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EXOGENOUS APPLICATION 0F ELICITORS INDUCES BIOCHEMICAL ALTERATIONS AND DEVELOPS TOLERANCE AGAINST YELLOW MOSAIC DISEASE IN MUNGBEAN

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ABSTRACT

Mungbean (*Vigna radiata*) is seriously affected by mungbean yellow mosaic disease (MYMD) caused by *Mungbean yellow mosaic India virus* (MYMIV) in the Indian subcontinent. The present study was conducted to evaluate the effectiveness of salicylic acid (SA) and benzothiadiazole (BTH) for the management of MYMD and to find their role in inducing alteration in different biochemical parameters in susceptible mungbean genotype VC3061A. Exogenous application of the elicitors resulted in delayed symptom development and reduced disease severity (DS). The severity index (SI) was higher on positive control plants while the minimum was observed in post combined application of BTH+SA, followed by BTH and SA application. Total phenolic contents (TPC) and malondialdehyde (MDA) increased significantly in virus inoculated plants of all treatments as compared to their healthy controls. SOD activity was increased significantly in BTH+SA treated plants but decreased in disease control and BTH treated virus inoculated plants. A significant decrease in catalase (CAT) activity, while an increase in peroxidase (POD) was observed in BTH+SA, treated virus inoculated plants. Protease and esterase activity were significantly increased in SA treated virus inoculated plants. Plant pigments exhibited decreased concentration in virus inoculated plants compared to non-inoculated plants under all the treatments except SA treated plants. Enhanced or suppressed levels of antioxidants suggest an association between constitutive induced levels of these enzymes. In this study, we also report for the first time the protein profiling of mungbean genotype VC3061A after the exogenous application of different combinations of elicitors. Proteomic analyses revealed the expression of two proteins phosphatase 2C 16-like isoform and capsid protein after MYMIV inoculation in SA and BTH+SA treated plants which may trigger signal transduction pathway and consequently induces resistance against MYMIV in *V. radiata* by activating PR protein.

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INTRODUCTION

Mungbean (*Vigna radiata* (L.) Wilczek) is considered as conventional, economic and ancient pulse crop in the world. In Pakistan, it is mainly grown in Punjab province, accounting for 88% area and 85% of the total mungbean production in the country. Due to different biotic and abiotic stresses its yield is low in Asian countries (Akhtar *et al.*, 2009 and Karthikeyan *et al.*, 2014) and among the biotic stresses, mungbean yellow mosaic disease (MYMD) caused by *Mungbean yellow mosaic virus* (MYMV) inflict heavy yield losses in all mungbean cultivating countries of Asia. The virus is not

transmitted by seed, sap or soil. In the field, MYMD is disseminated by an insect vector, whitefly, *Bemisia tabaci* (Honda *et al.*, 1983 and Jones, 2003). This disease was first described by (Nariani, 1960) who named the putative causal agent as MYMV. Mungbean yellow mosaic virus produces great yield losses in mungbean cultivating countries of Asia, including India and Pakistan (Biswas *et al.*, 2008). This disease in different leguminous species is known to be caused by *Mungbean yellow mosaic virus* (MYMV), *Mungbean yellow mosaic India virus* (MYMIV), *Dolichosyellow mosaic virus* (DYMV) and *Horsegram yellow mosaic virus* (HYMV). However, in Pakistan, the causal agent of MYMD is MYMIV. It belongs to family *Geminiviridae* and genus *Begomovirus* (Ilyas *et al.*, 2010). MYMIV-infection is particularly problemetic in summer mungbean cultivation due to high population of its insect vector, whitefly (Akhtar *et al.*, 2011).

The infected plant exhibits symptoms like chlorosis, reduction in internodes, deformed pods with small, shrivelled seeds and acute growth reduction with no yield or few pods (Kitsanachandee *et al.*, 2013). The best way to reduce the losses due to Yellow mosaic diseases (YMD) caused by MYMIV is to develop resistant varieties. Due to pathogen's changing nature, already resistant cultivars become susceptible. However, the use of safe chemicals to control plant pathogens are gaining importance. Applications of elicitors lead to the induction of many defense related molecules that initiate signaling for the activation of defense related genes which is completed by systematically induced resistance that prevent plants from further infection (Tariq *et al.*, 2018). In this study, the effectiveness of different elicitors (SA and BTH) against MYMD and modifications in various biochemical molecules that occur during the invasion of MYMIV and after application of SA and BTH were investigated.

MATERIALS AND METHODS

Plant material and treatment with elicitors

Mungbean susceptible genotype VC3061A was sown in the pots under insect proof cages covered with a muslin cloth in the net-house. After one month of germination, test plants of same sizes were divided into 8 sets and each set was comprised of three replicates. The details of the experimental sets are given in table 1. Solutions of BTH @ 336 mg/L (Novartis) and SA @ 138 mg/L (Merck) were freshly prepared at the time of their applications. To maintain the experimental plants in good conditions, plant protection measures were adapted and twice the experiment was repeated. After 21 days post inoculation, leaf samples were collected from each test plant for the ELISA test and various biochemical analysis. At the end of the experiment, yield related parameters were recorded.

Table 1. Detail of experimental sets and treatments combination.

Experimental set	Treatment	Description
Set 1	Healthy control (HC)	Plants sprayed with distilled water only
Set 2	Diseased control (DC)	Plants inoculated with MYMIV
Set 3	BTH+SA	Spray with the mixture of BTH+SA
Set 4	BTH+SA+MYMIV	Spray with the mixture of BTH+SA and subsequently inoculated with virus after three days of inducers application
Set 5	BTH	Spray with BTH only
Set 6	BTH +MYMIV	Spray with BTH and subsequently inoculated with virus after three days of inducers application
Set 7	SA	Spray with SA only
Set 8	SA+MYMIV	Spray with SA and subsequently inoculated with virus after three days of inducers application

Whitefly transmission of virus

Adult whiteflies were traped from the MYMIV-infected field and released on 4-5 weeks old plants under set 2, 4, 6 and 8 for an inoculation feeding period of 72-h (Akhtar *et al.*, 2011). A total of nine pots (with three pots per replicate containing five plants per pot) per treatment were inoculated by using a set of 50 whiteflies per plant. All the exposed plants were then sprayed with insecticide after 72-h to kill the released whiteflies standard agronomic practices were adapted

to keep the plants in good conditions. Data on different parameters including the percentage of plants infected, latent period (LP) and DS 60 days post transmission using the rating system described by Akhtar et al. (2011) given in Table 2 were recorded. Severity index (SI) was calculated using the formula;

$$SI = \frac{\text{Sum of all disease ratings}}{\text{No. of infected plants observed}} \times 5$$

Table 2. Disease scale for rating of mungbean yellow mosaic disease (MYMD).

