Uluslararası Tarım Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Agricultural Research 2020, Vol. 4 (1), 99-118
https://doi.org/10.29329/ijiaar.2020.238.11

Original article

Biochemical Alterations in the Leaves of Resistant and Susceptible Mungbean Genotypes Infected with *Mungbean Yellow Mosaic India Virus*

Areeha Suhail [a, b, *], Khalid Pervaiz Akhtar [b], Amjad Hameed [b], Najeeb Ullah [b], Imran Amin [c] & Ghulam Abbas [b]

[a] Pakistan Institute of Engineering and Applied Sciences, Nilore, Islamabad, Pakistan
[b] Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan
[c] National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan

Abstract

Mungbean (*Vigna radiata*), an important leguminous crop, is highly susceptible to yellow mosaic disease (YMD) caused by Mungbean yellow mosaic India virus (MYMIV), resulting in high yield penalty. Previously, different varieties of mungbean have been reported possessing resistance/ tolerance against MYMIV. However, the molecular events occurring during compatible and incompatible interactions between mungbean and MYMIV are yet to be explored. Therefore, in this study using MYMIV-resistant (NM-2016), moderately resistant (NM-2011) and susceptible genotype (VC-1647C), alterations in various biochemical attributes due to MYMIV infection were analysed and compared with healthy non-inoculated control plants for understanding the resistance mechanism. After MYMIV inoculation, the level of total phenolic contents (TPC) and total soluble proteins (TSP) increased significantly in the susceptible genotype. However, the level of Malondialdehyde (MDA) and Ascorbate peroxidase (APX) remained same in all the genotypes. Level of superoxide dismutase (SOD) and catalase (CAT) decreased in the susceptible genotype but CAT level increased in the moderately resistant genotype. Protease level decreased significantly in all the genotypes while esterase level increased in moderately resistant and susceptible genotype. Peroxidase (POD) increased only in moderately resistant genotype and Total Oxidant Status (TOS) increased significantly in the susceptible genotype. Due to MYMIV infection the level of all plant pigments decreased in all the genotypes tested. The comparative proteome analysis using SDS-PAGE resolved 22 peptides with molecular weight from 12.5 to 163 kDa. Differential expression of protein phosphatase 2C (PP2C) and Cytochrome b6 (Photosynthesis) in resistant and moderately resistant genotypes; Pectin acetyl esterase and Resistant specific protein-1(4) in resistant genotype and up-regulation of superoxide dismutase [Cu-Zn] and RuBisco by MYMIV may have triggered signal transduction pathway and consequently induced a resistance response against MYMIV in V. radiata by activating PR proteins.

Keywords: Biochemical, alterations, resistant, susceptible, mungbean, YMD.

Received: 05 April 2019 * **Accepted:** 12 September 2019 * **DOI:** https://doi.org/10.29329/ijiaar.2020.238.11

* **Corresponding author:**

Areeha Suhail, Pakistan Institute of Engineering and Applied Sciences, Nilore, Islamabad, Pakistan.
Email: areeha005@gmail.com

INTRODUCTION

Mungbean (*Vigna radiata* (L.) Wilczek) is one of the most important pulse crop in Pakistan and is grown on large scale because of its short duration, little water requirement and soil fertility improvement. Moreover, it is a rich source of proteins, amino acids, and minerals (Akhtar et al., 2009; Abbas et al., 2015) and its sprouts are also very popular in Asian cuisines (Kitsanachandee et al. 2013). Worldwide mungbean cultivation ranges from 2.5 to 5 million hectares annually. In Pakistan, it is cultivated both in spring and summer seasons, but mainly grown in summer. It is cultivated on about 2,085,000 hectares (85% cultivated in Punjab province alone). However, for the last two decades, its yield in Pakistan has been around 550 kg/ha, which is very low compared to many other countries, because of various constraints. In the field, mungbean crop is subjected to a number of biotic and abiotic stresses. The biotic constraints are causing hideous damage to legumes in farmers' fields substantially reducing yield, particularly in developing countries where small resources are devoted to their study (Johansen et al., 2000). Particularly, virus diseases have been a consistent problem in both summer and winter pulse crops in Pakistan (Bashir et al., 2006). The yield of mungbean is drastically affected by a number of viral diseases, widely distributed in Pakistan. These viruses include mungbean yellow mosaic virus (MYMV), mungbean yellow mosaic India virus (MYMIV), urdbean leaf crinkle virus (ULCV), cucumber mosaic virus (CMV), bean yellow mosaic virus (BYMV), and alfalfa mosaic virus (AMV) (Aftab et al., 1993; Bashir and Malik, 1988). Among the viral diseases, the most common and destructive one is the yellow mosaic disease (YMD), caused by Geminiviruses (genus *Begomovirus*, family *Geminiviridae*). In different leguminous species YMD is known to be caused by mungbean yellow mosaic virus (MYMV), mungbean yellow mosaic India virus (MYMIV), dolichos yellow mosaic virus (DYMV) and horsegram yellow mosaic virus (HYMV). However, only MYMIV is identified as the main pathogen causing YMD in Pakistan (Ilyas et al., 2010). Particularly, during summer season MYMIV adversely affect mungbean cultivation due to high population of its insect vector, a whitefly, *Bemisia tabaci* (Akhtar et al., 2011).

MYMIV has a bipartite genome (consisting of DNA A and DNA B, of 2726 and 2775 nucleotides, respectively) (Ilyas et al., 2010). It generally reduces mungbean yield by 85%, but in some cases, it can reduce yield up to 100%. It can infect the plant at all growth stages, but infection at an early vegetative stage results in plant death. This virus is not transmitted through seed or sap, but only through a whitefly (*B. tabaci*) vector. Disease symptoms, varies with the host species as well as the genetic makeup of each genotype. Symptoms due to MYMIV infection includes yellowing or chlorosis of leaves, followed by necrosis. In highly susceptible plants, the internodes are shortened, plants are severely stunted and give no yield or produce only few flowers, with deformed pods, producing small, immature and wrinkled seeds (Kitsanachandee et al., 2013). Management of YMD is mainly focused on the vector (*B. tabaci*) control and using resistant/tolerant varieties. Chemicals are ineffective (or temporarily effective) as well

as detrimental to the environment. The best way to reduce the losses due to YMD is to develop resistant varieties which is an environmentally safe and long-term solution to manage this disease (Kitsanachandee et al., 2013). The study of resistance and susceptibility is very difficult because of its transmission by vector, efficiency of transmission, acquisition period, persistence semi persistence nature of viruses and the interaction between host and the vector. Plants produce a large number of secondary metabolites to protect against invading pathogens. These secondary metabolites are either in activated form or as inactivated precursors. The inactivated precursors are activated by the host enzymes in response to pathogen attack or tissue (Hammond-Kosack, 2000). Furthermore, these metabolites also protect plants against UV radiations and oxidants (Lattanzio et al., 2006).

In order to develop a resistant variety of any crop, we need to know how the plant reacts to that disease or the defense mechanisms of the plant against that disease (at molecular level). A very little information is available regarding the involvement of biochemical barriers in YMD resistance in mungbean. In this view, present study was planned to investigate the alterations in various biochemical activities to understand mechanisms involved in resistance at biochemical level in resistant and susceptible mungbean genotypes after MYMIV infection using whitefly transmission.

