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Molecular Approach Coupled with Biochemical Attributes to Elucidate the Presence of DYMV in Leaf Samples of *Lablab purpureus*. L Genotypes

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Abstract A laboratory study was delineated to ascertain the impact and the extent of Dolichos yellow mosaic virus (DYMV) on biochemical constituents and various enzyme levels in the leaves of hyacinth bean. DYMV-infected leaves of all the genotypes used in the study revealed significant and consistent changes in activities of CAT, APX, PPO, DHAR, and MDHAR paralleled with a compelling hike in proline levels. Unlike that in non-infected leaves of the genotypes VRSEM-301 and VRSEM-749, VRSEM-894 and VRSEM-855, the enzyme level did not alter much which extended equally with increased phenolics, suggesting a well-coordinated generation of free radicals thereby suppressing oxidative stress in the latter. The genotypes were also evaluated at molecular level for elucidating the presence of the virus by using five sets of primer pairs. Two primers viz., DAC1 and DAC2 witnessed the presence of the virus in both non-infected and infected leaves. The difference in the appearance and/or disappearance of bands according to non-infected to infect reverberates the variation between genotypes in defense against infection.

Keywords Dolichos yellow mosaic virus (DYMV) · *Lablab perpureus* · Antioxidant enzymes · Photosynthetic pigments

Abbreviations

DYMV	Dolichos yellow mosaic virus
DHAR	Dehydroascorbate reductase
MDHAR	Monodehydroascorbate reductase
APX	Ascorbate peroxidase
PPO	Polyphenol oxidase
ROS	Reactive oxygen species

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CAT	Catalase
Chl a	Chlorophyll a
Chl b	Chlorophyll b
CAR	Carotenoid
Pro	Proline
DAS	Days after sowing

Introduction

Hyacinth bean (*Lablab purpureus* L.) has been predetermined as a multipurpose crop used for green vegetable, pulse, forage, soil improvement, soil protection, and weed control [19]. Besides, it also has a great potential as medicinal legume and imperative as therapeutic agents and in various chronic diseases [26]. The beans, including hyacinth bean, are also a great source of flavonoids and isoflavones such as dalbergioidin, phaseollidin, and vignafuran which ought to have divergent functions in plants, including protection against UV damage and pathogenic microbes, acting as pigments or co-pigments in influencing flower color, modulating auxin distribution, and as signal molecules to symbiotic microbes [8].

Many plant pathogens are known to infect hyacinth bean such as fungi, bacteria, nematodes, viruses that alter, crop growth, and productivity where environmental factors such as temperature and humidity play a compelling role in disease manifestation and severity [35]. The Dolichos yellow mosaic virus (DYMV) has only been recently discovered as a bipartite begomovirus belonging to the genus begomovirus and family geminivirdae [17]. The virus was proclaimed to have a very narrow host range consisting of only the host from which it was isolated, hyacinth bean (*Lablab purpureus*), and is characterized by faint chlorotic specks on leaf lamina, which later foster bright yellow mosaic patches with small islands of green tissue which are seldom deformed [21].

DYMV is transmitted by a vector whitefly (*Bemisia tabaci*) in the circulative persistent manner whose transmission is enhanced under high humidity and moderate to high temperature, which causes huge loss to the crop (20–25 %), and apart from the above factors, seeds also serve as one of the modes for virus conveyance [16]. Upon virus infection, there are spatial and temporal distortion in leaf chlorophyll content which can be applied as one of the attributes for screening the pathogen toxin response in the early stage of plant growth and development. Viruses depend on the host for their replication and other metabolic processes which instigate significant changes in their normal physiological processes such as loss of pigment contents, increasing respiration rates, soluble sugar, and starch accumulation and production level of enzymatic antioxidants. Among the enzymes, peroxidase and catalase are the first to show changes in their level of production during viral infections and divulged to increase in plant tissues under stress conditions, mechanical injuries, or attack by parasitic organisms [34].

Hyacinth bean is an important vegetable in the central and southwestern parts of India, and the species is largely cultivated as a system component of home gardens or mixed cropping schemes, whose specific contribution to the overall system is usually not recorded. It has been documented as part of traditional production systems, such as those based around irrigated agriculture in the oases of Oman, home gardens in Nepal, India, Bangladesh, and other tropical countries [10]. As this plant is of great economic importance in controlling soil erosion, managing soil fertility, and solving nutrient imbalances in livestock, systematic research with

aspiration have been needed. Therefore, the present study was extended to investigate differences in physiological and biochemical parameters in infected and non-infected leaves of hyacinth bean, as well as evaluation of genotypes at molecular level for validating the presence of viruses through PCR. In the future, we are planning to elucidate/study compatible and incompatible plant virus interactions through proteomics approach between hyacinth bean and DYMV.