Severity ratings	Symptoms	Severity index	Disease response
0	Complete absence of symptoms	0	Highly resistant
1	Few small yellow specks or spots on few leaves seen after careful observations	0.01-1.4	Resistant
2	Bright yellow specks or spots common on leaves, but no or minor reduction in yield	1.5-2.4	Moderately resistant
3	Mostly coalesced bright yellow specks or spots common on leaves, but no or minor reduction in yield	2.5-3.4	Moderately susceptible
4	Plants showing coalesced bright yellow specks or spots on all leaves, with no or minor stunting and set fewer normal pods.	3.5-4.4	Susceptible
5	Yellowing or chlorosis of all leaves on the whole plant followed by necrosis, shortening of internode, and severe stunting of plants with no yield or few flowers and deformed pods produced with small, immature and shrivelled seeds.	4.5-5.0	Highly susceptible

Confirmation of Virus Using TAS-ELISA and PCR

After 21 days of inoculation, leaf samples were taken from non-inoculated as well as whitefly-inoculated plants of all the genotypes to check the presence or absence of virus and for the analysis of levels of various biochemical processes. The virus in the inoculated plants was confirmed by polymerase chain reaction (PCR) and Triple Antibody Sandwich-Enzyme Linked Immunosorbent Assay (TAS-ELISA) as described (Kothandaraman *et al.*, 2016). ELISA plates were read at 405nm using a Microplate reader (Platos R496, AMP Diagnostic, BSE Austria) and visually for yellow colour development. For the detection of MYMIV, polymerase chain reaction (PCR) was performed. For PCR, total genomic DNA was extracted from young symptomatic and asymptomatic mungbean plant leaves as described (Kothandaraman *et al.*, 2016). PCR was performed by using primer pairs FLDNAAF (TGTGGGATCCATTGTTGAACGACTTTCCC) and FLDNAAR (CAATGGATCCCACATTGTTAGTGGGTTCAG) for the amplification of DNA A of MYMIV. PCR reaction of 20 µL include 10 µL 2X topsimple DyeMIX-nTaq (enzynomics, Korea), 1 µL template DNA, 0.5 µL each of forward and reverse primers (5pmol/ul) and 8 µL sterile ddH_2O. The PCR was carried out for initial denaturation at 94 °C for 5 min, 33 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 2 min and extension at 72 °C for 3 min and a final extension at 72°C for 10 min. The PCR amplified products were analyzed on 1% agarose gel.

Total phenolic contents (TPC)

TPC under different treatments was estimated among virus-inoculated and non-inoculated plants as described by (Ainsworth and Gillespie, 2007). For extraction, 100 mg of leaf sample was ground in 85% ice-cold methanol, incubated at room temperature for 48 hours and then centrifuged at 14,000 rpm for 20 minutes. The supernatant (100 µl) was then mixed with Folin–Ciocalteu (F-C) reagent [100 µl of 10 % (v/v)], vortexed and 800 µl of Na_2CO_3 (700 mM) was added. To determine TPC, different concentration of gallic acid was used to prepare a standard and then a linear regression equation was recorded.

Total soluble proteins

Total soluble protein (TSP) contents were measured using Bradford's method (Bradford, 1976). For TSP analysis, 0.5g of samples was crushed in the presence of 1.5 ml Phosphate buffer and centrifuged at 10000 g. To 5µl of this extract (supernatant) 1ml of Bradford reagent and 95 µl 0.1N NaCl was added and absorbance was taken at 595nm.

Enzyme assays

Malondialdehyde is produced through lipid peroxidation and it is measured by the levels of oxidants in cell (Zhang and Kirkham, 1994). Leaf sample (0.25g) was homogenized in 5 ml 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 g for 5 min. Then 1 ml of supernatant was mixed with 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) and was then heated at 95^{o}C for 30 min. After centrifugation for 10 min at 10,000 g, the supernatant absorbance at 532 nm was recorded and nonspecific absorption value at 600 nm was subtracted. An extinction coefficient of 155 mM^{-1} cm^{-1} was used to record MDA content. Superoxide dismutase (SOD) activity was estimated as described by (Dixit *et al.*, 2001). One unit of SOD activity was defined as the amount of enzyme which caused 50% inhibition of photochemical reduction of nitroblue tetrazolium (NBT) (Giannopolitis and Ries, 1977). For catalase (CAT) analysis, leaf samples were crushed in extraction buffer as described by Beers and Sizer (Beers and Sizer, 1952). The decrease in absorbance of the reaction solution at 240 nm was recorded every 30 seconds. An absorbance change of 0.01 unit min^{-1} was defined as one unit CAT activity. To estimate protease activity, casein digestion assay was used (Drapeau, 1976) while, esterase activity was studied as described by (Van Asperen, 1962). Leaf samples were triturated in potassium phosphate buffer (50 mM; pH 7.8) and then α–naphthyl acetate and β–naphthyl acetate were used as substrates to quantify the esterases (α and β). Peroxidase (POD) activity was measured using the method of Chance and Maehly (Chance and Maehly, 1955) while Ascorbate peroxidase (APX) activity was measured as described by Nakano and Asada (Nakano and Asada, 1981).

Total oxidant status (TOS)

Erel TOS method (Harma *et al.,* 2005) was used to record TOS. This method is based on ferrous ion oxidation to ferric ion in acidic medium and then ferric ion measurement by xylenol orange (Harma *et al.*, 2005).

Photosynthetic pigment content

The chlorophylls (*Chl a*, *b*) and carotenoids (x + c) were extracted in 80% (v/v) aqueous acetone and vacuum filtered through a Whatman No. 1 filter paper. Pigment measurements were quantified spectrophotometrically, and absorbance was determined at wavelengths of 663, 645, 505, 470 and 453 nm, respectively. Concentrations (mg g^{-1} f. wt.) of pigments were calculated by equations of (Lichtenthaler and Wellburn, 1983).

Protein profiling

For protein expression analysis, SDS-PAGE of the leaf samples was performed by using the method of (Laemmli, 1970) and (Hameed *et al.*, 2012). Soluble proteins from leaves were extracted by grinding the leaves in extraction buffer and then centrifuged at 14,000 rpm for 10 min. The obtained supernatant of each sample was mixed with the cracking solution (10 ml consisting of 0.01g bromophenol blue, 1g SDS, 2ml Marcaptoethanol, 5g sucrose, 1.5ml of 0.5M tris and 6.5 ml water) in the ratio 4:1. The mixture was vortexed and heated for 5 min in boiling water in a water bath to denature the proteins. Then equal quantities of samples along with the protein molecular weight marker were loaded in the 10% gels. After electrophoresis, gel was fixed in a solution (40% ethanol and 10% acetic acid) with constant agitation for 15 minutes. After fixing, the gel was washed with distilled water, stained with Coomassie brilliant blue dye G-250 and then de-stained in distilled water overnight. The gel was then photographed by UVI pro Platinum gel documentation system (UNI tech UK). Computerized gel analysis was performed using UVI pro Platinum 1.1 Version 12.9 for windows (copyright® 2004-2006).

Statistical analysis

All the collected data for each parameter was subjected to two way ANOVA (Analysis of Variance) using XL-STAT 2012 while, Tukey's HSD test (α=0.05) was used to compare mean for significance. The values are mean of three replicates ± standard error (SE) wherever applicable.