Materials and Methods

Plant Material

Three mungbean genotypes with known YMD resistance and susceptibility levels were grown in a net-house under insect free conditions. Seeds of these genotypes were kindly provided by the Mungbean and Lentil Group, Plant Breeding and Genetics Division, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The three tested genotypes were NM-2011 (moderately resistant); NM-2016 (Resistant) and VC-1647C (Susceptible). One month old test plants were divided into two sets for each genotype. They were placed separately under iron cages covered with muslin cloth. Each set consisted of three replicates (each replication contained 3 pots with five to six plants per pot). One set of each genotype was inoculated with MYMIV using whiteflies (*B. tabaci*) as described by Akhtar et al. (2011). The other set was left as healthy (non-inoculated) control.

Virus transmission and identification

For virus transmission whiteflies were collected from MYMIV-infected plants grown in the field. Four to five weeks old mungbean plants of each test genotypes were exposed to whiteflies for 72 hours inoculation feeding period (IFP), under fine mesh cages. A total of nine potted plants per test entry (three pots per cage representing one replication) were inoculated using about 50 viruliferous whiteflies per plants. After 72-h IFP, plants were sprayed with insecticides to kill the released whiteflies. Conventional agronomic practices and normal plant protection measures were adapted to keep the crop in good condition. The experiment was repeated for two times. Data was recorded on percentage of disease

transmission, mean latent period (average time required for first disease symptom appearance post inoculation), and average disease severity 20, 40, and 60 days post inoculation using the rating system described by Akhtar et al. (2011) (Table 1).

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 Immuno Sorbent Assay (TAS-ELISA) as described Kothandaraman et al. (2016).

For the detection of MYMIV using polymerase chain reaction (PCR) total DNA was extracted from young symptomatic and symptomless leaves of mungbean plants as described Kothandaraman et al. (2016). PCR was conducted using MYMIV specific primers: FLDNAAF (5'-TGTGGGATCCATTGTTGAACGACTTTCCC-3') and FLDNAAR (5'-CAATGGATCCCACATTGTTAGTGGGTTCAG-3') 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.

Table 1. Disease Scale for rating of Mungbean Yellow Mosaic India Virus (MYMIV)

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 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 shriveled seeds.	4.5-5.0	Highly susceptible

Severity index (SI) was calculated using following formula

$$SI = \left(\frac{\text{Sum of all disease ratings}}{\text{No. of infected plants observed}}\right) \text{x } 5$$

Total phenolic content (TPC)

TPC of different genotypes were compared among virus-inoculated and non-inoculated plants, by modified method of Ainsworth and Gillespie (Ainsworth and Gillespie, 2007), in which Folin-Ciocalteau (F–C) reagent was used. For the extraction of TPC, 0.1 g of leaf samples was ground in 85% ice-cold methanol, incubated at room temperature for 48 hours and then centrifuged (14,000 rpm) for 20 minutes. Then 100 µl supernatant was mixed with 100 µl of F-C reagent (10 %) and thoroughly vortexed and 800 µl of Na_2CO_3 (700 mM) was added. After incubation for 1 hour at room temperature, different concentrations of gallic acid were used to prepare a standard curve and a linear regression equation was calculated to determine TPC.

Total Soluble Proteins (TSP)

TSP contents were measured using Bradford's method (Bradford, 1976). For this purpose, 0.5g of leaf samples was crushed in a mini bead beater in the presence of 1.5 ml phosphate buffer (pH 7.0) and centrifuged at 10000 g. The supernatant was taken in a separate eppendorf tube and in 5µl of the extract 1 ml of Bradford reagent and 95 µl 0.1N NaCl was added. The absorbance was recorded at 595nm.

Enzyme assays

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) with minor modifications as described by Zhang and Kirham (1994). Briefly, first leaf sample (0.25 g) was crushed in 5 ml TCA (0.1%) and the obtained homogenate was centrifuged at 10,000 g for 5 min. Then to 1 ml of the supernatant, 4 ml of TCA (20%) containing TBA (0.5%) was added, heated for 30 min at 95 ^oC and centrifuged (10000 g) for 10 min. The absorbance of the supernatant was read (at 532 nm) and the value of the non-specific absorption (at 600 nm) was subtracted. Extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate 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). For catalase (CAT) estimation, selected plant parts were crushed in extraction buffer as described by Beers and Sizer (1952). Decrease in absorbance of the reaction solution at 240 nm was recorded after every 30 seconds. To estimate protease activity, casein digestion assay was used as described by Drapeau (1976). Using this method, one unit is the amount of enzyme, which releases acid soluble fragments equivalent to 0.001 (A_{280}) per minute (at 37^{o}C, pH 7.8). Esterase activity was measured according to the method of Van Asperen (1962). The selected plant parts were triturated in 50 mM potassium phosphate buffer (pH 7.8)

and the esterases (α and β) were recorded using α–naphthyl acetate and β–naphthyl acetate as substrates, respectively. Peroxidise (POD) activity was measured using the method of Chance and Maehly (1955) while Ascorbate peroxidase (APX) activity was measured as described by Nakano and Asada (1981). The reaction mixture consisted of 2.7 ml potassium phosphate buffer (25 mM) containing 2 mM EDTA (pH 7.0), 100 µl enzyme extract, 100 µl ascorbate (7.5 mM) and 100 µl H_2O_2 (300 mM). Ascorbate oxidation was recorded by the change in absorbance at 290 nm (ε¼2.8 mM/cm).

Total oxidant status (TOS)

TOS was measured using Erel TOS method (Erel, 2005). This method is based on the ferrous ion oxidation to ferric ion under acidic medium in the presence of different oxidative species and ferric ion measurement by xylenol orange (Harma et al., 2005).

Measurement of plant pigments

The chlorophylls (*Chl a, b*), lycopene and carotenoids (x + c) were extracted in 80% aqueous acetone (v/v) and vacuum filtered through a filter paper (Whatman No. 1). Spectrophotometrically, the pigments were quantified and absorbance were recorded at different wavelengths (663, 645, 505, 470 and 453 nm). Equations of Kichtenthaler and Wellburn (1983) was used to calculate the concentration of pigments (mg g^{-1} f. wt.).

Protein profiling

Leaf protein profiling was performed by following the protocol described by Hameed et al. (2012). Soluble proteins from leaves were extracted by grinding the leaves in 50mM phosphate buffer (pH 7.8) 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 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 using water bath to denature the proteins.

Protein profiling of the leaf samples was performed by SDS-PAGE as described by Laemmli (Laemmli, 1970). For this purpose, equal quantities of samples along with the protein molecular weight marker were loaded in the 10% gels. Electrophoresis was done at constant voltage (i.e. 100 V). After that, gel was fixed in a solution containing 40% ethanol and 10% acetic acid for 15 minutes with constant agitation on a shaker. After fixing, the gel was washed with distilled water (the water was changed every five minutes). The gel was then stained with Coomassie brilliant blue dye G-250 and then de-stained in distilled water over night. After that, the gel was 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

The data collected was separately analyzed for each parameter and subjected to two-way analysis of variance (ANOVA) using XL-STAT software. The means were then compared for significance using Tukey (HSD) test at $p<0.05$.

Results

Disease response

There were significant differences in the disease severity index (DSI) and in the latent period (LP) of the three genotypes depending upon their genetic makeup (Table 2). Disease severity was highest in case of susceptible genotype and much lower in the moderately resistant and resistant genotypes (Table 2).

Table 2. Response of mungbean genotypes against MYMIV through whitefly inoculation

Genotype	Latent Period (Days)*	Disease Severity Index*	Disease response	TAS ELISA absorbance values (A_{405nm}) after 1h
NM-2011	10^{ab}	1.87^{b}	Moderately resistant	1.34^{a}
NM-2016	13^{b}	1.19^{c}	Resistant	0.687^{b}
VC-1647C	7^{a}	4.23^{a}	Susceptible	2.240^{a}
Healthy control	------	----	-----	0.387^{b}

*Values sharing similar letters do not differ significantly at $P\leq 0.05$.

