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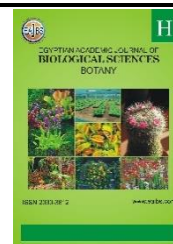
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Enhancing Tomato Cultivars Against Root-Knot Nematode Using Salicylic Acid and Their Impact on Protein Expression

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ABSTRACT

The defense strategies of plants against pathogen attacks included more than two kinds such as biochemical reactions, structural, hormonal reactions, and elicitors. On the other hand, pathogens in general have different ways and strategies that facilitate their infections and make them successful. Continuously, the interaction between tomato plants and root-knot nematodes causes many reactions involving both morphological symptoms and genetically on the molecular level. However, fundamental aspects of hormonal influences studies on plant nematode interactions are still complicated especially on the level of molecular genetics. Precise recognition of tomato-induced proteins is necessary for identifying plant-induced systemic resistance (ISR). In this paper, the target is the recognition of the protein profile changes accompanied by the biological changes through both processes of infection of two tomato cultivars (resistance and sensitive) with root-knot nematode and treated with two different concentrations of SA 0.1 and 5 mM. The results confirmed that induction of tomato roots infected with root-knot nematodes (RNK) and treated with Salicylic acid (SA) was significant especially in 5mM concentration of SA that appeared to have an excess of intensive bands in sensitive and resistant cultivars as compared to control roots. On the other hand, the bands were light in 0.1 mM concentration of SA.

INTRODUCTION

Tomato (*Solanum Lycopersicum*) is a major important economic crop for human beings although it was affected facilely by dangerous pathogens such as root-knot nematodes (*Meloidogyne* spp.). Tomatoes may provide an important proportion of the total antioxidants and have many uses either after processing or raw (Martinez-Valvercle *et al.*, 2002). Root-knot nematode (RKN) is one of the fatal infective agents, where it destroys the crop under severe infection with high densities populations (inoculums level) leading the end to total yield, destruction, and loss. Plant parasitic nematodes (*Meloidogyne* spp.) are obligate endoparasites nematodes and their infection affects negatively a number of plants and

agricultural crops causing huge damage in the grown fields (Abad *et al.*, 2008). Root-knot nematode (*Meloidogyne incognita*) causes extensive damage to tomato plants (Kamalvanshi *et al.*, 2004). One of the main essential structures in organisms is proteins, which catalyze biochemical reactions and are it very important to metabolism by merging every process into cells. The protein profile changes depend on both plant species and plant organs (Kong-Ngern, *et al.*, 2005). One of the biological analysis assays using shifts of protein bands detection is polyacrylamide gel electrophoresis (SDS-PAGE). These protein bands express hormonal changes or enzymes or any biotic stress (Ghasempour *et al.*, 1998; Gianello *et al.*, 2000, Ghasempour *et al.*, 2001, Ghasempour and Kianian 2002, Ghasempour and Maleki 2003). Many cultivars grown under various environments have reported changes in the electrophoretic profiles of the soluble proteins from the side of quantitative detection (Cloutier, 1983). Electrophoretic profiles of soluble proteins are a vital technique for genetic variability investigation for cultivars (Barta *et al.*, 2003). Salicylic acid (SA) has been illustrated as an endogenous signal used to activate most plant defense responses that enhance resistance to pathogens via impact on pathogen-related gene expression (Greenberg *et al.*, 1994 and Conrath *et al.*, 1995). The aim of this investigation is the participation of induced proteins and their expression impact on enhancing the ability of tomato cultivars against root-knot nematodes (RKN) (*Meloidogyne* spp.).

MATERIALS AND METHODS

Isolates of root-knot nematode (RKN) were collected from EL-Beheira Governorate (EL-Nubaria), Egypt. The samples were collected from infected eggplants and tomato plants. The samples were taken at a depth of 0-30 cm from growing areas. Root-knot nematodes were extracted from about 200 gm of sub-samples using sieving and flotation methods (Shepherd 1986). The extracted nematode suspension was stored in tubes at room temperature for one day until propagation.