RESULTS

Disease response

MYMIV was successfully transmitted in all the inoculated mungbean plants of different treatments, but delayed symptom development and low DS was observed in treated plants over DC (Table 3). Higher DS was recorded in virus-inoculated DC plants and was rated as susceptible. Plants treated with BTH, SA and combined application of BTH+SA followed by virus inoculation showed less disease than DC and behaved as moderately resistant and resistant, respectively (Table-3). Minimum DS was noticed post application of BTH+SA (2.22) followed by BTH spray (2.63), SA (2.63) and virus DC (3.83) plants with a

latent period of 13, 11, 11 and 7 days, respectively (Table 3).

Disease symptoms were first started as small chlorotic spots on virus-inoculated DC plants after 7 days of inoculation and after 20 days, these plants showed severe disease symptoms. However, in the case of BTH, SA and BTH+SA treated plants disease symptoms were initiated after 11, 11 and 13 days as small spots. After 20 days, BTH and SA treated plants expressed little more symptoms (light yellow spots on leaves) as compared to the combined spray of BTH+SA. No increase in DS was observed in these plants till the end of experiments (i.e. 45 days post inoculation).

Table 3. Response of mungbean susceptible genotype (VC3061A) against MYMD through whitefly inoculation under different treatments.

Elicitors treatment	Latent Period (Days)*	TAS ELISA absorbance values (A_{405nm}) after 1h	Disease Severity Index*	Disease response
SA+BTH	13^{b}	0.823^{c}	2.22^{b}	Resistant
BTH	11^{ab}	1.077^{bc}	2.63^{b}	Moderately resistant
SA	11^{ab}	1.307^{ab}	2.63^{b}	Moderately resistant
Disease control (DC)	7^{a}	1.757^{a}	3.83^{a}	Susceptible
Healthy control (HC)	-	0.357^{d}	-	-

*Values sharing similar letters do not differ significantly at P≤ 0.05.

The virus was detected in all the inoculated plants using ELISA. Dark yellow colour developed in DC plants while in the inducers treated plants comparatively light yellow colour was observed. The virus titer value was 0.823, 1.077, 1.307, 1.757 and 0.357 after BTH+SA, BTH, SA, DC and HC plants, respectively (Table 3). MYMIV presence was confirmed by using genus-specific primers in PCR, which amplified approximately 2.75 kb product (DNA-A of MYMIV) from virus inoculated plant samples but no amplification was found from non-inoculated plants.

Total phenolic content

The amount of TPC in virus-inoculated and non-inoculated plants differ with each other under all treatments (Figure 1A). There were non-significant differences for TPC among non-inoculated plants. However, after inoculation with the virus TPCs were increased significantly in all the treatments over their un-inoculated treated controls. The maximum amount of TPC was recorded in SA-treated virus-inoculated plants, followed by BTH-treated virus-inoculated plants. Lowest TPC was observed in the untreated virus inoculated plants.

Total soluble protein

There were no significant differences for TSP between MYMIV-inoculated and non-inoculated plants of susceptible mungbean genotype VC3061A while treated with different chemical elicitors (Figure 1B).

Enzyme assay

Malondialdehyde contents were found increased significantly in all the virus inoculated plants over their un-inoculated plants under all treatments (Figure 1C). However, Maximum MDA contents were observed in un-treated virus inoculated plants followed by BTH-treated virus-inoculated plants and BTH+SA-treated virus-inoculated plants. MDA content was lowest in SA-treated virus-inoculated plants. CAT activity was found higher in combined treatment of non-inoculated plants while among inoculated plants its activity significantly differed with each other (Figure 1D).

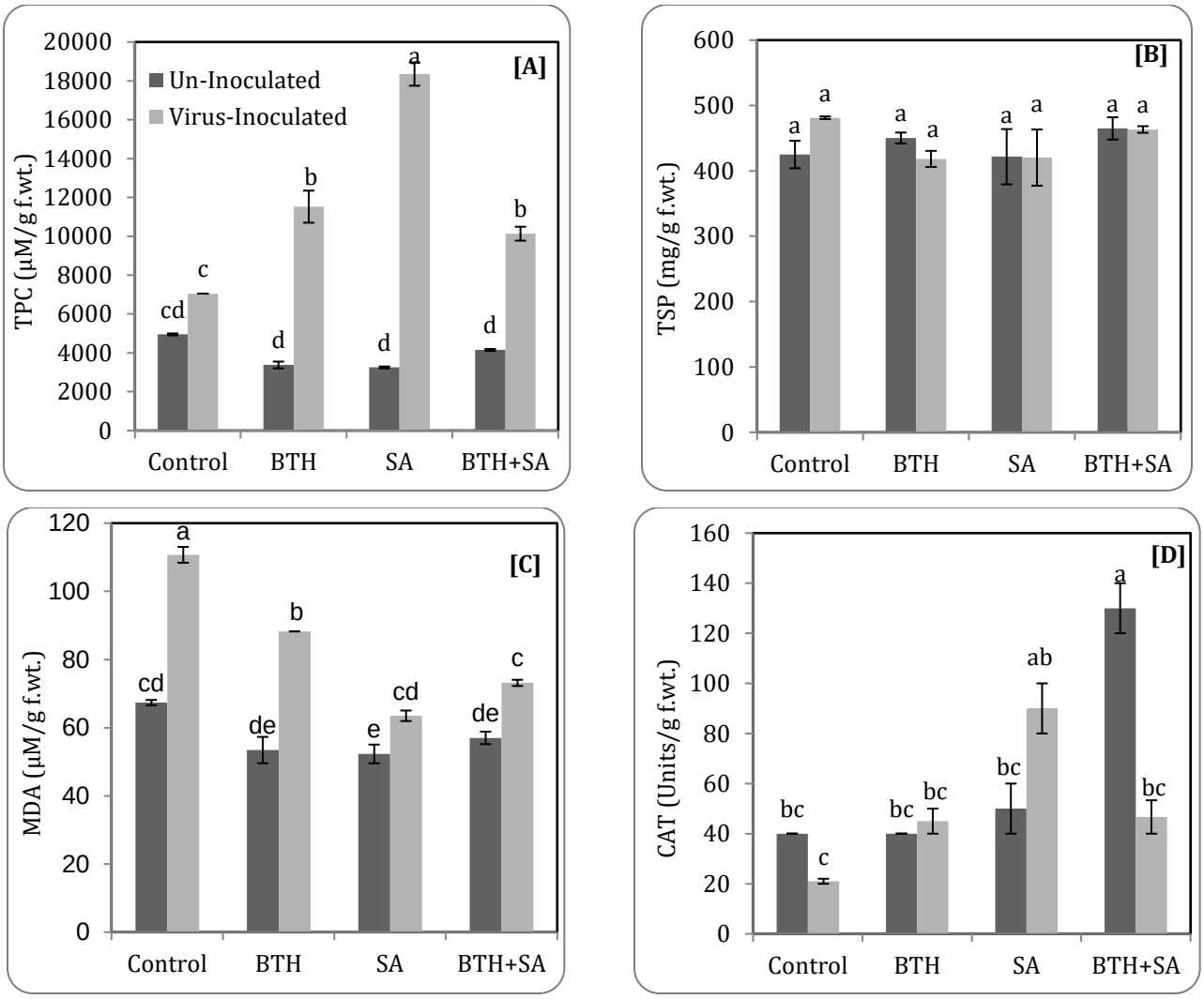

Figure 1A-D. [A] TPC, [B] TSP, [C] MDA, [D] CAT activity of non-inoculated and MYMIV-inoculated plants of susceptible mungbean genotype under different treatments of elicitors.

