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ORIGINAL ARTICLE

Physiological parameters correlated with Tomato Mosaic Virus inducing defensive response in *Datura metel*

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Tail moment unit

Abstract Programed cell death resembles a real nature active defense in *Datura metel* against TMV after three days of virus infection. This adaptive plant immune response was quantitatively assessed against Tomato Mosaic Virus infection by the following physiological markers; Chlorophyll-a (mg/g), Chlorophyll-b (mg/g), total protein (mg/g), hydrogen peroxide H_2O_2 (μmol/100 mg), DNA (μg/100 mg), RNA (μg/100 mg), Salicylic acid (μg/g), and Comet Assays. Parameters were assessed for asymptomatic healthy and symptomatic infected detached leaves. The results indicated H_2O_2 and Chlorophyll-a as the most potential parameters. Chlorophyll-a was considered the only significant predictor variant for the H_2O_2 dependent variant with a P value of 0.001 and R-square of 0.900. The plant immune response was measured within three days of virus infection using the cut-off value of H_2O_2 ($\leqslant$1.095 μmol/100 mg) and ($\leqslant$3.201 units) for the tail moment in the Comet Assay. Their percentage changes were 255.12% and 522.40% respectively which reflects the stress of virus infection in the plant. Moreover, H_2O_2 showed 100% specificity and sensitivity in the symptomatic infected group using the receiver-operating characteristic (ROC). All tested parameters in the symptomatic infected group had significant correlations with twenty-five positive and thirty-one negative correlations where the P value was <0.05 and 0.01. Chlorophyll-a parameter had a crucial role of highly significant correlation between total protein and salicylic acid. Contrarily, this correlation with tail moment unit was ($r = -0.930$, $P < 0.01$) where the P value was <0.01. The strongest significant negative correlation was between Chlorophyll-a and H_2O_2 at $P < 0.01$, while moderate negative significant correlation was seen for Chlorophyll-b where the

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P value < 0.05. The present study discloses the secret of the three days of rapid transient production of activated oxygen species (AOS) that was enough for having potential quantitative physiological parameters for defensive plant response toward the virus.

1. Introduction

The activated oxygen species (AOS) is a short production of the oxidative burst or the form of reactive oxygen species (ROS) like radical superoxide $O^{2*}\_$, H_2O_2, singlet oxygen $^1O_2$ and hydroxyl radical OH^* (Baker and Orlandi, 1995; Dicenta et al., 2000). The reason for this response could be due to the activation of a plasma membrane-associated NAD (P) H oxidase during the response of plants to pathogens (Jabs et al., 1996). The occurrence of $O_2^*\_$ dismutase spontaneously turned into H_2O_2 by peroxidase, urate oxidase, xanthine oxidase and glucose oxidase (Bolwell et al., 1998). It was indicated that AOS are critical mediators of programed cell death in opposite reactions during the hypersensitive response (Jabs et al., 1996). ROS overload due to pathogen attack needs to prevent the largest producers of ROS which the chloroplasts and the peroxisome are an excellent control of redox homeostasis (Mateo et al., 2004).

Chlorophyll-a (Ch1-a) interacts directly with the light requiring reactions by photosynthesis. The ratio of Ch1-a in the chloroplast is three-times Chlorophyll-b (Chl-b). Porphyrin is the only one of the functional groups bonded to Chl-b compared with Ch1-a. The spread of programed cell death is due to the emerged H_2O_2 and $O_2^*\_$ as the two key ROS that contribute together with the hormones salicylic acid (SA) and ethylene (Overmyer et al., 2003).

Light energy and photosynthetic flux are important determinant factors in the plant immune response to pathogens, and certain characteristics of its defense were shared with individual reaction to increased light intensity (Mateo et al., 2004). These changes occurred in Chl-a fluorescence parameters also, in foliar water status in addition to triggering of cell death as well as bursts of ROS associated with local and systemic signal transmission (Bechtold and Patterson, 2005).