Materials and Methods

Plant Materials, Crop Raising, and Data Recording

Seeds of ten hyacinth bean genotypes (VRSEM-855, 1003, 887, 894, 301, 749, 3, 186, 941 A and 7) was sown and raised at the experimental farm of Indian Institute of Vegetable Research, situated at longitude and latitude of 82052/E and 25010/N and altitude of 128.93 m above mean sea level at Varanasi, UP during the first week of August 2012. The experiment was conducted in triplicate in randomized block design superseded with all recommended agronomic practices to raise good crop and was obliterated with the use of pesticides during the entire period of delving. Both meteorological and data on disease prevalence was recorded on individual plant (0–3 scale) at 1 month interval starting from the last week of August 2012 (30 days after seed sowing) until the last week of August 2013. The leaf samples of non-infected and DYMV-infected leaves were collected on the last day of data recording from five random plants in each replication (three leaves/plant) and stored at −80 °C for further analysis. Severity of symptoms was examined using the following rating scale: 0=symptomless; 1= moderate chlorotic lesions and mosaic; 2=astringent lesions and mosaic, and 3=skewing and deformation. Disease severity values were calculated using the following formula according to Yang et al. [40].

Chlorophyll and Carotenoid

The chlorophyll and carotenoid were extracted in 80 % acetone [29]. The absorption of the extracts at wavelengths of 663 and 645 nm for chlorophyll and 480 and 510 nm for carotenoid was recorded with an SP 722E spectrophotometer. The concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids were then calculated using the following equations [1], and concentrations were expressed as milligrams per gram fresh weight:

$$\mathrm{Chl\,a} = 12.7A663 - 2.59A645$$
$$\mathrm{Chl\,b} = 22.9A645 - 4.67A663$$

Total Phenolic

Total phenolics were assayed according to the method as described earlier by Imeh and Khokhar [13]. Supernatants of the extracts were taken, and volume was made up to 1.58 ml with distilled water, followed by the addition of Folin-Ciocalteau (100 μl) reagent, and 20 % Na_2CO_3 (300 μl). The final aliquots were warmed for 30 min at 40 °C, cooled to initiate a complex redox reaction of phosphomolibdic acid with folin ciocalteau reagent in alkaline medium that resulted in the formation of a blue-colored complex whose intensities were

measured at 650 nm. The concentrations of phenolics in leaf samples were expressed as milligrams of catechol equivalent per gram fresh weight.

Total Flavonoids

Total flavonoid content was measured as per the method described earlier [6]. Dry tissue was powdered and extracted with 25 ml of 95 % of ethanol. An aliquot (2 ml) of this extract was mixed with 100 μl aluminum chloride (10 %), 100 μl Potassium acetate, and 2.8 ml distilled water. The test solution was vigorously shaken and incubated for 30 min, and absorbance was recorded at 415 nm. A standard calibration plot was generated (Fig. 2) using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as milligrams of quercetin equivalent per gram of the sample.

Total Protein

The estimation of protein was done according to the method describe earlier [18] where 500 mg of fresh leaf tissue was homogenized in 5 ml of 0.1 M Tris buffer and centrifuged at 5000 rpm. To the supernatant, 10 % trichloroacetic acid was added and mixed well, and the mixture was again centrifuged at 5000 rpm. The pellets were dried and dissolved in 0.1 N sodium hydroxide. The intensity of blue color supernatant was recorded at 650 nm by using BSA as standard. The concentrations of protein in the test samples were calculated and expressed as milligrams of protein equivalent per gram of the sample.

Total Sugar

For the determination of total sugar, both reducing and non-reducing sugar were measured separately by using DNS (dinitrosalicylic acid) as per the method described earlier [24]. Leaf sample (1.0 gm) were homogenized in 10 ml of 80 % ethanol using pestle and mortar and centrifuged at 10,000*g* for 20 min. The extract was boiled in boiling water bath for 10 min in order to remove ethanol, and the volume was maintained up to 10 ml with distilled water. For the supernatant (0.5 ml), 3.0 ml dinitrosalicylic acid and 1.0 ml of 40 % sodium potassium tartrate was added, heated for 5 min, and the absorbance was recorded at 520 nm. Non-reducing sugar was determined as per the method described earlier by Malhotra and Sarkar [20]. All the steps were the same as for reducing sugar, except the samples were neutralized with 1 ml of 1 N sodium hydroxide. Total sugar was expressed as grams per 100 g fresh weight.

Proline

Proline levels from hyacinth bean leaf samples were assayed according to Bates et al. [5]. Fresh leaf (0.2 g) was crushed in 5 ml of 3 % sulphosalycylic acid and centrifuged at 22,000*g* (5 min). For the supernatant (2.0 ml), 2 ml each of acid ninhydrin and glacial acetic acid was added and kept in a boiling water bath (100 °C) for 1 h. Finally, 4 ml of toluene was added thoroughly to the supernatant with vigorous stirring, and absorbance was recorded at 520 nm against blank (toluene) in an Elico SL-159 UV/VIS spectrophotometer (Perkin elmer, elico Ltd. China). L-Proline (Merck Millipore USA) was used for the preparation of the standard

curve, and the amount of proline was expressed in micrograms of proline per gram fresh weight.

Catalase

Catalase activity was assayed as earlier [22]. About 200 mg of fresh leaf tissue was extracted in 5 ml of 50 mm Tris NaOH buffer (pH 8.0) incorporated with 2 % (*w*/*v*) polyvinyl pyrrolidone (PVP), 0.5 % (*v*/*v*) Triton X-100, and 0.5 mm EDTA. The extracted solution was centrifuged at 22,000×*g* for 10 min at 4 °C, and dialyzed supernatant was used for enzyme assay. Absorption of the assay mixture of total volume (3 ml) containing 50 mm H_2O_2, 100 mm potassium phosphate buffer (pH 7.0), and 200 μl enzyme extract was recorded for 5 min at 240 NM (extinction coefficient of 0.036 mM^{-1} cm^{-1}) followed by a gradual decrease in absorbance at 25 °C. Catalase activity was expressed as micromoles of H_2O_2 oxidized per minute per milligram protein.