After virus inoculation, maximum CAT activity was observed in SA treated plants while minimum in DC plants. Results of SOD activity showed significant differences in all the treatments (Figure 1E). Maximum increase after virus-inoculation about three folds over their un-inoculated treatment was observed in case of BTH+SA treatment. It was interesting to note that SOD activity decreased more than four times in untreated virus inoculated as compared to their untreated un-inoculated plants. However, SOD activity was decreased significantly in DC and BTH treated plants but increased significantly in BTH+SA treated plants over their un-inoculated treatments. Non-significant change in the protease activity was observed for all the treatments except for SA-treatments (Figure 1F). Protease activity was significantly increased in SA-treated virus-inoculated plants over their SA-treated but un-inoculated plants. Similarly, Esterase activity was non-significant in all the virus-inoculated and non-inoculated plants under all treatments except for SA-treated virus-inoculated plants in which it was much higher compared to other treatments (Figure 1G). In case of POD, only in virus inoculated un-treated and BTH+SA-treated virus-inoculated plants significant increase was observed while in other treatment the effect was non-significant (Figure 1H). APX results showed its increased activity in all virus-inoculated treated plants as compared to their non-inoculated plants (Figure 1I). However, this increase was significant only in case of un-treated virus-inoculated plants.

Total oxidant status

There were no significant differences in TOS level in the virus-inoculated and non-inoculated treated plants

except in un-treated virus-inoculated plants (Figure 1J). The level of TOS was found to be increased significantly in un-treated virus-inoculated plants compared to all other treatments.

Photosynthetic pigment content

Photosynthetic pigments were significantly higher for all the un-inoculated plants as compared to the virus inoculated plants. After virus inoculation, *Chl a, b*, total chlorophyll and carotenoid pigments significantly decreased under all treatments over their non-inoculated treatments except for SA treatment in which they remained unchanged (Figure 2A to D). The maximum amount of *Chl a* content were recorded in un-treated un-inoculated plants, while minimum was recorded for un-treated virus-inoculated plants (Figure 2A). However, amount of *Chl b* contents was found similar in BTH, SA and BTH+SA treatments after infection with virus (Figure 2B). The maximum amount of carotenoid pigments was recorded in healthy control plants and BTH treated un-inoculated plants while minimum was recorded for un-treated virus-inoculated plants (Figure 2D). The amount of carotenoid pigments was recorded as statistically similar among all virus-inoculated treatments.

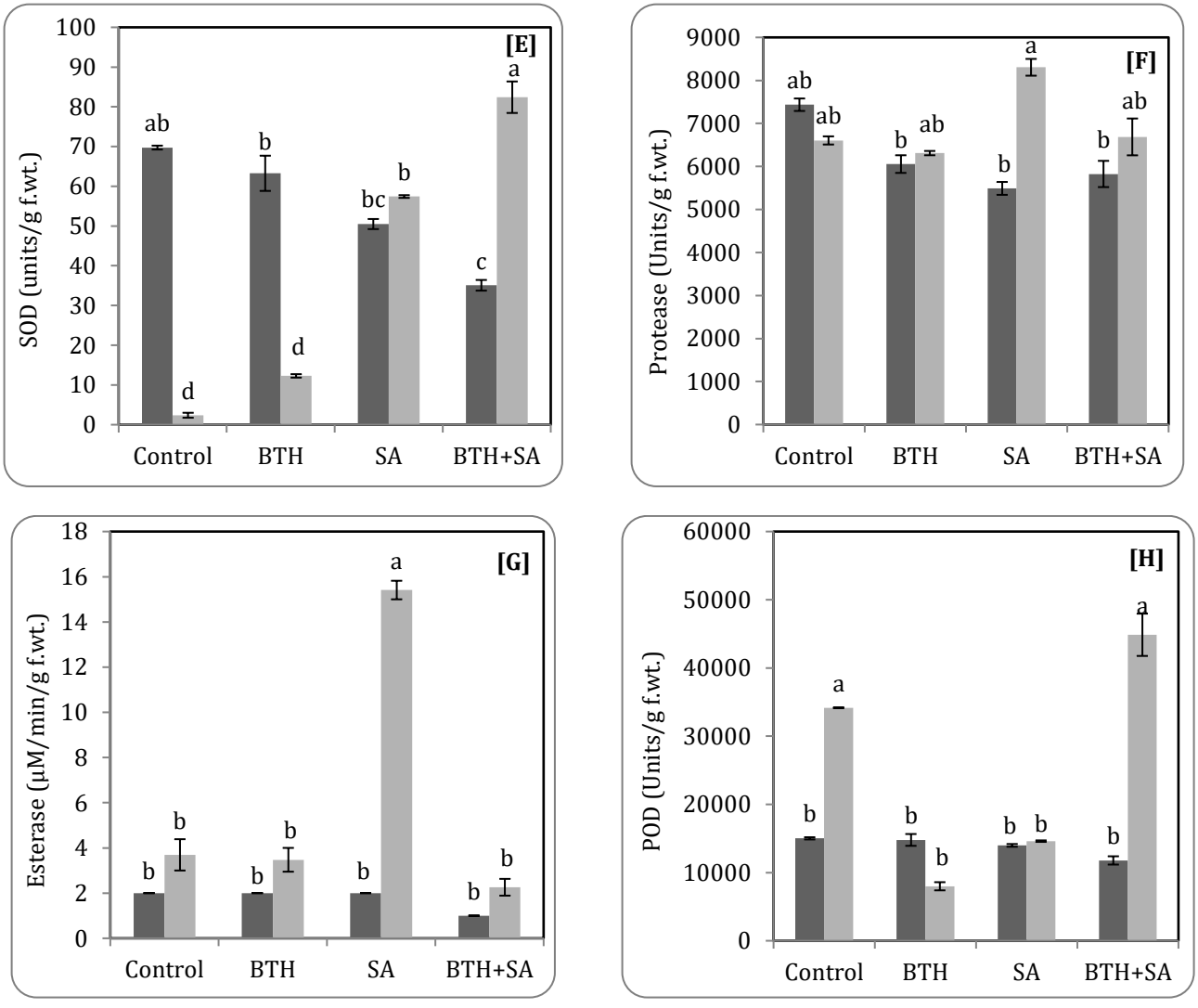

Figure 1 E-H. [E] SOD, [F] Protease, [G] Esterase and [H] POD activity of non-inoculated and MYMIV-inoculated plants of susceptible mungbean genotype under different treatments of elicitors.