Plants possess antioxidant system mechanisms that detoxify $O_2^*\_$ and H_2O_2, The primary components of these pathways include; carotenoids, ascorbate, glutathione and tocopherols which are non-enzymatic antioxidants. These components of the antioxidant defense system are found in different subcellular compartments (Hernandez et al., 2000). The first regulation of antioxidant systems is part of the signaling pathways. It is activating defense responses which lead to the reduction of tobacco mosaic virus (TMV), coat protein contents as well as suppression of TMV disease symptoms in tobacco plants (Gullner et al., 1999). It is speculated that a decrease in AOS scavenging capacity could be required before virus replication can take place and increase rapidly. Salicylic acid (SA) showed elevated catalase (CAT), glutathione reductase (GR) and peroxidase activities; when SA was applied on *Phaseolus vulgaris* L. before inoculation with white clover mosaic potexvirus. It inhibited virus replication and symptom development (Clarke, 2002). Apricot resistance to plum pox potyvirus evaluation can be referred to differences in assessment methods, the variability of isolates, or to the original plant material (Martínez-Gómez et al., 2000).

Salicylic acid acts as a signal in the operation leading to plants' adaptive responses to environs. It is found naturally in plants, and participating in the internal signals adjust the defensive response of plants against pathogens and alter the antioxidants, and chlorophyll concentrations (Purcărea and Cachiţă-Cosma, 2010). SA implementation on tomato should be performed within a minimum interval of eight days to maintain the SA level related to the increase in plant tolerance to environmental stress (Guzmán-Téllez et al., 2014). SA reached from 3.5 to 6.0 μg/g in infected cells with TMV 144 h after SA application. On the other hand, maximum SA concentrations were determined in TMV-infected tobacco, equal to 10 μg/g fresh weight, the level was elevated as the sample approached the site of infection (144 h after it) (Enyedi et al., 1992).

Oxidative stress was indicated as a result of plum pox virus (PPV) infection, by an elevation in lipid peroxidation and protein oxidation manufacture in peach leaves, which was tracked by the diaminobenzidine (DAB) peroxidase-coupled H_2O_2 probe. An alteration in chloroplast ultrastructure occurred by PPV infection, giving rise to dilated thylakoid membranes, a diminishing amount of starch in chloroplasts from palisade parenchyma, and elevation in the number and size of plastoglobules in control plants. Due to the production of oxidative stress, an antioxidative metabolism imbalance may relate to the progression of PPV infection and symptoms in peach trees (Hernández et al., 2004).

The outcome of the present study is to evaluate some potential physiological markers of the redox status of photosynthetic electron transport carriers, as well as chlorophyll, as a predictor variant that could play a role under the pressure of EEE for H_2O_2 while ROS signaling reflects the necessity of programed cell death induction. Could quantitatively assessment of H_2O_2 cutoff values indicate the plant immune response in the first three days of virus infection?

2. Materials and methods

2.1. Virus source and programed cell death for identifying antiviral activity

TMV strain obtained according to (Ara et al., 2014) was tested for antiviral activity in *Datura metel* plant leaves. The whole of the nine plants was used for the application was divided into two portions; each containing replicates for analysis. The virus extract source was mechanically inoculated onto the leaves of the symptomatic group while the second portion was treated as a control (asymptomatic group). Plant immune response against TMV was determined in the detached leaves with local lesions within three days.

2.2. Determination of H_2O_2

A method of ferrous ammonium sulfate/xylenol orange (FOX) that was modified (Halliwell and Gutteridge, 1999) was conducted on the leaf extracts to determine H_2O_2 contents. The amended assay mixture contained 250 lM ferrous ammonium sulfate, 100 lM sorbitol, and 100 lM xylenol orange in 25 mM H_2SO_4. The modified test includes 1% ethanol (EtOH), and was designated eFOX. The visible light spectrum, as well as complete UV, were monitored continuously with a diode array instrument (Ocean Optics S2000, Ocean Optics Inc., Dunedin FL) which had greatest sensitivity and stability by detecting the difference in absorbance between 550 and 800 nm. After at least 15 min from mixing the test solutions with the eFOX reagents; the color was stable for at least one hour.