The symptoms were more prominent on the susceptible genotype (VC-1647C), which included severe chlorosis, shortened internodes and stunted growth of plants. On the other hand few small yellow specks or spots on few leaves were seen after careful observing the resistant genotype but bright yellow specks or spots were common on the leaves of moderately resistant genotype.

ELISA and PCR results

ELISA results showed that the virus titer varied significantly among the tested genotypes depending upon their resistance/susceptibility levels. The virus titre ranging from 0.687 to 2.240 (A_{405nm} 1 h) was detected by ELISA and the highest titre was observed in the susceptible genotype (Table 2) The presence of MYMIV in these samples was further confirmed by PCR using a genus-specific primers pair, which amplified a full length component of DNA A of MYMIV of approximately 2.75 kb from virus inoculated plant samples but no amplification was found from non-inoculated plants of all test genotypes.

Total phenolic compounds

The level of TPC in non-inoculated plants of all the tested genotypes differed with each other. TPC were higher in moderately resistant genotype NM-2011, as compared to resistant NM-2016 and

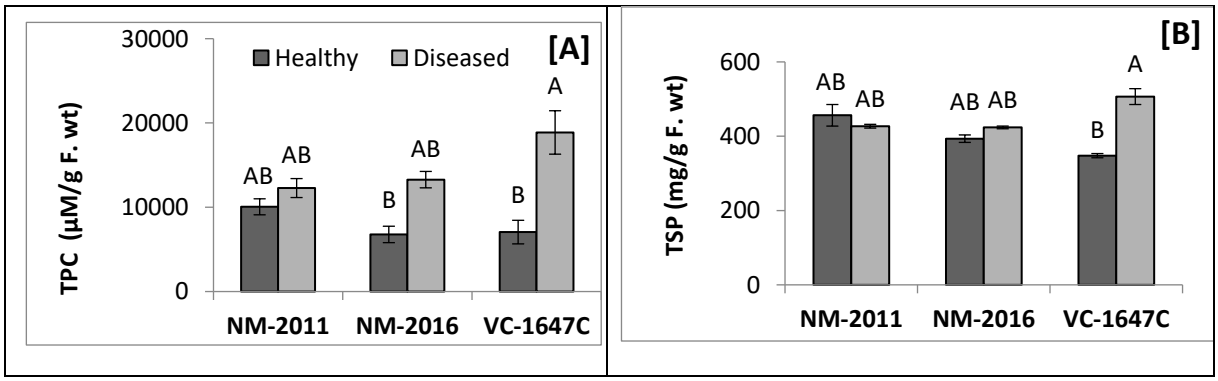
susceptible VC-1647C. The increase in TPC was significant only in the susceptible genotype VC-1647C (Fig 1A). The increase in TPC over their non-inoculated controls was 18.12% in moderately resistant genotype NM-2011, 48.96% in case of resistant genotype NM-2016 and 62.65% in susceptible genotype VC-1647C.

Total soluble proteins

The level of TSPs was relatively low in the susceptible genotype as compared to moderately resistant and resistant genotype. After inoculation with MYMIV, TSP level slightly decreased in moderately resistant genotype NM-2011 (6.4%) while increased in resistant genotype NM-2016 (7.71%) over their non-inoculated plants (Fig. 1B).

Enzyme assay

SOD activity was significantly higher in the non-inoculated plants of susceptible genotype (VC-1647C) compared to resistant genotypes (Fig 1C). Furthermore, there was no considerable change in SOD activity in MYMIV infected resistant genotypes compared to their non-inoculated plants. In non-inoculated plants the level of POD enzyme was statistically similar in all the tested genotypes. However, increase in POD level was observed upon MYMIV-infection in plants of the tested genotypes. Particularly, in moderately resistant genotype (NM-2011), the increase was significant (Fig. 1D). In case of CAT enzyme, its level was much lower in non-inoculated plants of moderately resistant genotype and higher in susceptible genotype. After MYMIV infection, the CAT level was significantly increased in moderately resistant genotype NM-2011 (71.4%) while decreased in other genotypes NM 2016 and VC-1647C (Fig. 1E). Based on results, protease activity was almost same before inoculation in all the tested genotypes but significantly decreased after infection (Fig. 1F). The decrease was more (26%) in susceptible genotype (VC-1647C). Esterase enzyme level was statistically similar in non-inoculated plants of all the tested genotypes (Fig. 1G). After inoculating MYMIV, a significant increase in esterase level was observed in moderately resistant genotype NM-2011 (74%) and susceptible genotype VC-1647C (65%). The observed MDA enzyme level was same in non-inoculated plants but changed after inoculation in all the genotypes.



