Dynamics of total proteins during stress alleviation in tomato infected with cucumber mosaic virus

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Background: Phytoalexins are the secondary metabolites produced in crop plants after the attack of necrotizing pathogens. They are aimed to support homeostasis in stressed plants. Proteins are also part of this regime that is manipulated according to the type of infection or environmental stress. Chemical elicitors substitute the traditional fungicides and safe to use. They also modify protein levels due to their direct or indirect influence on the defense pathways.

Methodology: An in vitro study was conducted on tomato seedlings inoculated with cucumber mosaic virus (CMV). Plants were treated with salicylic acid and BTH at different intervals to find out the alterations in the total soluble proteins. Purpose of study was to investigate the variation in total proteins during the viral attack and role of elicitors in this case.

Results: Results indicated that protein level was initially reduced (133.3 mg) in the inoculated plants prior to the application of elicitors. However, BTH alone or combined with SA significantly enhanced total soluble proteins (200.3 mg) in tomato leaves when applied on weekly intervals. Single application of Salicylic acid gave similar response as in case of non-inoculated plants, however weekly applied SA reduced the total proteins to much lower extent (83.6 mg) under the CMV stress.

Discussion: Modification of total proteins in a single tomato cultivar along with homogenous environmental factors indicates the active role of chemical elicitors in the defense pathways. BTH is a structural homologue to SA but expressed a remarkable functional contrast under viral attack particularly in solanaceous crops. This novelty in plant science is worthy of discussion.
Title: Dynamics of total Proteins during stress alleviation in tomato infected with cucumber mosaic virus; escorted by application of safe chemicals.

Short title: Protein variation in CMV infected Tomato

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ABSTRACT

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Introduction:

Tomato \textit{(Solanum lycopersicum)} belongs to family \textit{Solanaceae}. It is the 2nd important vegetables grown all over the world (Akhtar \textit{et. al.}, 2010). It has increased the interest for the fresh market due to its frequent use in a variety of processed foods (Georgé \textit{et. al.}, 2011). Phenolic, ascorbic acid and Carotenoids of tomato contain antioxidants properties. Its regular consumption can reduce the risk of prostate cancer (Giovannucci, 1999). Among the tomato viruses CMV is most destructive plant virus. Among the plant viruses it has broadest host range (Stevenson, 2004) and is serious pathogens for tomato cultivation in temperate climates (Kyriakopoulou \textit{et. al.}, 2000).

Among the chemical elicitors, BTH can activate SAR response against various plant viruses including CMV in tomato, and cantaloupe (Anfoka, 2000; Smith-Becker \textit{et. al.}, 2003), \textit{Turnip crinkle virus} and \textit{Tobacco mosaic virus} (Lawton \textit{et. al.}, 1996). SA has key role in the plant kingdom as being important part of signal transduction. In case of plant viruses, SA can induce the metabolic pathways that directly or indirectly interference with two general processes of replication and systemic movement of in the host plant (Murphy and Carr, 2002).

Total soluble proteins under biotic and abiotic stress indicate an active part of defense related compounds. They are reliable indicator of stress response in crop plants. However their quantification was studies during application of elicitors in the plants under stress.

MATERIALS & METHODS:

Tomato variety Nagina without special resistance against CMV was sown under insect free environment within the glass house. Eight treatments including positive and negative control were maintained in the pot experiment. One month old plant nursery was sown after 1 hour root dip into the solutions of the treatment formulations. While control plants were dip into the distilled water for same time. Treatment combinations were maintained as follows,

T1= Salicylic acid weekly foliar application, T2= Salicylic acid one foliar spray post inoculation
T3= BTH (Benzothiadazole-7-carbothioic acid S-methyl ester) foliar application on weekly basis, T4= BTH single foliar spray post inoculation, T5= BTH+ Salicylic acid weekly treatment
T6= BTH+ Salicylic acid single spray post inoculation, T7= Healthy control, T8= Inoculated control.

**Mechanical inoculation:**
Mechanical inoculation of plants was done after 1\(^{st}\) foliar spray of chemical elicitors. Inoculum source was the infected tomato leaves with typical shoestring disease ground in 0.02M phosphate buffer, pH 7.4 (1 g/ml); in a pestle and mortar. Later this homogenate was squeezed through a very fine muslin cloth. Young leaves of 4-5 week-old healthy tomato plants were dusted with 500-mesh carborandum powder and mechanically inoculated with the freshly extracted sap using cotton swabs. Later on, the inoculated Plants were rinsed gently in a stream of water to remove extra inoculum and kept under insect-free environment (Akhtar et. al., 2010).

**Virus Testing:**
Samples from tomato leaves were collected after the appearance of symptoms on the leaves. Virus testing was done through DAS-ELISA and protocols were followed as described by Hussain et. al. (2004) with commercial polyclonal antibodies to CMV (BIOREBA AG Switzerland).

- Wells of ELISA plate were coated with CMV antibodies diluted in coating buffer at 1:200.
  - The coated plate was incubated at 4\(^{\circ}\)C for overnight.
  - After incubation the plate was washed with PBS-Tween 3-4 times at 5-minute intervals.
  - Then wells were filled with the sap of CMV-infected tissue extracted in extraction buffer and two wells were filled with each of buffer, CMV negative and positive sample.
  - The plate was incubated for overnight at 4\(^{\circ}\)C and washed 3 times with PBST.
  - 200\(\mu\)l of enzyme conjugate diluted at 1:200 was added and plates incubated for overnight at 4\(^{\circ}\)C followed by washing as in step 3.
  - 200\(\mu\)l freshly prepared substrate buffer containing p- nitro phenyl phosphate (75ug/ml) was added to each well.
Incubation was done at room temperature for 30 minutes and reaction was observed visually for the development of yellow colour and read in reader at 405 nm.