Ascorbate Peroxidase

Ascorbate peroxidase (APX) was determined as per previously published procedure [27]. Leaf sample (200 mg) was extracted in 5 ml of potassium phosphate buffer (50 mm, pH 7.8) containing 1 % PVP, 1.0 mm EDTA, 1 mm phenylmethylsulfonylfluoride, and 1.0 mm ascorbic acid (AsA). The extract was then centrifuged at 22,000×*g* for 10 min at 4 °C, and the resulting supernatant was dialyzed with the same extraction buffer. The assay mixture (3 ml) contained 50 mm potassium phosphate buffer (pH 7.0), 0.2 mm H_2O_2, 0.5 mm AsA, 0.2 mm EDTA, and the enzyme. H_2O_2 was added last, and absorbance was recorded at 290 nm (extinction coefficient of 2.8 mM^{-1} cm^{-1}) up to 5 min. The enzyme-specific activity is expressed as micromoles ascorbate oxidized per minute per milligram protein.

Polyphenol Oxidase

Polyphenol oxidase (PPO; EC 1.14.18.1) activity was assayed as per previously described methods of Soliva et al. [37]. Leaf samples (200 mg) were crushed in 10 ml of (0.2 M) potassium phosphate buffer and centrifuged at 12,000 rpm (4 °C for 20 min). Catechol (1 ml) (0.05 M) and 0.2 M potassium phosphate buffer (3.5 ml) was incubated at 30 °C for 30 min and then 500 μl of enzyme supernatant was added. The assay was initiated at room temperature, and activity was measured by monitoring increase in absorbance for 3 min at 410 nm (extinction coefficient of 2.8 mM^{-1} cm^{-1}). The specific activity of the enzyme was expressed as micromoles catechol oxidized per minute per milligram protein.

Dehydroascorbatereductase

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was estimated according to the published methods of Roudriguez-Sanchez et al. [32] and Nakano and Asada [27]. Fresh leaf tissue (200 mg) was crushed in 5 ml of 25 mm sodium phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 15 min at 4 °C. An aliquot (2.0 ml) contained 2.5 mm reduced glutathione, 0.4 mm dehydroascorbate reductase, 25 mm sodium phosphate buffer (pH 7.0), and 0.2 ml enzyme extract. The gradual increase in DHAR activity was measured by monitoring absorbance for 3 min at 265 nm (extinction coefficient of 14 mM^{-1} cm^{-1}). The

specific activity of the enzyme was expressed as micromoles NADPH oxidized per minute per milligram protein.

Monodehydroascorbate reductase

The monodehydroascorbate reductase (MDHAR; EC1.6.5.4) activity was assayed according to the method described by Roudriguez-Sanchez et al. [32]. Fresh leaf tissue (200 mg) was extracted in 5 ml of HEPES-HCl buffer (100 mm, pH 7.6) and centrifuged at 12,000 rpm for 15 min; the supernatant was used as enzyme extract. The rate of NADPH oxidation in the assay mixture (2 ml) containing 0.2 ml enzyme extract, 100 mm HEPES-HCl buffer (pH 7.6), 0.15 mm NADPH, and 2.5 mm AsA were recorded by a monitoring transition in absorbance at 340 NM (extinction coefficient of 6.2 mM^{-1} cm^{-1}), and the enzyme-specific activity was expressed as micromoles ascorbate oxidized per minute per milligram protein.

DNA Extraction and PCR-Based Confirmation

The DNA was extracted from healthy and diseased (showing characteristic symptoms of yellow mosaic disease) samples of Dolichos by cetyl trimethyl ammonium bromide (CTAB) method with some modifications as described by Doyle and Doyle [7]. The quantity and quality of the isolated DNA were determined using Dyna Quant 200 Fluorimeter, 0.8 % agarose gel stained with ethidium bromide (etbr). Five previously reported sets of primers [36] designed from available sequence data of DoYMV (AY309241) were used for confirmation of resistant and susceptible lines (Table 1). The PCR amplification was carried out in a 25 μl reaction mixture, containing 15 ng genomic DNA, 25 mm magnesium chloride, 10 mm dinucleotides Tri phosphates, 10× assay buffer, 0.4 μm primer, and 0.6 U of Taq polymerase. Amplification was performed in dome-shaped capillary tubes in Bio-Metra Thermal Cycler—programmed as one cycle of initial denaturation at 94 °C for 5 min, 36 cycles each of denaturation at 94 ° C for 1 min annealing at 55–60 °C for 1 min, and elongation for 2 min at 72 °C and a final elongation at 72 °C for 10 min. The amplified products were separated by electrophoresis in 2.5 % (*w*/*v*) agarose gels, stained with ethidium bromide and photographed under ultraviolet light with Alfa InfoTech.