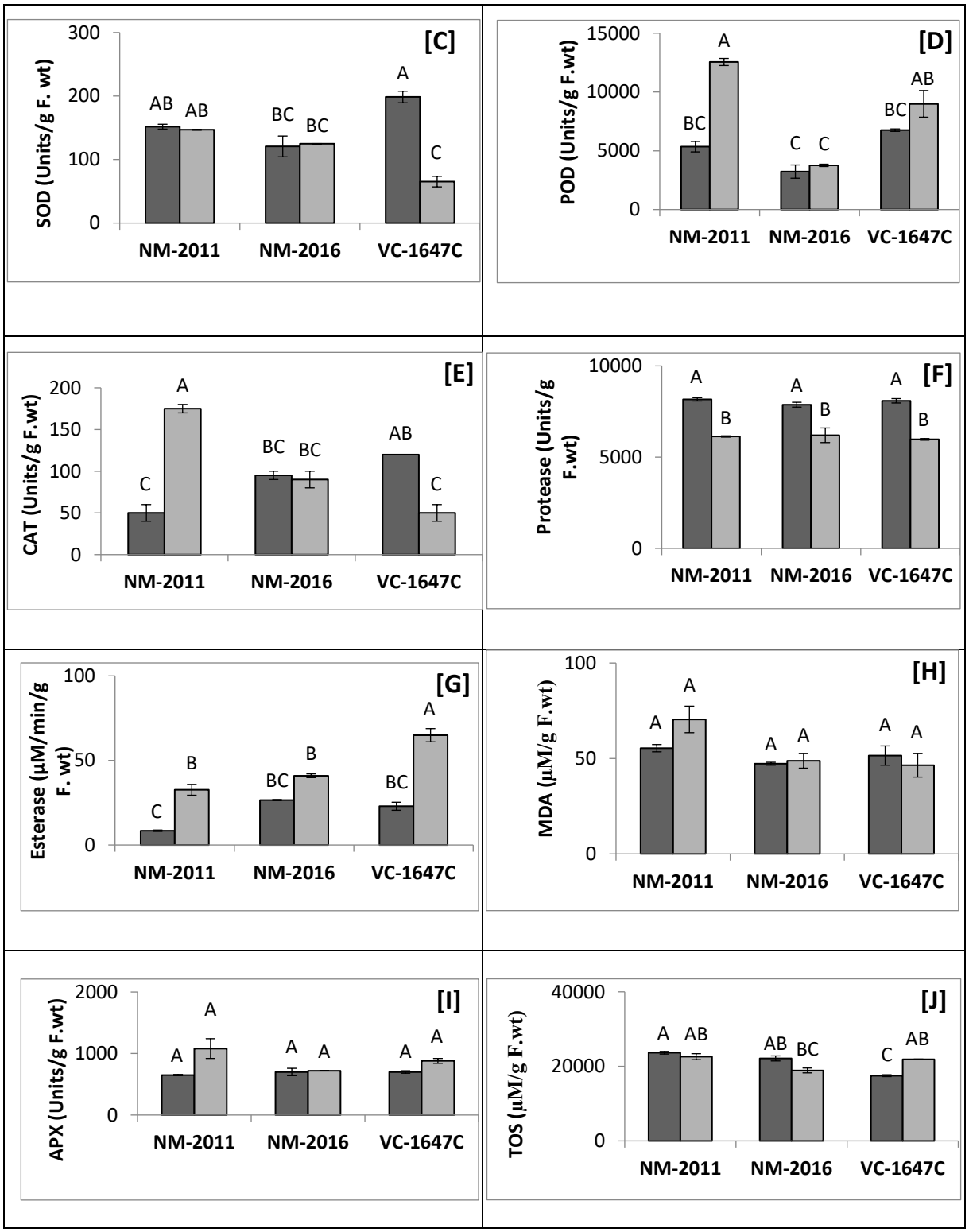


Figure 1. [A] TPC, **[B]** TSP, **[C]** SOD, **[D]** POD, **[E]** CAT, **[F]** protease, **[G]** esterase, **[H]** MDA, **[I]** APX and **[J]** TOS of non-inoculated and MYMIV infected plants of resistant, moderately resistant and susceptible mungbean genotypes. Different letters on the top of bars indicate significant differences between genotypes at $P \leq 0.05$.

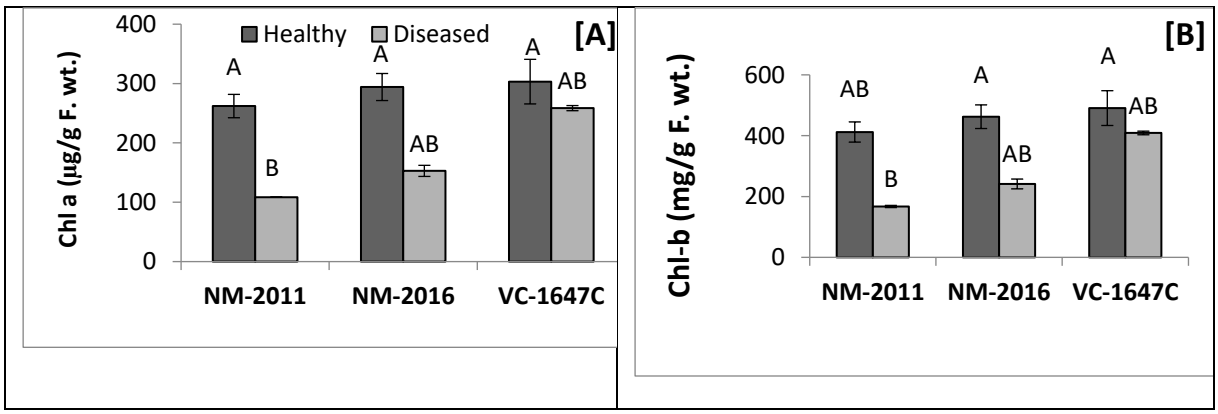
Interestingly, its level increased non-significantly in genotypes possessing resistance but decreased in the susceptible one (Fig 1H). The level of APX enzyme was almost same in non-inoculated plants of all mungbean genotypes. After virus inoculation, its level was increased non-significantly in all the tested genotype (Fig. 1I). APX level was increased by 66% in moderately resistant genotype (NM-2011), 2.8% in resistant genotype (NM-2016) and 25% in susceptible genotype (VC-1647C).

Total oxidant status

TOS in the leaves of non-inoculated plants of the susceptible genotype (VC-1647C) was significantly lower than the non-inoculated plants of moderately resistant (NM-2011) and resistant genotype (NM-2016). However, TOS level was decreased non-significantly in MYMIV infected moderately resistant (4.4%) and resistant (14.5%) genotype, while it increased significantly (25%) in case of susceptible genotype VC-1647C (Fig. 1J).

Photosynthetic pigment content

Chlorophyll a (Chl a) contents were statistically similar in non-inoculated plants but decreased significantly in virus inoculated plants of these genotypes (Fig. 2A). This decrease was significant for moderately resistant genotype NM-2011 (58.6%) while non-significant for resistant genotype NM-2016 (48%) and susceptible genotype VC-1647C (14%). *Chl b* level decreased non-significantly in inoculated plants of all the tested genotypes (Fig. 2B). This decrease was most prominent in NM 2011 (59.4%). Non-inoculated plants of susceptible genotype (VC-1647C) showed higher amount of total chlorophyll followed by resistant (NM-2016) and moderately resistant (NM-2011) genotype. Upon MYMIV-inoculation, significant decrease was observed in resistant genotype NM-2016 (47.9%) and moderately resistant genotype NM-2011 (61.9%) but non-significant in susceptible genotype VC-1647C (15.9%) (Fig. 2C). Total carotenoids were statistically similar in non-inoculated plants and decreased upon MYMIV inoculation in all the genotypes. This decrease was significant only in moderately resistant genotype NM-2011 (Fig. 2D).