The reaction was stopped by adding 50µl 3M NaOH to each well.

**Sample Extraction and biochemical Analysis:**

For extraction and estimation of enzymes and other biochemical parameters leaf and fruit samples from glass house were ground in extraction buffer specific for different enzymes/biomolecules and centrifuged at 15,000×g for 20 min at 4°C. The supernatant was separated and used for assay.

**Total soluble proteins:**

For protein estimation in different parts of maize, 5 µL of supernatant and 95 µL 150 mM NaCl were mixed with 1.0 mL of dye reagent (100 mg). Coomassie Brilliant Blue G-250 dye was dissolved in 50mL of 95% ethanol and 100 mL 58% (w/v) phosphoric acid and dilute to one litter and the mixture was left for 5 min to form a protein dye complex. Then, the absorbance was measured at 595 nm (Bradford, 1976).

**Statistical analysis:**

All experiments were conducted in triplicates using completely randomized design (CRD). The significance was ascertained by analysis of variance and Tukey (HSD) test at p<0.05 and where applicable at P<0.01 using XL-STAT software.

**RESULTS:**

**ELISA:**

Presence or absence of virus was confirmed by typical viral symptoms and ELISA. ELISA plate was assessed visually. Positive reaction (yellow colour) was observed with CMV-infected (symptomatic) plants but negative reaction indicated the absence of virus as in case of healthy (-ve) control. However, negative reaction was rare due to unchecked aphid feedings in tunnel throughout the cropping season. Mechanical inoculation was also successful and all treated samples positively indicated the presence of CMV. Colour development was due to conversion of p-Nitro phenyl phosphate into p-Nitro phenol phosphate. Positive and negative control samples were also loaded as standards available in the ELISA kit.
Total soluble Proteins (mg g\(^{-1}\) fresh weight):

Proteins are assumed to play key role in plant metabolic system and defense activity against the infections. They include pathogenesis related proteins as well. According to the results protein level was slightly decreased after mechanical inoculation of CMV. Among the treatments, (B+SA)\(_W\) (200.33 mg) and BW (200.33 mg) induced higher level of total proteins. While SA1 (169 mg) demonstrated similar level of proteins; as in case of non-inoculated plants. But B1 (113.67 mg) and SA-w (83.67 mg) reduced the total protein concentration in tomato leaves to much lower extent.

DC= positive control, HC= negative control, SA= salicylic acid, B= BTH, BSA= BTH+Salicylic acid, W= weekly sprays, 1= single spray
Discussions:

In the present studies we have discussed the dynamic progress of protein contents while alleviating the CMV infection in tomato with application of chemical elicitors. It is generally observed that protein contents show a great diversity while crops experience the biotic or abiotic stress. Our results indicated the minor decrease in total proteins during initial challenge by cucumber mosaic virus (CMV). Previous studies on tomato support our findings where protein level was reduced after salt stress artificially induced by NaCl (Doganlar et. al., 2010). Tomato also responds with increased level of heat shock proteins and other secondary metabolites under heat stress. However, increase or decrease in the protein contents is not limited to the stress but the genetic variations (susceptible or resistant varieties) are also an important factor that indicates the level of proteins in tomato (Vishwanath et. al., 2011; Amini and Ehsanpour, 2005). While, our conclusions were drawn from tomato var. Nagina. It had no special resistant against CMV.

Moreover, our experiment was based on the application of safe chemicals under biotic stress. Protein levels were significantly increased in our treatments particularly BTH and BTH+SA when applied on weekly basis. Increased expression of proteins in BTH treated tomato plants under CMV infection has been previously documented that was accompanied with reduced vegetative characters (Kalogirou, 2012). Similarly it was observed that combined treatment of these elicitors also expressed higher level of proteins in tomato. Our conclusion is supported by the work of Thakur and Sohal (2014); investigated different concentrations of SA and BTH on physico-chemical parameters of Brassica juncea and B. napus up to four consecutive weeks. Total soluble protein increased among all the treatments particularly in combine application of SA and BTH. It was also suggested that the combinations of elicitors promoted growth and metabolic activities. Similar conclusion was drawn from the rice cultivars treated with foliar application of BTH and SA to combat sheath blight disease. It induced a large amount of defense related proteins and stress tolerance while applied in combination.

Another significant observation from the results is minimum amount of proteins in the host plants treated with SA on weekly basis. Excessive application of SA might alleviate or inhibit the stress response imposed by the mechanical injuries and CMV or both. Previous studies on tomato leaves artificially wounded and treated with SA and acetylsalicylic acid (ASA) have
shown to inhibit proteinase inhibitor synthesis (Doherty et al., 1988; Peda-Cortés et al., 1993). These chemicals were shown to be potent inhibitors of systemin-induced and jasmonic acid (JA)-induced synthesis of proteinase inhibitor mRNAs and proteins in tomato leaves. Such inhibitions are not restricted to wounding but SA also inhibits the Synthesis of Proteinase inhibitors induced by Systemin and Jasmonic Acid in Tomato Leaves (Steven et al., 1995).

**Conclusion:** Total proteins show significant alterations in the infected plants. However, chemical elicitors alter their quantitative production by interacting with defense pathways. Moreover, BTH show an altered response versus salicylic acid, though considered its structural analogue. Further investigations may help to find out the mechanisms involved in the BTH induced defense pathways and Molecular mechanism involved in vegetative stress imparted by it.

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