1. Tomato Plants and Nematode:

The two types of tomato cultivars; "Thuria" as a resistant cultivar and "380" as a sensitive cultivar were grown in the greenhouse of the Faculty of Agriculture, Alexandria University, Egypt. These cultivars were obtained from EL-Nubaria as saplings to assay that experiment. The tomato saplings were germinated in 36-cm plastic pots (1:3 mixtures of an autoclaved sterile sand and loam) about 80 total numbers of saplings were divided into 40 for sensitive cultivars and 40 for resistant cultivars.

2. Pathogenicity Assay and Induces of Tomato Root Genes:

After three weeks each cultivated pot was infected with the stock suspension of *Meloidogyne* spp. which was isolated previously and maintained on sensitive and resistant tomato cultivars. Five-week-old tomato saplings were inoculated with 2000 freshly hatched second juveniles (J2) per plant where, that (J2) was isolated from the collected infected roots for hatching at room temperature (Shukla *et al.*, 2018). After three weeks of the plant inoculation two SA concentrations, 0.1 and 5 mM were used for induction of plant systemic acquired resistance where the process was accomplished near tomato roots of both cultivars resistant and sensitive. The roots of infected treated plants have been collected carefully, at four intervals after inoculation and induction; 24h, 48h, 72h, and 7d dpi (day post-induction) treatments of both sensitive and resistant cultivars and the root systems washed free of soil and were used for the next step to compare with the control ones.

3. Extraction and Quantification of Total Soluble Proteins to Prepare Samples:

The protein samples were isolated from tomato roots infected with RKN and treated with two different concentrations of SA for both sensitive and resistant cultivars for all intervals and the control ones. Analysis of samples was accomplished by: approximately two grams of infected treated root tissues were homogenized in 2.5 ml of protein extraction

buffer consisting of (0.6 ml of 0.5 M Tris-HCl, pH 6.8, 2 ml of 10% SDS, 5 ml of 10% glycerol, 2 mM EDTA, 1 ml of 1% bromophenol blue and 0.5 ml of 1mM β -mercaptoethanol) with a prechilled mortar and pestle under the ice-cold condition to dissociate the proteins to their subunits. The homogenates were centrifuged at 13000 rpm for 20 min at 4°C. The supernatant was collected and mixed with three volumes of cold acetone. The samples were kept at -20°C for 20 min followed by centrifugation at 13000 rpm for 15 min at 4°C. The supernatant was removed and the pellets were air-dried at room temperature. Thereafter, the pellets were resuspended in a small volume of distilled sterile water and centrifuged at 13000 rpm for 5 min 4°C. Finally, the supernatant was prepared for use in protein fractionation with the dye binding technique of (Bradford 1976) and SDS-PAGE assay.

4. Protein Profile Analysis Using Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) Assay:

Different polypeptides of induced infected treated tomato roots of two cultivars (sensitive and resistant) were analyzed and performed on SDS-PAGE (12% W/V separating gel and 5% w/v stacking gel) where about 10 μ l of clear supernatant was mixed with gel loading dye (with SDS) and was fractionated on the polyacrylamide gel. A preparatory gel slab was prepared by clamping two clean glass plates with plastic spacers. To avoid leakage later, the bottom was taped carefully. 12% resolving gel was injected into the gel slab (between the glass plates) using a 50 ml plastic syringe. 12% polyacrylamide gel mixture consisted of 0.1 ml of 10% ammonium peroxy disulfate and 10 μ l of N.N.N1.N1-tetramethylene- ethylenediamine (TEMED) in 10 ml of 12% gel solution, 4 ml of 30% bisacrylamide, 2.5 ml of 1.5 M Tris-HCl pH 8.3, 100 μ l of 10% SDS, 3.3 ml of distilled sterile water. After that 5% stacking gel was injected (between glass plates) using a 50 ml plastic syringe. The components of 5% stacking gel were (0.83 ml of 30% bisacrylamide, 0.63 ml of 1 M Tris-HCl pH 6.8, and 50 μ l of 10% SDS. Freshly prepared 50 μ l of 10% ammonium persulfate, 3.4 ml of distilled water, and 5 μ l of TEMED were added. As soon as, completely filled with the gel mixture, a gel comb was inserted between the glass plates, and the gel was allowed to polymerize. The electrophoresis was conducted on a vertical slab gel PAGE unit (Bio-Rad), where the gels were run at a constant power of 80 mA for 3-6h till the tracking dye reached the end of the gel. After the electrophoresis, gels were washed to remove excess SDS and the separated proteins were stained with 0.1% Coomassie brilliant blue G250 in a mixture of methanol acetic acid distilled water in the ratio of 40:10:50. The gels were transferred in the detaining solution with these components of mixture methanol: glacial acetic acid: distilled water in the ratio 40:10:50 without dye. The relative molecular weight of each protein was characterized using a standard protein marker. The separated bands were visualized under visible light and photographed using the BIORAD Gel Documentation system (Bio-Rad Laboratories, USA).