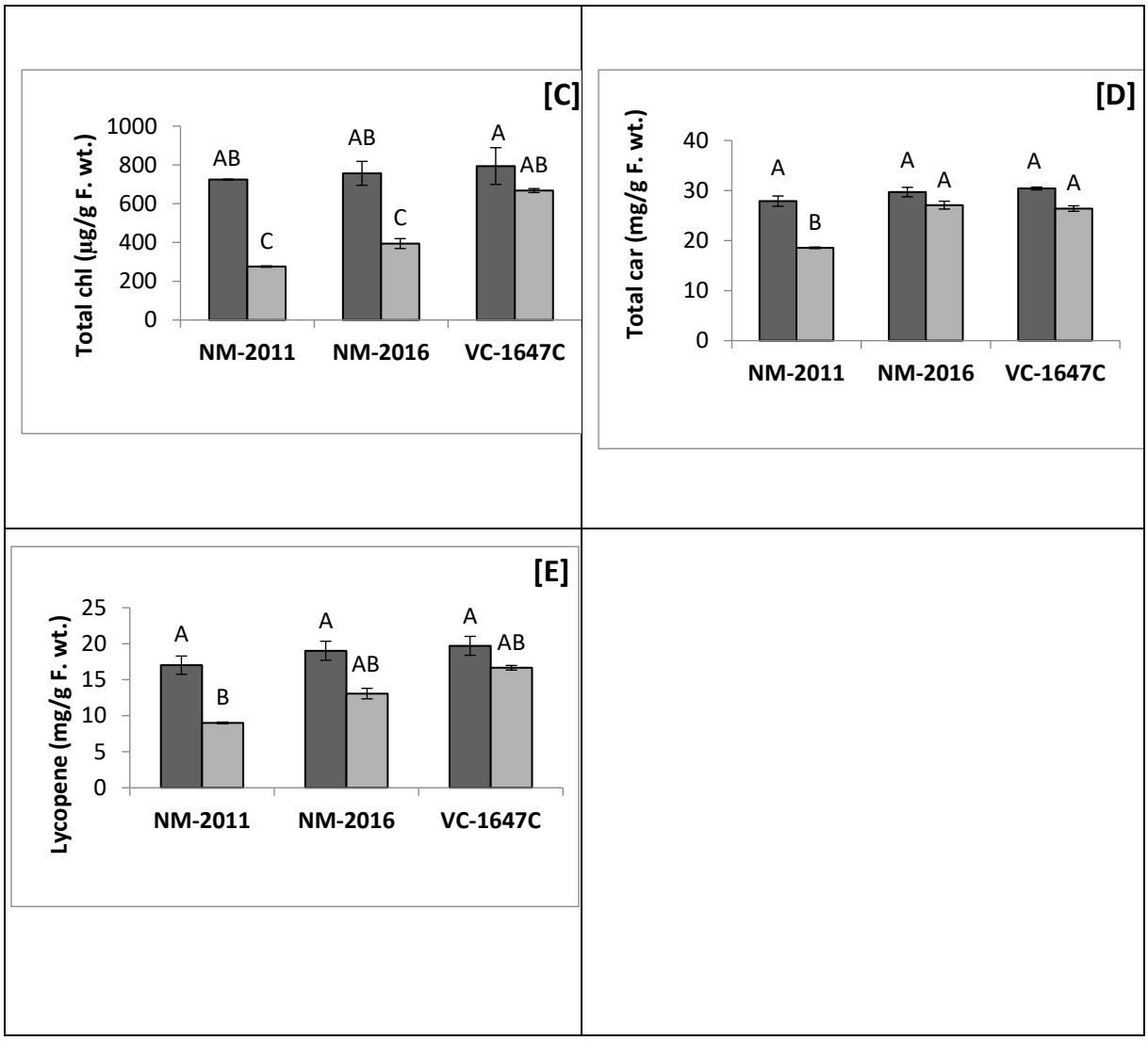


Figure 2. [A] *Chl a*, **[B]** *Chl b*, **[C]** total chlorophylls, **[D]** carotenoids and **[E]** lycopene of non-inoculated and MYMIV infected plants of resistant, moderately resistant and susceptible mungbean genotypes. Different letters on the top of bars indicate significant differences between genotypes at $P \leq 0.05$.

Lycopene

A decrease in lycopene concentration was recorded in all the genotypes after inoculation. This reduction was significant in moderately resistant genotype while non-significant in resistant and susceptible genotype (Fig. 2E).

Protein profiling

Mungbean leaf proteins were resolved into 22 detectable bands using SDS-PAGE (Fig. 3).

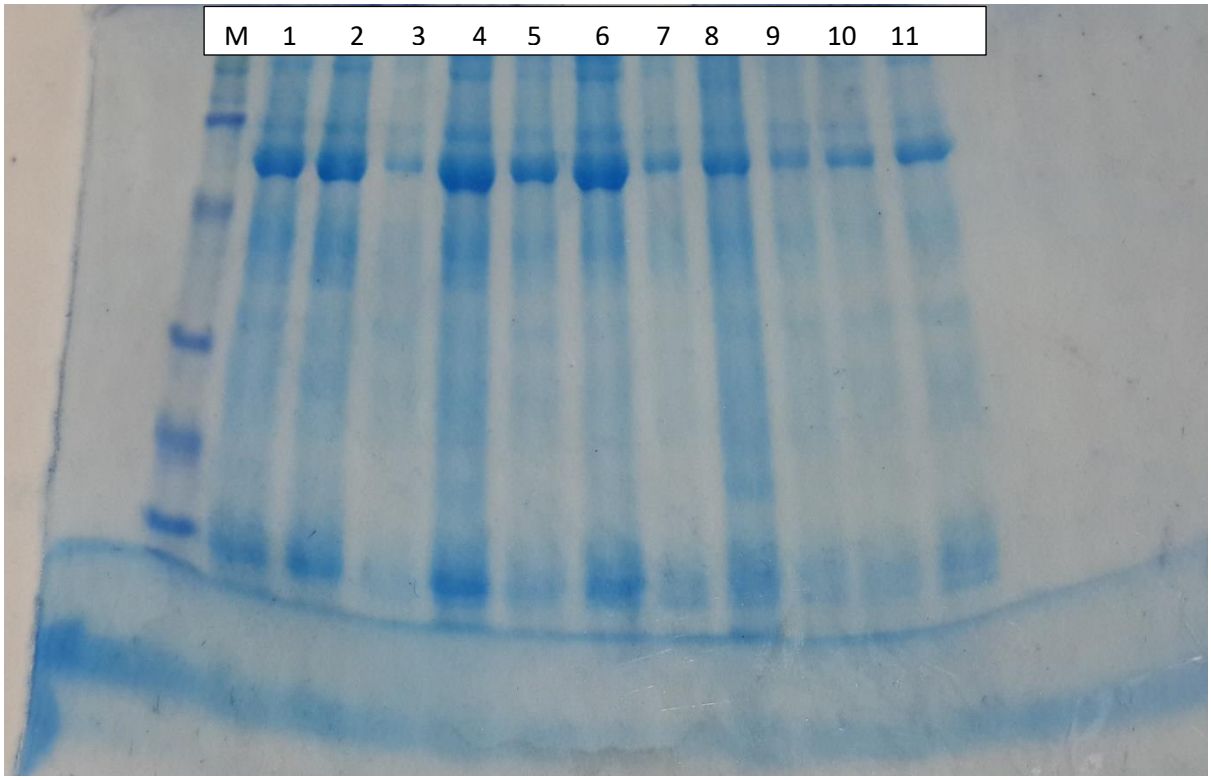


Figure 3. Protein profiling using SDS-PAGE

M: Protein Molecular Weight Marker, Lane 1-2: NM-2011 (D), Lane 3-4: NM-2016 (D), Lane 5-6: VC-1647C (D), Lane 7-8: N,-2011 (H), Lane 9: NM-2016, Lane 10-11: VC-1647C (H). Where d= diseased, H= healthy (non-inoculated)

Molecular weights of these proteins ranged from 12.58 kDa to 163 kDa (Fig. 4).

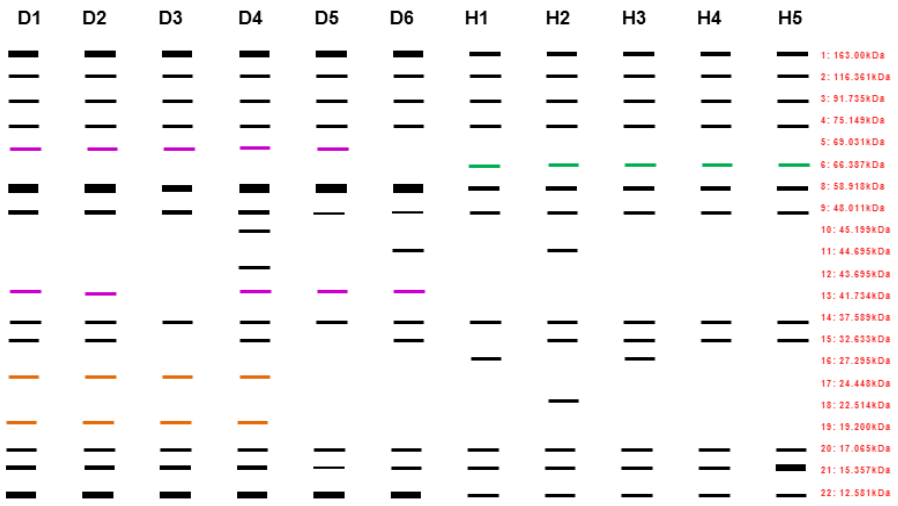


Figure 4. Histogram based on Electrophoretic data of resistant and susceptible genotypes of Mungbean. D1, D2= NM-2011 (MYMIV inoculated); D3, D4= NM-2016 (MYMIV inoculated); D5, D6= VC-

1647C (MYMIV inoculated); H1, H2= NM-2011 (non-inoculated); H3= NM-2016 (non-inoculated); H4, H5= VC-1647C (non-inoculated)

A list of proteins identified in present study and a histogram is presented in Table 3.