2.3. DNA and RNA

The leaf powder using liquid nitrogen was suspended with 5 ml of 0.3 N NaOH and held at 30 °C for 18 h. The sediment was obtained by centrifugation after washing once with 5 ml of 0.3 N NaOH. The extract and wash were combined up to 10 ml with 0.3 N NaOH, then acidified to pH 1 with 15% PCA, at 4 °C for 40 min, and then centrifuged. The DNA–protein sediment was re-suspended using 2 ml of water and then by the addition of 2 ml of 1 N perchloric acid, held at four °C for 20 min, centrifuged, and the supernatant added to the RNA fraction, then repeated until the optical density (OD) of the solution indicated the absence of RNA nucleotides. The final RNA extract was measured to the nearest whole volume with water. The final DNA extract was made up to 5 ml with 0.5 N perchloric (Ogur and Rosen, 1950). The OD of DNA extract was measured directly with 0.5 N perchloric as a blank at 250, 260, 265, 280, and 310 mμ. The OD at 260 mμ of a solution of hydrolyzed nucleic acid containing one μg of phosphorus per ml was calculated for RNA and DNA. RNA = 0.346 (in the presence of 0.04 or 0.2 N perchloric), DNA = 0.290 (in the presence of 0.5 N perchloric).

2.4. Determination of salicylic acid

Leaf samples were frozen in liquid nitrogen for fine grinding and weighing of 0.05 g, and then placed in a 1.5 ml Eppendorf tube. 1 ml of the extraction solution (10% methanol, 1% acetic acid, and 89% distilled water) was added using an automatic pipette (PRO Accumax 100–1000 μl). The Eppendorf tube was applied to vibration for 20 s on a vortex mixer (Scientific Products S.A.) and degassed for 5 min, followed by centrifugation for 10 min at 10,000*g*. The SA assay on the filtering results by LC Agilent chromatograph, model 1120 performed and managed by EZChrom Elite Compact Software, ver. 3.3.0B (Agilent 2005–2008 MR). The chromatographic separation was conducted in a Phenomenex Luna C18 100 × 20 mm at 35 °C column. Two solvent gradients of 100% A (94.9% H2O, 5% CH_3CN, 0.1% CHOOH), to 100% B (5% H_2O, 94.9% CH_3CN, 0.1% CHOOH) were used. A wavelength of 250 nm was detected and conducted at a flow rate of 0.6 ml min^{-1} for 10 min(Guzmán-Téllez et al., 2014).

2.5. Chlorophyll analysis

One leaf punch per Eppendorf was a flash freeze in liquid N_2 (Punches with a borer that gives 0.5 cm 2 leaf disks). White re-useable plastic pestles were pre-freezed in liquid N_2, and leaf disk in Eppendorf was ground using a plastic pestle and 1 ml 80% acetone was added, rinsing off the last traces of chlorophyll from the pestle. All samples were pulse spanned and taken to the spectrophotometer for detection. Chlorophyll concentration can be measured by this formula: chlorophyll concentration in μg/ml = (OD645 × 20.2) + (OD663 × 8). This value was then multiplied by 2 to obtain chlorophyll concentration per cm^2 or by dividing the weight in mg to have total chlorophyll concentration per mg (Arnon, 1949).

2.6. Protein quantitation

According to the Lowry method (Lowry et al., 1951); 0.1 ml of 2 N NaOH was added to 0.1 ml of sample and hydrolyzed at 100 °C for 10 min in a water bath. The hydrolysate was kept to cool at room temperature and 1 ml of freshly mixed complex-forming reagent was added and the solution kept at room temperature for 10 min. 0.1 ml of Folin reagent was then added using a vortex mixer. At room temperature the mixture was kept for another 30–60 min for the detection of protein concentrations (Sapan et al., 1999).