RESULTS AND DISCUSSION

1. Protein Detection of Tomato Roots Infected with RKN And Induced by SA In Comparing with Control Using SDS-PAGE Assay:

The protein was extracted from tomato roots infected with RKN on intervals and induced by SA and also from control ones. Then these isolated samples of protein were separated on 12% SDS-PAGE analysis. The results confirmed that there were changes in different bands of infected plants compared with control samples as shown in Figure (1). These changes were related to the interaction between plants and pathogens.

The characterization of total soluble root proteins by SDS-PAGE indicated that the number of bands had increased in both the (sensitive and resistant) cultivars upon transition from control to infected treated cultivars with a 5 mM concentration of SA. The resistant

and sensitive cultivars had shown more bands as compared to the control plant. The results of electrophoresis illustrated in Figure (1) appeared significant changes under SA induction, especially in resistant cultivars that; presented high-intensity bands with molecular weight at 84 kDa. Most of the extracted fractionated proteins migrated in the range from 84 to 10 kDa with a total number of eight bands that presented heterogeneity among both sensitive and resistant cultivars under both the conditions of infection with RKN and induction with SA. The highest number of protein polypeptides showed induction of at least five different proteins with molecular weights of 23, 49, 73, 75 and 84 kDa in resistant cultivars and three bands of molecular weights 23, 75 and 84 kDa were observed in sensitive cultivars. While the lowest expressed bands were indicated in sensitive cultivar infected treated roots after 24h from treatment with 5mM SA that was on the level of disappeared three bands. But there was one different band at molecular weight 49 kDa as compared with control and other sensitive cultivars at the different intervals at the same concentration of 5mM SA. Similar results were reported by lipid peroxidation detection in shoots of potato infected with cyst nematodes during intervals where; there was an increase of that enzyme after 24h to 7 days and also up to 35 days post-infection with nematodes (ELKobrosy *et al.*, 2018). In contrast, other results mentioned that a high concentration of ethylene treatments 5mM revealed low gene expression on levels of the roots infected with cyst nematodes (ELKobrosy *et al.*, 2022).