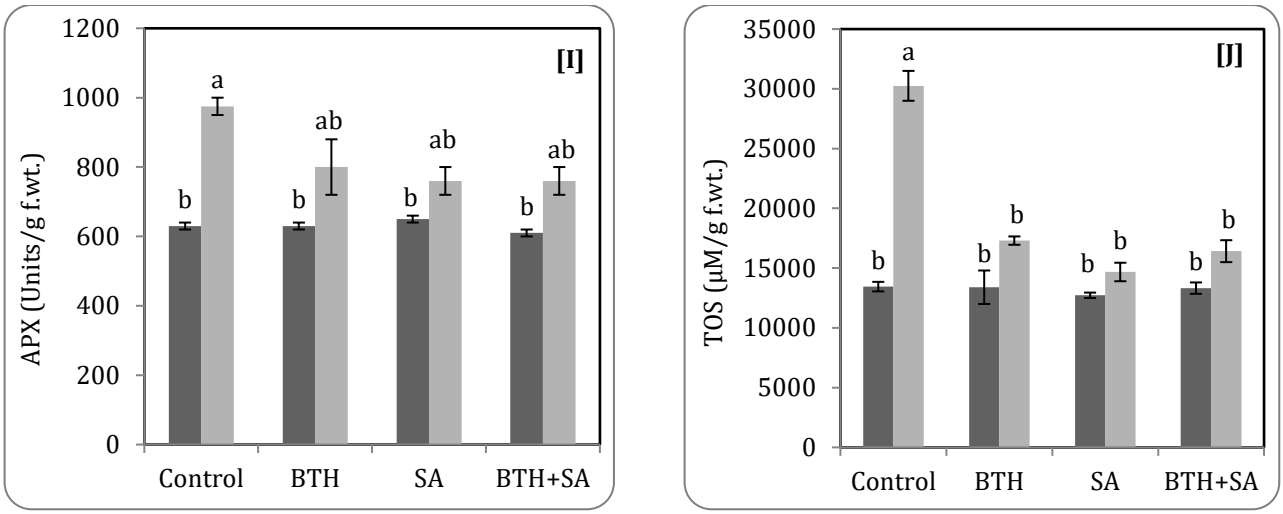

Figure 1 I-J. [I] APX and [J] TOS activity of non-inoculated and MYMIV-inoculated plants of susceptible mungbean genotype under different treatments of elicitors.

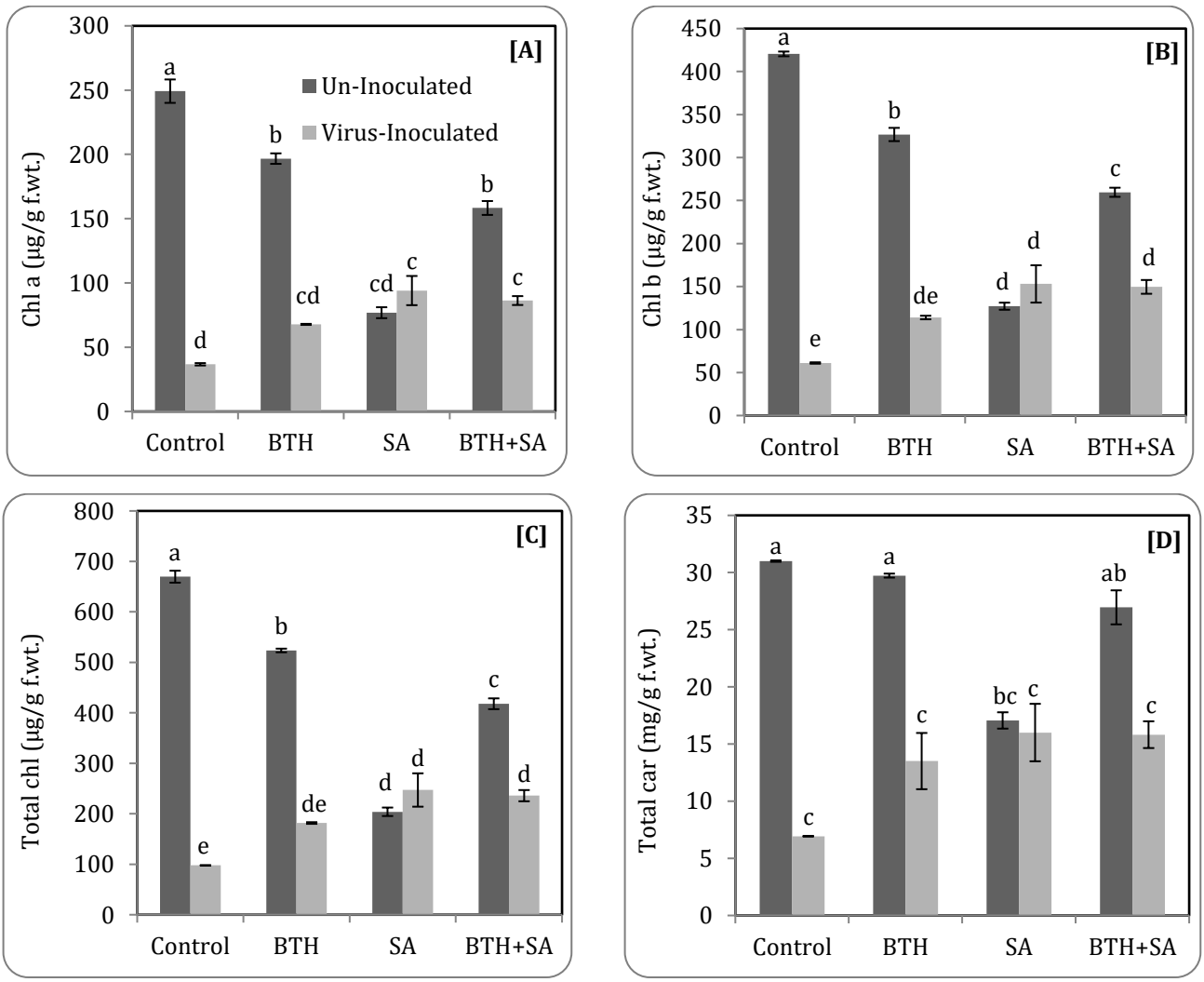

Figure 2A-D. [A] *Chl a*, [B] *Chl b*, [C] Total chlorophylls, [D] Lycopene contents of non-inoculated and MYMIV-inoculated plants of susceptible mungbean genotype under different treatments of elicitors.

Lycopene

Amount of lycopene contents in virus-inoculated and non-inoculated plants were significantly different except for SA treatment. Lycopene contents were significantly higher in all the un-inoculated plants as compared to the virus-inoculated plants under all treatments except for SA treatment (Figure 2E).

After MYMIV inoculation, lycopene contents were significantly decreased in all the treatments over their non-inoculated treatments except for SA treatment in which they remained unchanged. However, the amount of lycopene contents was significantly higher for all elicitor treated virus inoculated plants as compared to untreated virus-inoculated plants.

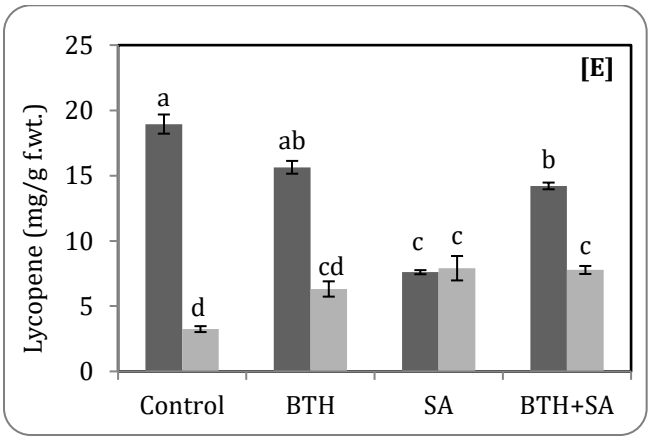

Figure 2E. Total lycopene contents of non-inoculated and MYMIV-inoculated plants of susceptible mungbean genotype under different treatments of elicitors. Different letters on the top of bars indicate significant differences between genotypes at $P \leq 0.05$.