Table 1 Details of the primers used for detection of DYMV in hyacinth bean

S.N	Primer ID	Sequence 5′–3′	Expected size of amplified DNA fragments
1	DAC1F	GTG CGT GAA TCC GTC ATT TCG	1100 bp
	DAC1R	ATA CAC CGA TGA CGT GGC AAT	
2	DAC2 F	ATA CAT ATT CGC AAC CGC GTA	500 bp
	DAC2R	C CA CGT TCG TCC TAT AAA GAG	
3	DAC3 F	GCA ACC GCG TAA TAC ATT GTT	380 bp
	DAC3R	AAC GAT TCG GCG TAA GCG AAT	
4	DAC4 F	CTC AGA TTG TGG TAC TGG AAC	390 bp
	DAC4R	TGTAAG AAG CTC TCG AGC AAC	
5	DAV2 F	GCT CCG TGG AGT CTT ATT TAG	400 bp
	DAV2R	ATG GTA CAT CGC GTG AAC GAT	

Statistical Analysis

The experiment was arranged in a complete randomized design (CRD), and the results were statistically analyzed using SPSS (1988) software. The mean comparisons among treatments were determined by Duncan's multiple range tests at the 5 % level of probability.

Result and Discussion

Peak Period for Incidence of DYMV

Attack of virus and other abiotic stresses are often associated with increased reactive oxygen species (ROS) levels. Prolonged viral infection inevitably causes ROS overproduction, which perturbs plant metabolism, leading to oxidative damage to the cellular components which prevails its productivity [11]. The responses of varieties varied greatly with respect to their level of resistance and susceptibility ranged from 20 to 100 %. The data of temperature and humidity for experiencing the peak period of DYMV incidence has been represented in Fig. 1a. Initially, all the genotypes were free from yellow mosaic disease, but as the growth progresses, the genotypes started showing yellow mosaic disease symptoms. Temperature between 36.8 and 42.6 °C and 18.8 to 26.4 °C respectively, with RH of 80 to 97 %, contemplated the impact of DYMV (range 20 to 100 %) in all the varieties. Lakshminaryan et al. [16] ascertained that spread of virus in *Vigna radiata* plants was varietal and vector-dependent and also remembrances of the susceptibility trend of genotypes of *Plectranthus barbatus* with respect to white fly remained similar to that of YMV (temperature and RH) which support our findings.The genotypes VRSEM-941 and VRSEM-3 were the first to show yellow mosaic disease symptoms (10 %) in the months of October/November where maximum/minimum temperature was 32.2/21.2 °C coupled with maximum relative humidity at 99.1 and 82 %. The growth of disease was stagnated from Nov 2012 to Feb 2013 but gradually expanded during the day and night temperature in March (31.2 °C) in all the genotypes (20–100 %). The result ascertained manifested that at 36.8 °C temperature and humidity of 97 %, the DYMV had a peak incidence (Figs. 1b and 2).

Effect of DYMV Incidence on Photosynthetic Pigment

All infected genotypes exhibited significant reduction in photosynthetic pigment contents (chlorophyll a, chlorophyll b, carotenoids, and total pigment) compared to non-infected genotypes (Fig. 3g). The percent of reduction in total pigment content of the infected genotypes ranged between 6.1–64.7 %. In infected genotypes, a significant reduction in chlorophyll b, about 21.2 to 41.21 % as compared to chlorophyll a was observed indicating that chlorophyll b is more sensitive to viral attack. Similarly, the carotenoid content (Table 2) was also significantly lower in all infected genotypes. The percent of reduction reached 43.9 and 78.1 % in infected genotypes. In non-infected genotypes, VRSEM-894 and VRSEM-749 possessed the higher carotenoid contents (43.9–78.1 %) compared to their corresponding counterparts. The high concentration of chlorophyll in VRSEM-1003, 855, 887, and 307 may be due to surpassing of chlorophyll and contraction in size of stomata juxtapose to susceptible cultivars as scrutinized by Awasthi [3]. The decrease in the ratio might be due to the generation of ROS causing damage to photosystem II, which in turn suggests that plants