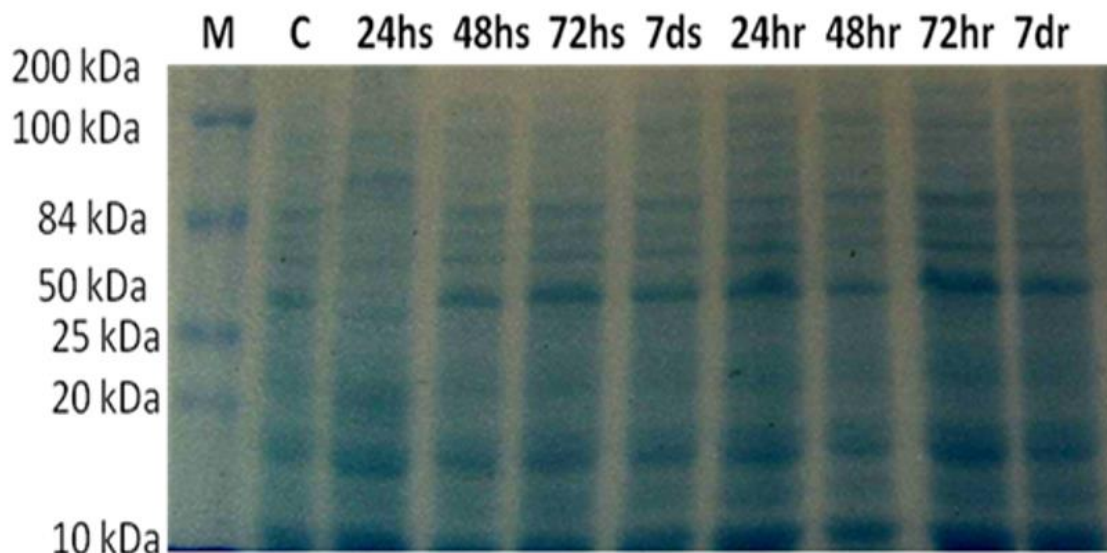


Fig. 1: 12% SDS-PAGE analysis of protein, where M, 200 kDa stander marker protein, C, Control, (24hs, 48hs, 72hs, 7ds) hours and days after infection with RKN and treatment with 5mM SA for sensitive cultivars, (24hr, 48hr, 72hr, 7dr) hours and days after infection with RKN and treatment with 5mM SA for resistant cultivars.

In contrast, comparing the protein profiles between control plants and those treated with 0.1mM SA concentrations using SDS-PAGE pattern showed that only a few changes in the pattern of proteins were induced. Also, the intensity of some other protein bands decreased dramatically as shown in Figure (2). In the control roots appeared three bands and were very light at molecular weights 20, 84 and 100 kDa and those bands disappeared totally after 24 & 48 h of treatment with 0.1% SA in sensitive cultivar where became achromatic bands on the gel. On the other side, two of them were observed again in an intensive band at molecular weights (84 and 100 kDa) after 72h of treatment with 0.1% SA in addition, appearing of one other new band may correspond to a new protein at molecular weight 25 kDa as compared to control. That newly expressed protein (25 kDa) was appeared also after 7 days post inoculation under RKN biotic stress and treatment with SA. The two newly

expressed proteins were observed at molecular weights 25 and 25 kDa in the resistant cultivars at different intervals with different intensities of bands. Similar results presented that, the highest number of protein polypeptides was found in infected eggplant with nematodes and treated (seven bands), while the lowest number of bands was observed in the infected only eggplants and root dipping 45 min (one band) (Ramadan and Soliman, 2020). Approximately four bands appeared in control (untreated) plants at molecular weights 43, 47, 53, and 55 kDa under normal conditions but were absent in infected eggplants with nematodes (Ramadan and Soliman, 2020).

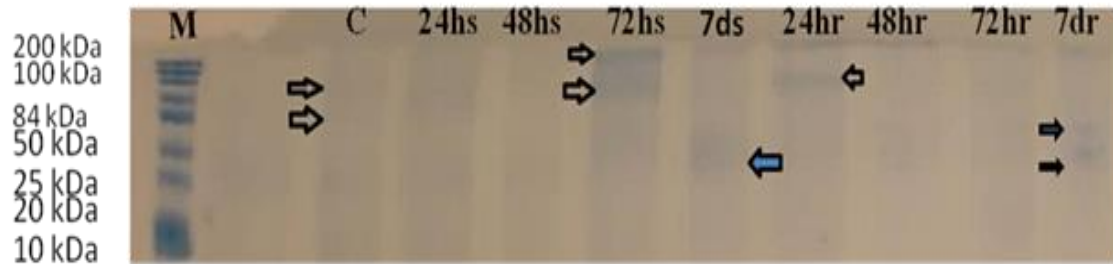


Fig.2: 12% SDS-PAGE analysis of protein, where M, 200 kDa stander marker protein, C, Control, (24hs, 48hs, 72hs, 7ds) hours and days after infection with RKN and treatment with 5mM SA for sensitive cultivars, (24hr, 48hr, 72hr, 7dr) hours and days after infection with RKN and treatment with 0.1 mM SA for resistant cultivars.

Conclusion:

To abolish the effects of RKN infection, plants may change their protein accumulation using two different concentrations of SA. Comparative proteomic analysis was applied to investigate the protein profiles under SA. In order to distinguish biotic responses from developmental changes in protein accumulation in both control and infected roots were collected at four intervals to minimize experimental error. Alterations induced in sensitive and resistant proteins under the induction of protein patterns using SA treatment were studied by one –dimensional SDS-PAGE. The target of this investigation was to elevate the efficacy of two different concentrations of SA (0.1mM and 5mM) aimed to detect novel expressed proteins. These induced proteins may be considered promising candidates for determination markers in the screening of resistant plants against root-knot nematodes.

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