Protein profiling

Leaf protein profiling of mungbean genotype (VC3061A) of virus inoculated and un-inoculated plants in different treatments were performed by SDS-PAGE. Peptides that were resolved on 10% gel ranged from 17kDa to 134kDa with 22 noticeable peptides (Table 4, Figure 3, 4). A 17.662kDa representing cytokinin specific binding protein was only observed in DC plants; two peptides of 18.9 and 28.9kDa representing protein phosphatase 2C 16-like isoform and a capsid protein were only detected in virus inoculated SA and BTH+SA treated plants. A peptide of 19.1kDa representing NAD (P) H-quinone oxidoreductase subunit I, the chloroplastic protein was observed in both healthy and virus inoculated SA treated plants and in BTH+SA treated healthy plants.

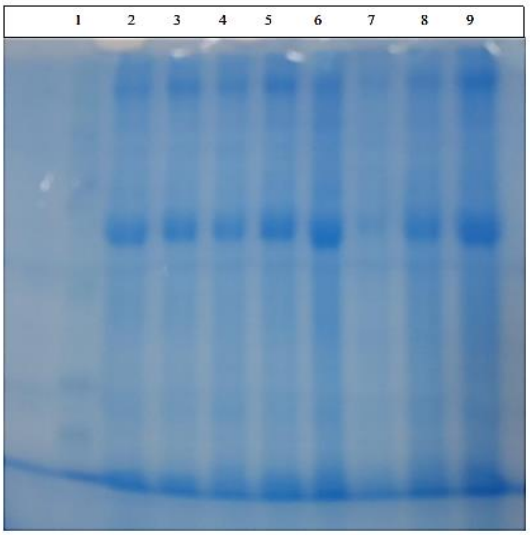

Figure 3. Protein profiling using SDS-PAGE: A representative diagram showing the leaf proteins of mungbean genotype treated with elicitors. Gel sequence from Left to right Lane 1: Protein molecular weight marker, Lane 2: Healthy control, Lane 3: BTH, Lane 4: SA, Lane 5: SA+BTH, Lane 6: Disease control, Lane 7: BTH + V, Lane 8: SA + V, Lane 9: SA+BTH + V.

Similarly, 29.3kDa likely ribose-5-phosphate isomerase 3 chloroplastic and 31.3kDa representing carboxypeptidase were observed in HC, BTH and BTH+SA treated plants. Two peptides of 30.6kDa and 61.7kDa representing ribulose-phosphate 3-epimerase and Maturase K, respectively, were reported in virus inoculated plants under all treatments and BTH treated healthy plants only. Four peptides of 23.7, 32.6, 33.5 and 47.1kDa representing 30S ribosomal protein S4 chloroplastic, 30S ribosomal protein S5 (*Arabidopsis thaliana*), chloroplast drought-induced stress protein and rubisco activase chloroplast precursor (*V. radiata*), respectively, were reported under all treatments except for virus inoculated BTH treated plants.

RuBisCO large subunit-binding protein subunit alpha (61.6kDa) was spotted in untreated and BTH treated HC plants. Nine peptides of 27.1, 35.1, 40.6, 43.9, 45.7, 72.1, 79.1, 82.3 and 134.5kDa representing cytosolic ascorbate peroxidase(*V. unguiculata*), photosystem II oxygen-evolving complex protein 1 precursor (*Solanum lycopersicum*), replication-associated protein, **o**mega-3 fatty acid desaturase endoplasmic reticulum, NAD (P) H-quinone oxidoreductase subunit H chloroplastic, **a**cid beta-fructofuranosidase, DNA-directed RNA polymerase subunit, photosystem I P700 chlorophyll an apoprotein A2 and 134kDa protein respectively, were spotted in all the treatments.

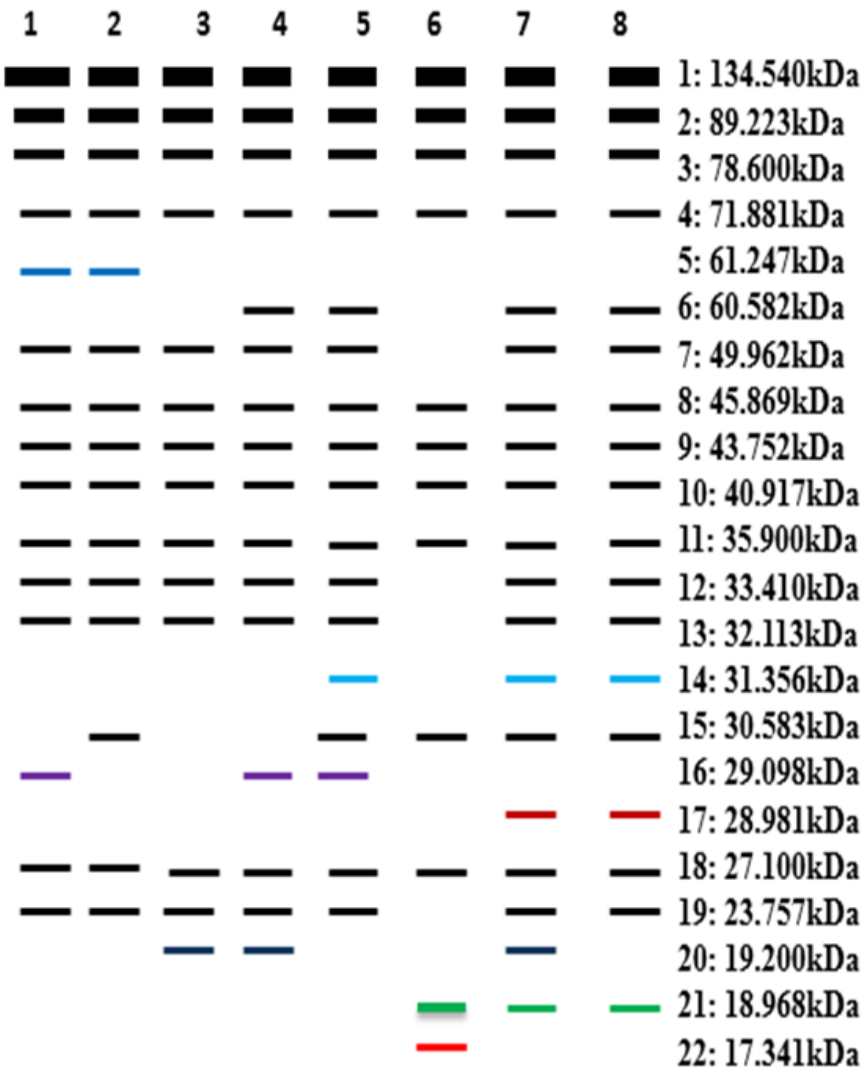

Figure 4. Histogram based on Electrophoretic data of non-inoculated and MYMIV-inoculated plants of susceptible mungbean genotype under different treatments of elicitors. Gel sequence from Left to right Lane 1: Healthy control, Lane 2: BTH, Lane 3: SA, Lane 4: SA+BTH, Lane 5: Virus inoculated control, Lane 6: BTH + V, Lane 7: SA + V, Lane 8: SA+BTH + V.