Table 3. Possible protein families searched in different databases having similar mol. Wt. as detected in present study

Sr No.	Protein Name	Accession No.	Function	Mol. Wt. (kDa)
1	Chloroplast photosystem II subunit X	C1K5D2	Photosynthesis	12.235
2	Superoxide dismutase [Cu-Zn]	F2WVP3	Metal ion binding, SOD activity (converts superoxide to molecular oxygen and H_2O_2	15.256
3	Pathogenesis-related protein 10	Q2VU97	Defence Response, response to biotic stimulus	16.189
4	Protein phosphatase 2C 16-like isoform	A0A141G753	metal ion binding, protein serine/threonine phosphatase activity	18.613
5	Ascorbate Peroxidase	B2ZFL8	Responds to Oxidative Stress	22.416
6	Cytochrome b6	A0A0F7RPG9	Photosynthesis	24.160
7	ATP synthase subunit a,	D3J8G7	translocation of protons across the membrane	27.377
8	Carboxypeptidase	Q41691	serine-type carboxypeptidase activity	32.591
9	Photosystem II protein D1	A0A0F7RND6	Photosynthesis	38.967
10	Replication-associated protein	Q9YPS2	Plays important role in replication of MYMV.	40.677
11	Pectin acetyl esterase	Q41695	Alters physical properties of cell wall.	43.823
12	Alanine: glyoxylate aminotransferase	C1IGP4	Transaminase activity	44.276
13	Resistant specific protein-1(4)	Q8GSG3		45.813
14	RuBisco	O98997	Photosynthesis, Respiration	47.902
15	Cytochrome c oxidase subunit 1	A0A0U4ZGT0	Component of the respiratory chain that catalyses the reduction of oxygen to water. Subunits 1-3 form the functional core of the enzyme complex. CO I is the catalytic subunit of the enzyme.	57.452
16	Maturase K	A0A0F7RNM9	Usually encoded in the trnK tRNA gene intron. Probably assists in splicing its own and other chloroplast group II introns.	61.719
17	Polyphenol Oxidase	A0A0S3Q2G5	Binds 2 copper ions per subunit	66.718
18	Ku-70 like protein	A7MAS2	DNA helicase activity (ATP dependent), damaged DNA binding, tolemeric DNA binding	71.259
19	RNA helicase	Q9M6R6	Helicase activity	77.423
20	Lipoxygenase	S5XAM1	Involved in growth and development, pest resistance, and senescence or responses to wounding	97.417
21	Salt overly sensitive 1	S5LPS7	Solute: proton antiport	116.096

Salt overly sensitive protein (116 kDa), RNA Helicase (77.4 kDa), Cytochrome c oxidase subunit 1 (57.5 kDa), ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic (48 kDa), Photosystem II protein D1 (38 kDa), Carboxypeptidase (32 kDa), Superoxide dismutase [Cu-Zn] (15 kDa) and Chloroplast photosystem II subunit X (12 kDa) were detected in all the samples. Other bands detected in all samples were of molecular weight of 91.735 kDa (may be lipoxygenase [97.417kDa], 17.06 kDa (can be of Pathogenesis-related protein 10 [16.19 kDa] and an unidentified protein of 163kDa. A band of molecular weight 69 kDa (may be Ku70-like protein [71.2 kDa]) was spotted only in the infected samples of all test genotypes. Two proteins, Cytochrome b6 (24 kDa) and protein phosphatase 2C 16-like isoform (19 kDa) were spotted only in the infected samples of moderately resistant and resistant genotypes. Bands for protein Polyphenol Oxidase (66 kDa) was detected only in the non-inoculated plants of all the genotypes. A band for replication-associated protein (41 kDa) of MYMIV was noticed in infected plants of all the genotypes. Pectin acyltransferase (43.8 kDa) and resistant specific protein (45.8 kDa) were detected only in non-inoculated plants of resistant genotype. One protein, Alanine: glyoxylate aminotransferase (44.4 kDa) was detected in infected plants of susceptible genotype and non-inoculated plants of resistant genotype. Another protein, ATP synthase subunit a (27.3 kDa) was spotted in non-inoculated plants of moderately resistant and resistant genotypes each. Ascorbate peroxidase (22.4 kDa) was detected only in non-inoculated plants of moderately resistant genotype.

Discussion

Several defence strategies are adapted by plants to handle and coop pathogen induced stresses. Like other pathogens, viral infections in plants also cause many alterations in physiology, biochemistry and metabolic processes. These changes in plants appeared as disease symptoms during viral infection (Tecsi et al., 1996). The present study investigates the appearance of epidemiological symptoms characteristic to YMD infection in V. radiata with respect to alterations in biochemical changes those occur during compatible and incompatible host-virus interactions. Severe and prominent YMD symptoms appeared on the susceptible varieties having high virus titre compared to resistant varieties tested. In our study, based on biochemical analysis increase in phenolic contents was recorded in all the genotypes, but was significant only in the susceptible genotype. However, in another study, in incompatible interactions between V. mungo and MYMIV, TPCs were only increased in resistant genotypes (Kundu et al., 2013). Generally, increase in TPC is associated with the increase in resistance of a plant (Velazhahan and Vidhyasekaran, 1994). But Junqueira et al. (2004) pointed out that resistance is not only related to the presence of phenolic compounds and they may have other functions in plant tissue. No significant alteration in TSP level was observed in resistant genotypes but its level increased significantly in susceptible genotype upon MYMIV inoculation. Normally, in susceptible genotypes after infection with pathogens the TSP level is low because of high susceptibility, but in our study it was

reverse. Earlier similar results for phytoplasma infection in chickpea and mungbean were observed by Nasir et al. (2017) and Hameed et al. (2017). SOD concentration in the susceptible genotype decreased significantly after MYMIV infection. Similar SOD isoenzyme patterns was noted by Siddique et al. (2014) in different Gossypium sp. in compatible and incompatible interactions after cotton leaf curl Burewala virus inoculation. No change in SOD in incompatible interactions could be a strategy of the mungbean plants to restrict the infection of virus. Activity of POD increased in all the genotypes after MYMIV inoculation, but maximum increase was observed in moderately resistant genotype. Upon MYMIV-inoculation, CAT activity increased significantly in the moderately resistant genotype and decreased in the susceptible genotype. Earlier studies reported that CAT works antagonistically with the POD and our results are in compliance to this fact as activity of CAT and POD increased almost equally in the moderately resistant genotype. In other studies, CAT activity was also reported to decrease in resistant plants upon attack by a pathogen (Patel et al., 2011; Siddique et al., 2014). Interestingly, level of Protease decreased almost equally in the diseased plants, irrespective of the resistance level of genotypes confirming their non involvement in the resistance or susceptibility of mungbean plants. Similar reduction in protease activity was observed in resistant and susceptible tomato cultivars when subjected to Alternaria leaf blight disease (Hameed et al., 2010). Under present study, maximum increase in esterase was observed in susceptible genotype compared to others. This low level of esterase might play a role in resistance of mungbean plants against MYMIV which needs to be further investigated to elucidate its role in plant defense mechanisms. MDA is an indicator of cellular damage at the cell membrane by pathogenic infection (Aly et al., 2012). Similarly, APX maintain ROS homeostasis in plant cell which either destroy the invading pathogens directly or activate expression of defense related gene cascade (Kitsanachandee et al., 2013; Kundu et al., 2013). However, in present study, we did not find significant differences in MDA and APX level in healthy and diseased mungbean genotypes plants which shows that these enzymes are not involved in contributing resistance or susceptibility of mungbean plants to MYMIV. Significant decrease was recorded in TOS level in resistant genotypes but increased in the susceptible genotype upon MYMIV inoculation. Yellow spots appearance on MYMIV infected plant leaves indicates that chlorophyll is affected. In almost all virus infected plants where chlorotic and necrotic symptoms appear, there is a reduction in net photosynthetic rate and hence in the chlorophyll content (Venkatesan et al., 2010). In the present study, Chl a, b and carotenoids decreased in MYMIV infected plants of the three mungbean varieties tested. Previously, Kundu et al. (2013) observed decrease in these pigments in MYMIV infected V. mungo plants. Similar reduction in green pigments were observed in mesta plants infected by yellow vein mosaic disease and cotton infected by CLCuV (Chatterjee and Ghosh, 2008; Siddique et al., 2015). The decrease in lycopene concentration in diseased plants was most prominent in both moderately resistant and resistant genotypes. This reduction in lycopene might induce resistance in mungbean plants against YMD.

