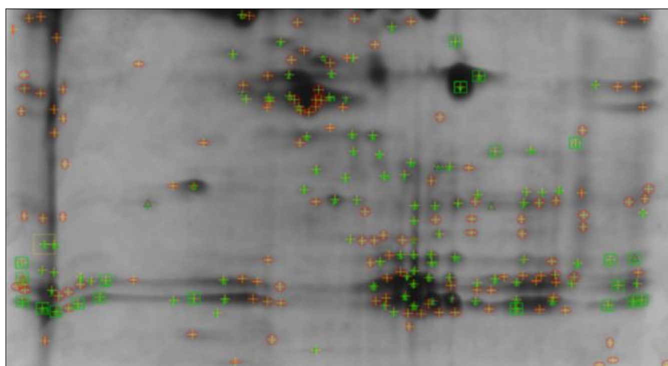


Plate 6: Protein profile by 2 – D electrophoresis for AT – 3 (Treated)

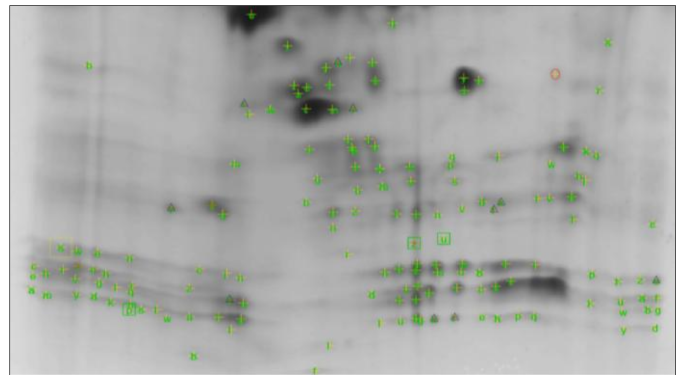


Plate 7: Protein profile by 2 – D electrophoresis for Sl – 120 (Control)

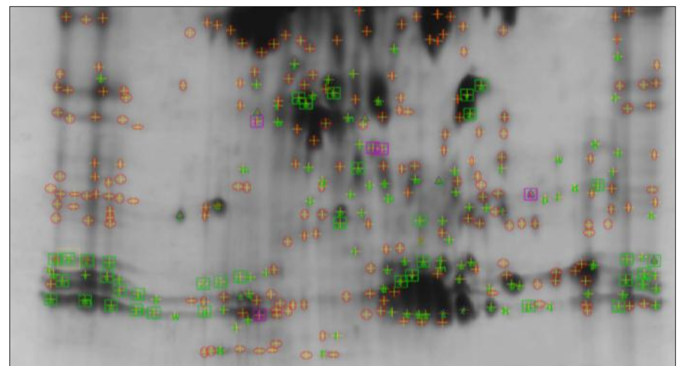


Plate 8: Protein profile by 2 – D electrophoresis for Sl – 120 (Treated)

Summary and Conclusion

The present investigation entitled "Proteomics study during Root Knot Nematode (*Meloidogyne incognita*) Infection in Tomato (*Solanum lycopersicum* L.)" was undertaken to enhance understanding regarding the proteomics changes that takes place in the tomato seedlings under root knot biotic stress. Better understanding about the exact mechanism of plant response towards such biotic stress can help to improve screening strategies for the selection of resistant cultivars. Two tomato cultivars AT 3 (root knot susceptible) and SL 120 (root knot resistant) were grown under sterile and root knot nematode inoculated soil (3000 J_2 stage larvae per plant). 45 days after infection, the tomato plants were carefully uprooted and the root systems washed free of soil and had been used for various biochemical and molecular analysis. The statistical experimental design followed was Completely Randomized Design (CRD). Isozyme analysis for polyphenol oxidase (PPO), peroxidase (POX) and catalse (CAT) revealed one cultivar specific isoform of PPO while two stress induced isoform of POX. The characterization of root proteins by SDS-PAGE had indicated that number of bands had increased in both the cultivars upon transition from control to stress environment and resistant cultivar had shown more number of bands as compare to susceptible cultivar. Protein profiling through 2-D gel electrophoresis had shown total of 506 protein spots out of which 166 proteins were found to be differentially expressed in both the cultivars (susceptible and resistant) under both the conditions (control and disease). Overall 26 proteins were found to be specifically expressed in resistant cultivar (SL 120) only and absent in susceptible one

(AT 3) under both control and disease conditions and 29 proteins seem to be differentially expressed under disease condition in susceptible cultivar AT 3; which were totally absent in AT 3 (Control). These expressed proteins could be considered as promising candidates for identification markers in the screening of resistant genotype against the root knot nematode via marker assisted selection in the breeding programme for the development of the root knot resistant variety.

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