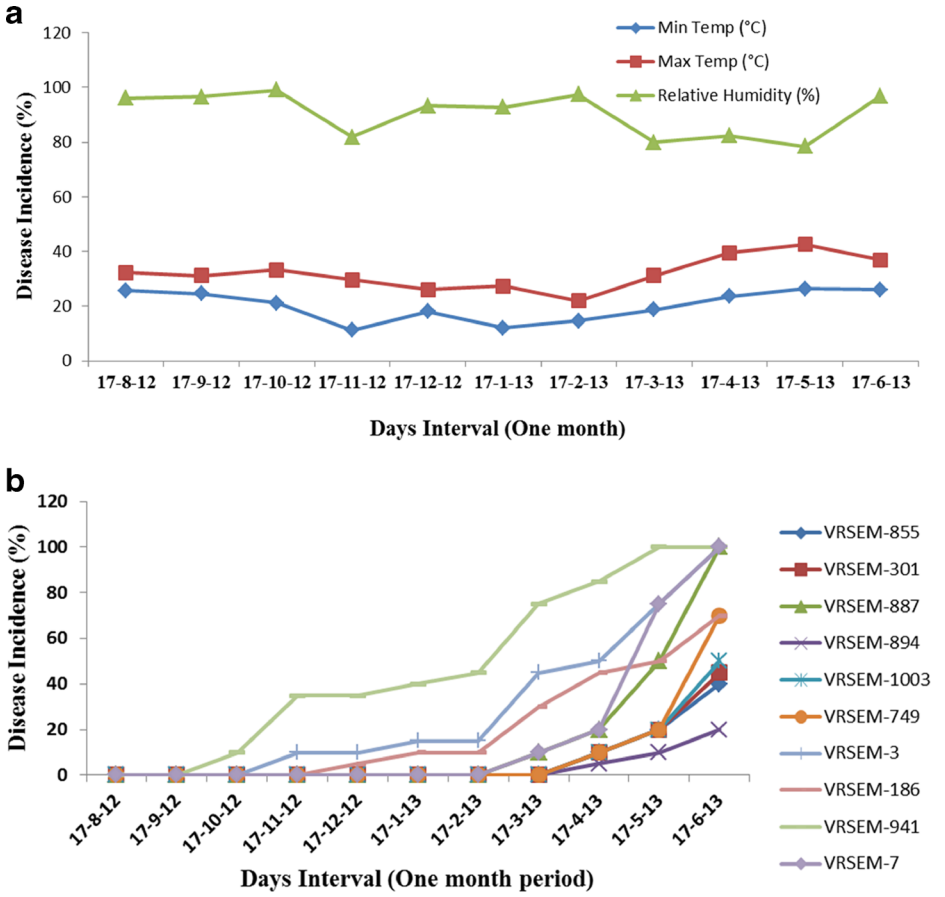

Fig. 1 **a** Curve showing changes in mean maximum/minimum temperature and relative humidity during entire period of disease progression in field condition. **b** Disease progress curve of different hyacinth bean genotypes for ten consecutive months of disease scoring

failed to capture the light energy, and photosynthesis eventually was decreased or abated as ascertained by Barka et al. [4].

Protein and Sugar Content

Results in (Table 2) also showed that the total protein content changes regarding viral infection in all hyacinth bean genotypes thereby confirming the accumulation of protein as a response to viral infections. In non-infected leaves, the increase in total soluble proteins was about 33.28 to 42.30 %, respectively, as compared to infected ones. In genotypes viz., VRSEM-860 and VRSEM-186 the viral infection caused an increase from 15.61 to 27.04 % of the total soluble protein content. A similar trend was observed in protein content due to fungal infection in coconut [28]. During host-pathogen interaction, amino acids act as a substrate for the pathogen or they may have a fungistatic effect through their involvement in metabolic reactions associated with disease resistance [25].

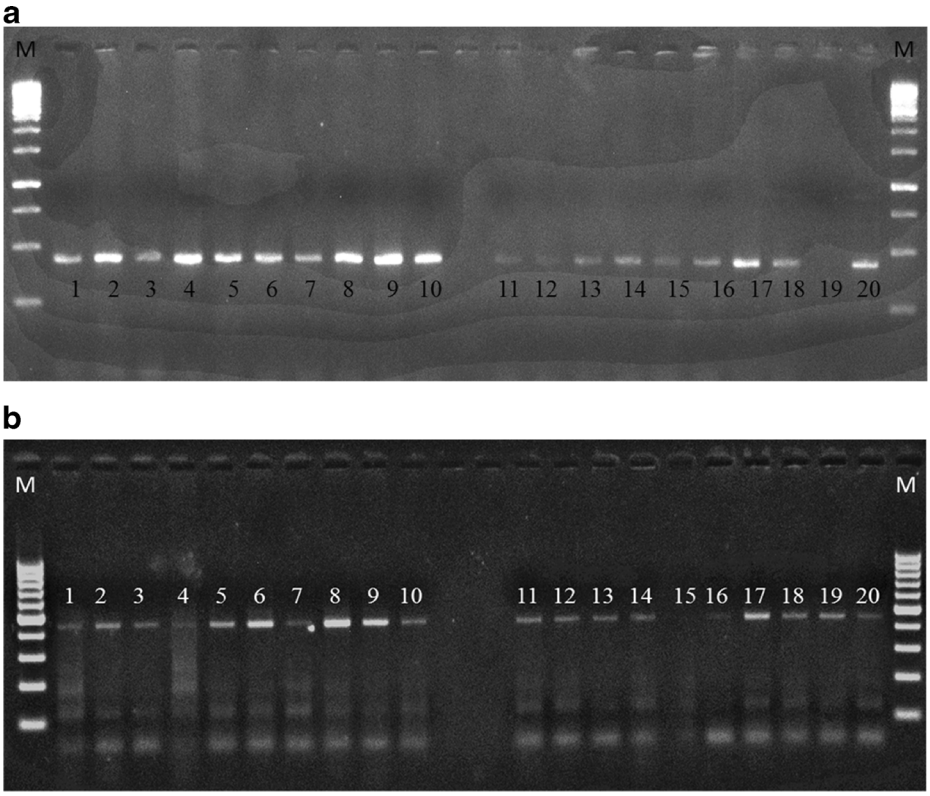

Fig. 2 **a** PCR amplification of coat protein gene of DYMV by primer DAC-1 in symptomatic and non-symptomatic leaves of hyacinth bean. **b** PCR amplification of coat protein gene of DYMV by primer DAC-2 in symptomatic and non-symptomatic leaves of hyacinth bean