Table 4. Possible protein families searched in different databases having similar mol. Wt. as detected in the present study.

Protein	Function	Molecular Wt. (kDa)	Accession #
Cytokinin specific binding protein	A cytosolic protein that specifically binds with the hormones from cytokinin group, Cis-zeatin 1 by carbonyl oxygen	17.622	Q9ZWP8_VIGRA
Protein phosphatase 2C 16-like isoform	Key players in signal transduction pathways and their substrate is serine/threonine.	18.613	A0A0F7RNS9_VIGRA
NAD(P) H-quinone oxidoreductase subunitI,chloroplastic	Play role in photosynthesis and respiration by transferring electron form NAD(H) to quinones	19.056	A0A0F7RNS9_VIGRA
30S ribosomal protein S4, chloroplastic	rRNA binding protein, play role in translational accuracy	23.757	D3J8F3_VIGRA
Cytosolicascorbate peroxidase(*Vigna unguiculata*)	This protein respond to oxidative stress, and have peroxidase activity.	27.100	Q41712_VIGUN

Capsid protein	Enclosed viral ssDNA into geminate particle and transport it into and out of the cell nucleus and not required for systemic infection	28.981	Q534Y2_MYMVV
Probable ribose-5-phosphate isomerase 3, chloroplastic	It converts ribose-5-phosphate to ribulose 5-phosphate.	29.306	Q9S726
Ribulose-phosphate 3-epimerase	Energy/pentose phosphate	30.632	
Carboxypeptidase	Serine-type carboxypeptidase activity	31.356	Q41689_VIGRA
30Sribosomal protein S5[*Arabidopsis thaliana*]	Protein synthesis	32.645	RR5_ARATH
Chloroplast drought-induced stress protein	Respond to oxidative stress, cell redox homeostasis.	33.505	F2VQI2_VIGRA
Photosystem II oxygen-evolving complex protein 1 precursor [*Solanum lycopersicum*]	Play key role in photosynthesis	35.15	T06368
Replication-associated protein	It is necassaryl for the replication of viral ssDNA. The closed circular ssDNA genome is first transformed into a superhelical dsDNA. Rep binds a particular region at the genome origin of replication.	40.677	REP_MYMVV
Omega-3 fatty acid desaturase, endoplasmic reticulum	Biosynthesis of the polyunsaturated fatty acids, part of lipid metabolism.	43.996	FAD3E_VIGRA
NAD (P) H-quinone oxidoreductase subunit H, chloroplastic	NDH tranfers electrons from NAD (P)H:plastoquinone, via FMN and iron-sulfur (Fe-S) centers, to quinones in the photosynthetic chain and possibly in a chloroplast respiratory chain.	45.752	A0A0F7RPH9_VIGRR
Rubisco activase, chloroplast precursor [*Vigna radiate*]	Activation of RuBisCO (ribulose-1,5 bisphosphate carboxylase/oxygenase; involves the ATP-dependent carboxylation of the epsilon-amino group of lysine leading to a carbamate structure.	47.092	RCA_VIGRR
Maturase K	Probably assists in splicing its own and other chloroplast group II introns.	61.719	Q1PG41_VIGRA
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	This protein binds RuBisCO small and large subunits and is implicated in the assembly of the enzyme oligomer. ATP binding	61.606	RUB2_BRANA
Acid beta-fructofuranosidase	This protein is involved in sucrose metabolism, a part of Glycan biosynthesis.	72.167	P29001-1
DNA-directed RNA polymerase subunit	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates.	79.081	**(D3J8G4_VIGRA)**

Photosystem I P700 chlorophyll a apoprotein A2	PsaA and PsaB bind P700, the primary electron donor of photosystem I (PSI), as well as the electron acceptors A0, A1 and FX. Oxidized P700 is reduced on the lumenal side of the thylakoid membrane by plastocyanin.	82.397	**D3J8F6_VIGRA**
134kDa Protein	ATP binding, hydrolase activity, RNA binding	134.54	**D0EWY7_9VIRU**

DISCUSSION

The purpose of the present study was to examine the effectiveness of different elicitors (SA and BTH) against MYMD and to find out various biochemical changes those occur during infection and after the exogenous application of chemical elicitors. In this study, it was observed that MYMIV caused severe symptoms in mungbean susceptible genotype VC3061A. However, exogenous treatment of this genotype with different elicitors (BTH, SA and BTH+SA) not only delayed symptom expression but also reduced DS leading the plant response from susceptible to resistant phase. Moreover, the combined application of SA and BTH prevented the mungbean against unbearable damage of MYMD that occurred in case of untreated plants. However, few studies were reported to manage MYMD in V. mungo (black gram; urdbean) using an exogenous application of SA (Kundu *et al.*, 2011). However, in this study we have evaluated single as well as the combined application of SA and BTH for the management of YMD in mungbean. Combined application of BTH+SA was found effective in reducing YMD severity as previously reported by Farooq et al. (2018). The feasibility of exogenous elicitors (BTH, SA and BTH+SA) treatment as a complementary strategy for MYMD management was thus confirmed under the present study. Virus infections in plants cause many alterations in physiology, biochemistry and in metabolic processes (Fraser, 1987). These changes in plants appeared as disease symptoms during viral infection (Tecsi *et al.*, 1996). Many defense policies are redesigned by plants to cope with microbial stresses (Kundu *et al.*, 2011). Indeed, the production of secondary metabolites modifies carbon growth function but also affect manufacturing of energy input and storage (Hammerschmidt, 1999). Plants synthesize phenolic compounds for natural coloring, growth, reproduction and as a resistance towards many pathogens (Lattanzio *et al.*, 2006). Phenols are highly active group of molecules with anti-microbial activity that accumulate in plants after infection of pathogen (Nasir *et al.*, 2017). Based on our results, the amount of TPC in virus-inoculated plants increased significantly in all treatments over their un-inoculated treatments and maximum increase was recorded in SA-treated virus-inoculated plants. This increase in phenolic compounds in virus-inoculated plants and particularly maximum increase in SA-treated virus-inoculated plants indicated that phenolic compounds were activated in these plants upon MYMIV infection playing a significant role in plant defense mechanism. TSPs are reported playing a key role in plant defense mechanisms and these bio-molecules functions as pathogen recognition proteins (PR proteins), cellular signaling and defense signal amplification (Souza *et al.*, 2017). There were no significant differences for total proteins present between virus-inoculated and un-inoculated plants of susceptible mungbean genotype VC3061A treated with different chemical elicitors. For no change in total protein concentration in case of disease it has been assumed that pathogen infection reduced the synthesis of ribulose-1, 5-bisphosphate (RuBP), the major leaf protein (Bertamini *et al.*, 2003). MDA act as a marker of lipid peroxidation and under stressful circumstances, the level of lipid peroxidation has been widely used as an indicator of ROS mediated deterioration of cell membranes. Lipid peroxidation in diseased plants indicates the damage caused by pathogen infection and the development of severe symptoms. This fact is proved under present study as an increase in MDA contents was more in DC showing severe symptoms as compared to other treatments that showed relatively less disease. (Durner and Klessig, 1995) suggested that SA inhibits CAT and the resulting H_2O_2 burst is part of the pathogenesis signal transduction chain. We found that CAT activity significantly decreased in BTH+SA treated plants after virus-inoculation as compared to their un-treated non-inoculated control plants. These results revealed that low CAT activity led to a temporary increase in H_2O_2 levels. High levels of H_2O_2 showed direct involvement in ROS causing pathogen destruction