Proteomic analysis of the tested genotypes has revealed an early accumulation of the defence/stress and pathogenesis related proteins during host-pathogen interaction. Protein profiling detected bands of Superoxide dismutase [Cu-Zn] (15 kDa) in all the samples, but the band observed was lightest in the diseased plants of susceptible genotypes while were darker in the resistant genotypes. These results are at par with the biochemical analysis, which showed that this ROS scavenging enzyme significantly decreased upon inoculation of the MYMIV. Ascorbate peroxidase (22.4 kDa) was detected only in non-inoculated plants of moderately resistant genotype. Cytochrome b6 (24 kDa) and protein phosphatase 2C 16-like isoform (19 kDa) were observed only in the virus infected samples of moderately resistant and resistant genotypes. Previous studies have revealed the role of protein phosphatase 2C 16-like isoform in the development of resistance in tobacco plants against tobacco mosaic virus (Hu et al., 2009) and in mashbean against MYMIV (Kundu et al., 2013). Similarly, Cytochrome b6 plays a role in photosynthesis and its presence in the virus-inoculated plants of resistant genotypes indicates its role in resistance. ATP synthase subunit a (27.3 kDa) was spotted in one non-inoculated sample of both resistant genotypes. Its presence in non-inoculated samples of both resistant genotypes and absence in the susceptible genotype indicates that it might play a role in defending the plant from the pathogen. Photosystem II protein D1 (38 kDa), was spotted in all the test samples suggesting that this protein is not affected by MYMIV. This protein was also reported in V. mungo species upon inoculation of the same virus where it was found to be the primary target of pathogenesis (Kundu et al., 2013). Similarly, a band of 41 kDa for replication-associated protein (Rep) was spotted in virus-inoculated samples of all the genotypes. This protein belongs to MYMV and is necessary for single-stranded DNA (ssDNA) virus replication. It also displays activities including nucleotidyl transferase, origin-specific DNA cleavage, helicase and ATPase (Karthikeyan et al., 2004). A protein, Alanine: glyoxylate aminotransferase (44.4 kDa) presence in some non-inoculated and virus-inoculated plants of resistant and susceptible genotypes respectively, shows that it does not have a prominent role in imparting resistance to the plant. However, further studies are needed to elucidate its role in mungbean. Two proteins, Pectin acyltransferase (43.8 kDa) and resistant specific protein (45.8 kDa) were detected only in non-inoculated plants of resistant genotype. As resistant specific protein name indicates, it imparts resistance to the plant. While Pectin acyltransferase protein is involved in protection or repair of plant cell wall against the damage caused by pathogen. Presence of these proteins in virus infected mungbean plants of resistant genotype indicates its positive role in the defence against MYMIV. RuBisco (48 kDa) was detected in all the samples tested but its bands were relatively thicker in diseased samples of moderately resistant and resistant genotypes compared to susceptible genotype. These results show that this protein is up-regulated by MYMIV imparting resistance in mungbean. These results are at par with another study on mashbean with same pathogen (Kundu et al., 2013). Polyphenol Oxidase (66 kDa) was found only in the non-inoculated plant samples and its absence in virus-inoculated plants indicates that it is a chief target of MYMIV. Ku70-like protein (71.2 kDa) was detected in diseased

samples of all the genotypes. This protein repairs the damaged DNA and is also involved in tolemeric DNA binding (Qi et al., 2014). The presence of this protein only in the diseased samples compliments its function. RNA helicase (77.4 kDa) with almost same intensity was spotted in all the samples suggesting that this protein is not affected by MYMIV (Mourad et al., 2000). Lipoxygenase (97 kDa) was detected in all the samples tested but darker in the diseased plant samples suggesting that this protein is up-regulated by MYMIV. It plays a role in growth and development, pest resistance and wound healing. Band of salt overly sensitive protein (116 kDa) were present in all the samples, but were much clear in inoculated plants than in the non-inoculated ones. This protein is found only in mungbean and is probably activated under some stress conditions suggesting that upon virus inoculation the protein is up-regulated. In addition to these, an unidentified protein of 163kDa was also detected in all the test samples. The bands in all the samples were very thick and clear, but were more prominent in the virus-inoculated plants. It means that this protein is up-regulated by MYMIV. However, in protein databases (Uniprot and NCBI), no mungbean protein of 163 kDa was found. Further studies should be carried out to find the structure and function of this protein.

Conclusions

In summary, MYMIV infection alters the biochemical parameters of tested genotypes which probably play an active role in resistance against MYMIV. However, significant decrease or increase in SOD, CAT and POD in resistant and susceptible varieties after infection with virus suggests their correlation between constitutive induced levels of these enzymes and plant resistance that could be considered as biochemical markers for studying plant-virus compatible and incompatible interactions. SOD activity was not changed after infection with virus in resistant and moderately resistant genotypes but decreased significantly in susceptible genotype. Thus, these phenomena could be a strategy of the plants of resistant genotypes to restrict virus colonization, because the excess ROS can be removed. In this study, we have also reported for the first time the protein profiling during compatible and incompatible interactions between MYMIV and *V. radiata*. Proteomic analyses revealed that expression of protein phosphatase 2C (PP2C) and Cytochrome b6 (Photosynthesis) in resistant (NM-2016) and moderately resistant (NM-2011); expression of Pectin acetyl esterase and resistance specific protein-1(4) in resistant genotype (NM-2016) and up-regulated of Superoxide dismutase [Cu-Zn] and RuBisco by MYMIV may trigger signal transduction pathway and consequently induces resistance response against MYMIV in *V. radiata* by activating PR proteins.

REFERENCES

Abbas, G., A. Hameed, M. Rizwan, M. Ahsan, M. J. Asghar and N. Iqbal (2015). Genetic Confirmation of mungbean (*Vigna radiata*) and Mashbean (*Vigna mungo*) interspecific recombinants using molecular markers. Front. Plant Sci., 6, 1107

Aftab, M., S. Asad, K. Khokhar, M. Ayub and T. Butt T (1993). Effect of *Mungbean yellow mosaic virus* on the yield and growth components of asparagus bean. Pak. J. Phytopathol., 5, 58-61

Ainsworth, E. A. and K.M. Gillespie (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nat. Protoc., 2, 875-877

Akhtar, K. P., G. Sarwar, G. Abbas, M. J. Asghar, N. Sarwar and T. M. Shah (2011). Screening of mungbean germplasm against *Mungbean yellow mosaic India virus* and its vector *Bemisia tabaci*. Crop Protec., 30, 1202-1209

Akhtar, K. P., R. Kitsanachandee, P. Srinives, G. Abbas, M. J. Asghar, T. Shah, B. Atta, O. Chatchawankanphanich, G. Sarwar and M. Ahmad (2009). Field evaluation of mungbean recombinant inbred lines against mungbean yellow mosaic disease using new disease scale in Thailand. Plant Pathol. J., 25, 422-428

Aly, A. A., M. Mansour, H. I. Mohamed and K. A. Abd-Elsalam (2012). Examination of correlations between several biochemical components and powdery mildew resistance of flax cultivars. Plant Pathol. J. 28, 149-155

Bashir, M. and B. A. Malik (1988). Diseases of major pulse crops in Pakistan—a review. Int J Pest Manag., 34, 309-314

Bashir, M., Z. Ahmad and S. Mansoor (2006). Occurrence and distribution of viral diseases of mungbean and mashbean in Punjab, Pakistan. Pak. J. Bot., 38, 1341

Beers, R. F. and I. W. Sizer (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem., 195, 133-140

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-254.