In infected leaves, there was a significant decrease in reducing sugar and total sugar content (Fig. 3h), and DYMV seems to be the plausible factor causing the decrease (Fig. 3h). Virus-infected leaves of VRSEM-855, VRSEM-894, and VRSEM-3 showed a slight increase of 22.5 and 30 % in non-reducing sugar content. Interestingly, the total sugar content seems to be contracted in infected leaves. Among non-infected leaves of the genotypes, VRSEM-894 and VRSEM-1003 had the highest increase, 55.66 and 66.35 %, of soluble and total sugar contents respectively. These results are supported by the previous findings of Jaypal and Mahadevan [14] who described that a decrease in sugar levels may be caused by the rapid hydrolysis of sugars through enzymes secreted by the pathogen. The co-existence of free-sugars and phenols results in glycosylation of phenols by sugars, articulating phenolic glycosides, which are more soluble in the cell sap, thus provide more efficiency in the resistance expression [39].

Phenol and Flavonoid Content

Results in Table 2 showed the variation in total phenolic compound of hyacinth bean genotypes in response to infection with DYMV. The total phenolic content seems to be higher in non-infected leaves than in infected ones. A gradual increase in total phenolic contents of about 56.76 to 58.98 % (Table 2) was noticed in non-infected ones. The genotype VRSEM-

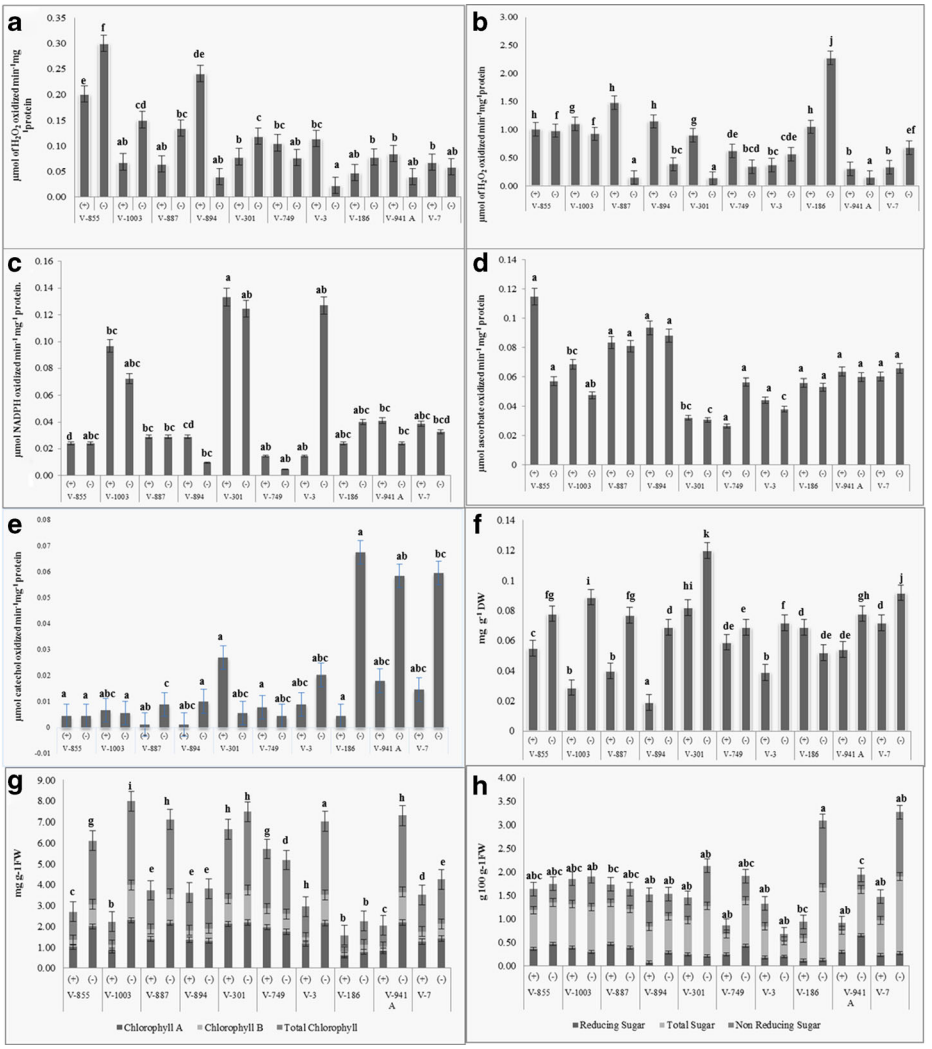

Fig. 3 **a** Peroxidase (POX), **b** catalase (CAT), **c** dehydroascorbate reductase (DHAR), **d** monodehydroascorbate reductase (MDHAR), **e** polyphenol oxidase (PPO), **f** flavonoids, **g** chlorophyll, and **h** sugar concentrations in ten hyacinth bean genotypes with (+) or without (−) infection with DYMV. Each value represents the mean±standard error (n=3). *Means with different letters* above bars were significantly different at the 0.05 level according to Duncan's multiple range test

887 had a significantly higher amount of total phenols followed by VRSEM-7 exhibiting an increase of about 49.01 % as compared to other genotypes. Rivero et al. [31] propounded that an accumulation of phenolic compounds in response to stress would be attributed to the activation of phenylalanine ammonia lyase (PAL) [9]. They also enumerated that many kinds of plant phenolic compounds have been considered to be preeminent for cell acclimatization against stress in plants. Phenolic compounds act as a substrate for many antioxidant enzymes thus it mitigates the stress injuries, and especially, oxidized forms are toxic to certain pathogens and may take part in the plant defense mechanisms [30].