at infection site which is completed by induction of resistance by the expression of defense genes in plant cells (Barna *et al.*, 2012). Similarly, it is interesting to note that SOD activity decreased more than four times in untreated virus inoculated plants and increased about three folds in case of BTH+SA treated plants as compared to their un-inoculated plants. Many studies revealed that up-regulation of SOD decreased the CAT level which causes the increment in H_2O_2 level in plants leading to defense mechanisms activation (Kundu *et al.*, 2013). Proteases play key roles in plants, maintaining protein quality control and degradation of particular proteins in response to biotic and abiotic stimuli (Chini *et al.*, 2007). Present results showed that there was no significant change in the protease activity for all treatments except for SA treatments. From this it is assumed that there may be chances that proteases are more sensitive to virus infection (Kazemi *et al.*, 2010). We also recorded that esterase activity in SA-treated virus-inoculated plants was much higher as compared to other treatments which shows the role of SA in induction of resistance against MYMIV. PODs are antioxidants that are involved in plant defense mechanism against pathogen attack by detoxifying the harmful reactive oxygen species. In present study increase in POD activity in DC and BTH+SA treated virus-inoculated plants were recorded. In response to pathogen infection, POD is induced, and its activity was higher in resistant than the susceptible plants (Mydlarz and Harvell, 2006). Similarly, increased activity of POD was observed in several plant pathosystems (Dieng *et al.*, 2011 and Siddique *et al.*, 2015). APX also plays key role in the detoxification of the reactive oxygen species (Asada, 1992). In our study, the increase in APX activity was recorded in all the virus-inoculated plants as compared to their non-inoculated plants. But, the increase was significant only in case of un-treated virus-inoculated control plants. However, the low level of CAT and APX activities are needed to maintain a higher level of hydrogen peroxide. As previously reported that reduction in CAT and APX activities lead to the increased H_2O_2 level by blocking the two H_2O_2 degrading pathways, thus enhancing plant resistance (Durner and Klessig, 1995). In our study, the reduction was observed in CAT activities in MYMIV inoculated mungbean plants but the APX level increased in inoculated plants treated with BTH+SA which contradict with earlier findings. However, the APX level increased non-significantly compared to control. Similarly, Farooq *et al.* (2018) observed an increase in CAT, POD and SOD activities in MYMIV inoculated SA & BTH treated plants than in the infected control. We also observed the level of TOS in virus-inoculated and non-inoculated plants among all treatments was statistically at par except for un-treated virus-inoculated plants. When plants are exposed to any kind of stress biotic or abiotic, reactive oxygen species are produced above their normal level to fend off stresses. These results suggest that elicitors play a role in plant defense by inducing higher oxidants to cease pathogen spread. Photosynthesis is one of the main physiological processes important for plant growth (Arfan *et al.*, 2007) and is badly affected by viral infection (Radwan *et al.*, 2007). In response to MYMIV infection, we found that the contents of *Chl a*, *b*, total chlorophyll and carotenoids decreased upon MYMIV infection. Reduction in photosynthetic pigments was also indicated by the yellowish appearance of the infected plant leaves. *Chl b* molecules are found more sensitive to virus infection (Nasir *et al.*, 2017). This decrease in *Chl b* was noticeable in virus inoculated plants because of the inability of susceptible genotype to up-regulate the synthesis of *Chl b* molecules (Siddique *et al.*, 2015). According to Zechmann *et al.* (2003) viruses decrease the photosynthetic rate in infected leaves through inhibition of photosystem II activity and decrease in chlorophyll content. Interestingly, in our study these pigments content remains unchanged in SA-treated and MYMIV inoculated plants. SA act as inducer of defense signaling and is known to indirectly protect chlorophyll molecules by improving carotenoid molecules (Radwan *et al.*, 2007). Proteins act at the front end of nearly all biological processes. Therefore, detection and quantification of protein expression becomes very important for functional analysis of proteins. Proteomic analysis in our study revealed an early accumulation of the defense/stress related proteins and pathogenesis related proteins during host-pathogen interaction. Beside other proteins observed in present study the two proteins namely; protein phosphatase 2C 16-like isoform and a capsid protein that enclosed viral ssDNA into geminate particle and transport it into and out of the cell nucleus but not required for systemic infection respectively, were only observed in virus inoculated SA and BTH+SA treated plants. These treatments also delayed symptom expression and significantly reduced DS and virus

inoculum over DC showing the induction of resistance under these conditions. Function of protein phosphatase 2C 16-like isoform is to bind metal ions and has a protein serine/threonine phosphatase activity. Its expression in susceptible genotypes after the exogenous application of SA and BTH+SA treated plants inoculated with virus may trigger signal transduction pathway and consequently induces resistance response against MYMIV by activating PR proteins. Enhanced resistance due to this protein has also been reported earlier in transgenic tobacco plants against *tobacco mosaic virus* (Hu *et al.*, 2009) and in mashbean against MYMIV (Kundu *et al.*, 2013).

CONCLUSION

Based on our study findings, the combined application of BTH and SA was found more effective to induce resistance in mungbean against MYMIV with reduced DS. The exogenous application of elicitors and MYMIV infection alters the biochemical parameters of tested genotypes which probably play an active role in resistance against MYMIV. However, significant increase in TPC, CAT, protease and esterase in SA treated plants while the increase in TPC, MDA, and POD in BTH+SA treated plants after MYMIV infection suggests their correlation between constitutive induced levels of these enzymes and plant resistance could be considered as biochemical markers for studying plant-virus interactions. Furthermore, increase in SOD activity while reduction in CAT level at same time in BTH+SA treated plants after MYMIV inoculation reveals a subsequent increase in H_2O_2 level which starts signaling in plants for the induction of defense mechanisms against pathogen infection. In this study, we have also reported for the first time the protein profiling after the exogenous application of different combinations of elicitors during MYMIV and *V. radiata* interaction. The expression of proteins namely phosphatase 2C 16-like isoform and capsid protein after MYMIV infection in elicitors (SA and BTH+SA) treated plants may consequently induces resistance by activating PR proteins. Future prospects include the application of safe chemicals at different doses using more mungbean genotypes under open field conditions.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

AUTHORS CONTRIBUTIONS

Performed the experiments: S. Tariq. Conceived and designed the experiments: K.P. Akhtar. Wrote the paper: S. Tariq, K.P. Akhtar, N. Ullah. Analyzed data: S. Tariq, K.P. Akhtar, A. Hameed, N. Ullah, I. Amin. Contributed reagents/materials: G. Abbas, M.J. Asghar.