Chance, B. and A. Maehly (1955). Assay of catalases and peroxidases. Methods Enzymol., 2, 764-775.

Chatterjee, A. and S.K. Ghosh (2008). Alterations in biochemical components in mesta plants infected with yellow vein mosaic disease. Braz. J. Plant Physiol., 20, 267-275.

Dixit, V., V. Pandey and R. Shyam (2001). Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J. Exp. Bot., 52, 1101-1109.

Drapeau, G.R. (1976). Protease from *Staphyloccus aureus*. Methods Enzymol., 45. 469-475.

Erel, O. A. (2005). A new automated colorimetric method for measuring total oxidant status. Clin. Biochem., 38, 1103-1111

Hameed, A., K.P. Akhtar, M. Y. Saleem and M. Asghar (2010). Correlative evidence for peroxidase involvement in disease resistance against Alternaria leaf blight of tomato. *Acta* Physiolog. Plant., 32, 1171-1176

Hameed, A., M. Qureshi, M. Nawaz N. Iqbal (2012). Comparative seed storage protein profiling of mung bean genotypes. Pak. J. Bot., 44, 1993-1999

Hameed, S., K. P. Akhtar, A. Hameed, T. Gulzar, S. Kiran, S. Yousaf, G. Abbas, M. J. Asghar and N. Sarwar (2017). Biochemical changes in the leaves of mungbean (*Vigna radiata*) plants infected by phytoplasma. Turk. J. Biochem., 42,591-599

Hammond-Kosack, K. (2000). Responses to Plant Pathogens In "Biochemistry and Molecular Biology of Plants" p 1102-1156 Ed BB Buchanan, W Gruissem and RL Jones. Pp1367 American Society of Plant Physiology, Rockville, Maryland, USA.

Harma, M., M. Harma and O. Erel (2005). Oxidative stress in women with preeclampsia. The *Am. J. Obstet. Gynecol.*, 192, 656-657

Hu, X., H. Zhang, G. Li, Y. Yang, Z. Zheng and F. Song (2009). Ectopic expression of a rice protein phosphatase 2C gene OsBIPP2C2 in tobacco improves disease resistance. Plant Cell Rep., 28, 985-995

Johansen, C., J. M. Duxbury, S. M. Virmani, C. L. Gowda, S. Pande and P. K. Joshi (2000). Legumes in rice and wheat cropping systems of the Indo-Gangetic Plain-Constraints and opportunities. International Crops Research Institute for the Semi-Arid Tropics

Ilyas, M., J. Qazi, S. Mansoor, R. W. Briddon (2010). Genetic diversity and phylogeography of begomoviruses infecting legumes in Pakistan. J. Gen. Virol., 91, 2091-2101

Junqueira, A., I. Bedendo and S. Pascholati (2004). Biochemical changes in corn plants infected by the maize bushy stunt phytoplasma. Physiol. *Mol. Plant Pathol.*, 65, 181-185

Karthikeyan, A., R. Vanitharani, V. Balaji, S. Anuradha, P. Thillaichidambaram, P. Shivaprasad, C. Parameswari, V. Balamani, M. Saminathan and K. Veluthambi (2004). Analysis of an isolate of *Mungbean yellow mosaic virus* (MYMV) with a highly variable DNA B component. Arch. Virol., 149, 1643-1652.

Kichtenthaler, H. and A. Wellburn (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvent. *Biochem. Soc.* Trans., 603, 591-593

Kitsanachandee, R, P. Somta, O. Chatchawankanphanich, K.P. Akhtar, T. M. Shah, R. M. Nair, T. S. Bains, A. K. Sirari, Kaur and P. Srinives (2013). Detection of quantitative trait loci for *Mungbean yellow mosaic India virus* (MYMIV) resistance in mungbean (*Vigna radiata* (L.) Wilczek) in India and Pakistan. Breeding Sci., 63, 367-373

Kothandaraman, S. V., A. Devadason and M. V. Ganesan (2016). Seed-borne nature of a begomovirus, *Mungbean yellow mosaic virus* in black gram. *App. Microbiol. Biotech.*, 100, 19*25-1933*

Kundu, S, D. Chakraborty, A. Kundu and A. Pal (2013). Proteomics approach combined with biochemicalattributes to elucidate compatible andincompatible plant-virus interactions betweenVigna mungo and Mungbean Yellow MosaicIndia Virus. Proteome Sci., 11, 11-15

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685

Lattanzio, V., V. M. Lattanzio and A. Cardinali. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. in Phytochemistry: Advances in research Trivandrum, Kerala, India: Research, Signpost, 66, 23-67

Mourad, G., R. Emerick and A. Smith (2000). Molecular cloning and sequencing of a cDNA encoding an isoleucine feedback insensitive threonine dehydratase/deaminase of Arabidopsis line GM11b (Acc no. AF177212) (PGR 00-020). Plant Physiol., 122, 619

Nasir, F., K.P. Akhtar, A. Hameed, S. Yousaf, T. Gulzar, N. Sarwar, T. M. Shah and S. Kiran (2017). Biochemical alterations in the leaves of different Desi and Kabuli type chickpea genotypes infected by phytoplasma. Turk. J. Biochem., 42, 409-417

Patel, S. J., R. Subramanian and Y. S. Jha (2011). Biochemical and molecular studies of early blight disease in tomato. Phytoparasitica 39:269-283

Qi X, Li MW, Xie M, Liu X, Ni M, Shao G, Song C, Yim AK-Y, Tao Y, Wong, F-L (2014) Identification of a novel salt tolerance gene in wild soybean by whole-genome sequencing. Nat. Commun., *5*

Siddique, Z., K. P. Akhtar, A. Hameed, N. Sarwar, I. U. Haq and S. A. Khan (2014). Biochemical alterations in leaves of resistant and susceptible cotton genotypes infected systemically by *Cotton leaf curl Burewala virus*. J. Plant Interac., 9, 702-711

Siddique, Z., K. P. Akhtar, A. Hameed, I. Haq, M. Ashraf, N. Sarwar and M. Khan (2015). Physiological response of cotton leaf curl Burewala virus-infected plants of tolerant and susceptible genotypes of different *Gossypium* species. J. Plant Pathol., 97, 483-490

Tecsi, L. I., A. M. Smith, A. J. Maule and R. C. Leegood (1996). A spatial analysis of physiological changes associated with infection of cotyledons of marrow plants with cucumber mosaic virus. Plant Physiol., 111, 975-985

Van Asperen, K. (1962). A study of housefly esterases by means of a sensitive colorimetric method. J. Insect Physiol. 8, 401-416

Velazhahan, R. and P. Vidhyasekaran (1994). Role of phenolic compounds, peroxidase and polyphenol oxidase in resistance of groundnut to rust. Acta Phytopathol. Entomol. Hung.,29, 23-29

Venkatesan, S., R. Radjacommare, S. Nakkeeran and A. Chandrasekaran (2010). Effect of biocontrol agent, plant extracts and safe chemicals in suppression of *Mungbean yellow mosaic virus* (MYMV) in black gram (*Vigna mungo*). Arch. Phytopathol. Plant Protec., 43, 59-72

Zhang, J. and M. Kirkham (1994). Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. Plant Cell Physiol., 35, 785-791.