Table 2 Levels of protein, phenol, carotenoid, flavonoid, proline and hydrogen peroxide in hyacinth bean genotypes without (−) or with (+) infection with DYMV

Genotypes	DYMV Infection	Carotenoids (mg g^{-1}FW)	Protein (mg g^{-1}FW)	Phenol (mg g^{-1}FW)	Proline (μg g^{-1}FW)	H_2O_2 (μm g^{-1}FW)
V-855	(+)	0.37±0.03bcd	84.1±0.2a	3.25±0.07b	1.09±0.08a	12.7±0.16f
	(−)	1.08±0.08g	144.6±0.3j	4.15±0.08fgh	0.48±0.08a	13.7±0.12g
V-1003	(+)	0.38±0.03bcd	106.3±0.4efg	3.46±0.06bc	1.37±0.09a	19.5±0.13i
	(−)	1.51±0.08h	135.1+0.4ij	5.25±0.09k	0.95±0.08a	2.1±0.08b
V-887	(+)	0.70±0.02f	96.5±0.3e	4.34±0.09hi	2.08±0.10a	16.5±0.13h
	(−)	1.19±0.08g	118.6±0.3g	9.19±0.08m	0.95±0.08a	29.3±0.07l
V-894	(+)	0.61±0.02cde	128.9±0.4h	2.10±0.07a	1.91±0.09a	9.7±0.09d
	(−)	0.47±0.02ef	213.1±0.4k	3.08±0.08a	1.06±0.10a	20.7±0.10j
V-301	(+)	0.98±0.02g	67.8±0.2bc	3.60±0.09bcd	1.18±0.09a	11.7±0.09ef
	(−)	0.96±0.03f	109.9±0.3efg	3.57±0.08cde	0.89±0.07a	21.9±0.09k
V-749	(+)	0.78±0.02f	130.3±0.4hi	4.87±0.09jk	6.25±0.10a	13.5±0.07g
	(−)	1.03±0.09g	95.2±0.4e	4.09±0.08efg	1.28±0.08a	19.8±0.07i
V-3	(+)	0.38±0.02bcd	115.1±0.4fg	7.90±0.07l	3.08±0.10a	9.5±0.10d
	(−)	1.14±0.09g	98.8±0.2ef	4.62±0.10hi	2.19±0.07a	6.4±0.09c
V-186	(+)	0.15±0.03a	99.0±0.3e	4.75±0.08ij	3.43±0.07a	0.32±0.09a
	(−)	0.28±0.02ab	71.5±0.5bc	4.45±0.07ghi	3.08+0.10a	12.2±0.10a
V-941 A	(+)	0.39±0.02ab	54.2±0.4b	4.47±0.12hi	2.51±0.07a	17.1±0.10h
	(−)	1.28±0.07bcd	53.1±0.4a	3.85±0.09def	4.60±0.10a	12.4±0.11ef
V-7	(+)	0.37±0.02bcd	75.5±0.3cd	5.48±0.08k	4.39±0.11a	9.4±0.09d
	(−)	0.47±0.02de	59.4±0.2b	9.24±0.08m	2.71±0.06a	10.1±0.09e

Each value represents the mean of three replicates±standard error. Means with different letters were significantly different at the 0.05 level according to Duncan's multiple range test

The flavonoids were observed to be less in infected leaves, and the content decreased sharply with the concomitant increase in disease infection. From the date of flavonoid analysis, it can be inferred that there was a significant increase in flavonoid content in non-infected leaves. Increase in flavonoid (Fig. 3f) content in healthy leaves was about 22.09 to 49.74 % higher being recorded in VRSEM-7. These results are substantiated with earlier findings of Sengooba et al. [33] in legumes where he reported decrease in flavonoids content with the progression of age and infection with Alternaria blight in cluster bean. However, flavonoid had a less significant role in plant defense as compared to total phenols and other dihydroxy phenols [2].

Pro and Hydrogen Peroxide Content

The results showed that infection with DYMV increased significantly the proline in the leaves of all genotypes compared to non-infected genotypes. Proline contents significantly inflated in all infected leaves which were about 66.76 to 81.98 % (Table 2) in VRSEM-941 and VRSEM-749, respectively. There was a significant increase in the hydrogen peroxide level (Table 2) in healthy leaves with the exception of two genotypes viz., VRSEM-1003 and VRSEM-3, in which infected leaves had a high level of hydrogen peroxide. Pro accumulation occurs normally in cytosol in response to biotic and abiotic stress where it has been shown to contribute to osmotic adjustment, and buffering cellular redox potential was suggested to play

a conspicuous role as stressed signal molecule [12]. Infection with yellow mosaic disease significantly increased pro content in infected and non-infected leaves of all genotypes (Table 2). The protraction of ROS is one of the earliest cellular responses following successful pathogen recognition and is commonly associated with normal plant biochemical processes such as pro accumulation with membrane destruction, protein inactivation, and DNA mutation [38]. The maximum percentage of increase reached to 43, 46, and 53 % in VRSEM-887, VRSEM-301, and VRSEM-894 genotypes, and minimum (6 and 8 %) was observed in VRSEM-855 and VRSEM-7 genotypes, respectively.

Enzyme Activities

The infection of DYMV significantly increased the activity of peroxidase (POX) in infected leaves of most genotypes except VRSEM-186 (Fig. 3a). The decrease in the activity of peroxidase in VRSEM-186 was about 30 % as compared to its non-infected leaves. The maximum percent of the increase (64 %) was observed in VRSEM-1003. POX and PPO are crucial in the defense mechanism against pathogens [15], as they oxidize phenolic compounds to Quinones, causing exaggeration in antimicrobial activity [23]. Thus, an agglomeration of phenolic compounds and high activity of antioxidant enzymes in these plants may emulate a component of defense signals stimulated by bioagent and hormonal inducers, which turned on the defense system in tomato against pathogens. Similar to POD activity, the activity of enzyme PPO in infected and non-infected genotypes increased significantly with a gradual decline thereafter (Fig. 3e). The significant elevation of 94, 76, and 70 % was observed in VRSEM-186, VRSEM-7, and VRSEM-941. The activity of CAT in general increased in both infected and non-infected leaves; however, the increase was always significantly higher in non-infected ones as compared to infect ones (Fig. 3b). The maximum percent of increase reached 114 % in VRSEM-186 genotype followed by 92 % in VRSEM-887, while VRSEM-855 and VRSEM-1003 showed significant reduction in their activity both in infected and non-infected leaves.

The DHAR activity did not differ significantly between infected and non-infected leaves of all the genotypes (Fig. 3c); although, the magnitude of DHAR activity was highest in infected leaves. The maximum percent of increase reached to 51 % in VRSEM-855 and 39 % in VRSEM-1003. In VRSEM-749, non-infected leaves exhibited significantly higher DHAR activity, which was about 54 % higher as compared to their corresponding counterpart. The unified semblance of GPX, APX, DHAR, MDHAR, and CAT helps facilitate ROS detoxification mechanism in plant cells [7]. The specific activity of MDHAR (Fig. 3d) increased in both infected as well as in non-infected leaves of all the genotypes. Non-infected leaves showed significantly higher (89 %) MDHAR activity compared with infected ones. The maximum percent increase (77 %) was observed in VRSEM-894 followed by 26 % in VRSEM-1003 despite no significant reduction in activity in the rest of the genotypes. DHAR regenerates ascorbic acid from the oxidized state and regulates the redox state of cellular ascorbic acid, which is critical for ROS-mediated tolerance to oxidative stress. In the present study, DHAR and MDHAR in infected genotypes exhibited time-dependent elevation. However, this relationship did not hold well for non-infected ones, although the levels in infected groups were marginally similar as compared to their pedants, and enhanced activities of DHAR and MDHAR were recorded in the infected leaves of VRSEM-855 and VRSEM-301. Transgenic plants of *Arabidopsis thaliana* has been reported to overexpress DHAR and MDHAR that showed a higher level of ascorbic acid with or without aluminum (Al) stress, and

it has also been noted that overexpression of DHAR and MDAHR protected tobacco plants against drought, salt, and osmotic stress [11].

PCR Based Confirmation of DYMV

The presence of DYMV in infected leaves and in non-infected leaves were analyzed by PCR using five sets of primer pairs. The primers (DAC1F/DAC1R; DAC2F/DAC2R) were amplified in both infected and non-infected genotypes thereby ascertaining the presence of 300 and 500 bp DNA fragment of coat protein (Fig. 2a, b). No amplification was contemplated with the primer pair's viz., DAC3F/DAC3R, DAC4F/DAC4R and DAV2F/DVA2R, in all the genotypes and with the negative control (PCR buffer and PCR water).

Conclusion

The present study consummates that the genotypes VRSEM-941 A, VRSEM-7, VRSEM-894, and VRSEM-855 manifesting lowest decrease percent of photosynthetic pigment and pro along with the escalation in total phenol, total sugar, and total flavonoid contents can be considered as tolerant genotypes. This accretion may lead to the formation of the phenol complex, which may not limit attack, but reduce or set of consequences on the plant strength by adjusting its physiology to fend the effects of diseases. The inflation in phenolic compounds was convoyed by the percent of increase in PPO which oxidize phenolic compounds into quinones. The same genotypes were also delineated by the increase in POX and CAT which scavenged ROS produced thereby increasing the ability of plant defense against infection. It could also be concluded that the genotypes viz., VRSEM-3, VRSEM-186, VRSEM-301, and VRSEM-1003 were characterized by decrease in proline content as these genotypes were also characterized by reduction in phenolic compounds. The behavior of these genotypes was also accompanied with the low content of POX and CAT so they can be contemplated as susceptible genotypes. It could render that in any case, higher proline accumulation in diseased tissue as noted in the present study might be related to pathogenic disorder. So, we could explicate that the degree of susceptibility of genotypes show a correlation with changes of various biochemical parameters.

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Compliance with Ethical Standards

Contribution Dr Nagendra Rai—Experiment design and manuscript correction, Mr. Krishna Kumar Rai—performed all biochemical, molecular experiment, and preparation of manuscript, Dr V. Venkataravanappa—recorded the meteorological data and DYMV incidence data, Dr Sujoy Saha—manuscript correction.

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