2.7. Single Cell Gel Electrophoresis (SCGE) or Comet Assay

Isolation of nuclei was performed based on microelectrophoresis in the individual leaves. A small petri dish including 200 μl of cold 400 mM Tris–HCl buffer, pH 7.5 on the ice was used (Juchimiuk et al., 2006). The prepared slides were placed on ice for 5 min and the coverslip removed. While on the slide 110 μl of LMP agarose (0.5%) was placed and a coverslip mounted again. A horizontal gel electrophoresis tank 238 containing 300 mM NaOH, 1 mM 239 EDTA, pH > 13 was used for placing and the slides were incubated with plant cell nuclei for 15 min. *SCGE* was operated at 16 V, 300 mA for 30 min 16 V, 300 mA at 4 °C. In every slide, 50 randomly chosen cells were analyzed. A fluorescence microscope was utilized with an excitation filter of 546 nm and a barrier filter of 590 nm, in addition to computerized image analysis system (Komet Version 3.1, Kinetic Imaging, Liverpool, UK). The tail DNA (TD, %) and tail moment (TM) were used as plant immune response parameters.

2.8. Statistical analysis

Nonparametric tests including the Mann–Whitney *U* test for univariate analysis were used (Ruxton, 2006). Moreover, Kruskal–Wallis analysis of variance for multivariate was conducted for comparisons (Gibbons and Chakraborti, 2011) as well as nonparametric Spearman's rank test for correlation analysis. The area under the receiver-operating characteristic curve (AUROC) was analyzed for the cutoff values of the selected five parameters to differentiate between symptomatic and asymptomatic groups (Bewick et al., 2004). All statistical tests were performed using Statistica 8.0; a $P < 0.05$ was considered significant (StatSoft, 2001; Fetoni et al., 2008).

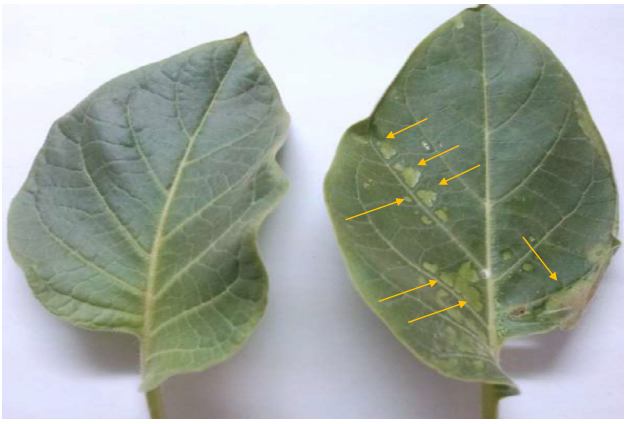

Figure 1 Detached leaves of *Datura Metel* plants. Asymptomatic (left) and Symptomatic (right) with TMV infection. Plant Programed Cell Death in response to virus infection modulated by redox status of the plastoquinone (PQ) pool.

3. Results

3.1. Evaluation of twelve physiological parameters in Datura plants having local programed cell death with TMV

TMV virus isolate induced local lesion immune response in *D. metel* leaves (Al-Huqail et al., 2014; Ara et al., 2014) after three days in Fig. 1. During this short period after virus infection, plant defense response was stimulated and assessed in the detached leaves. Significant values between symptomatic and asymptomatic groups of twelve physiological potential parameters in Table 1 and Fig. 2 were illustrated, where P value < 0.05. Five independent parameters only gave rise to a distinguished response in the symptomatic group as follows; H_2O_2 (μmol/100 mg) 255.12%, untailed cells (%) 311.54%, tail length (μm) 251.89%, tail DNA (%) 201.29% and tail moment units 522.40%.

3.2. Specificity and sensitivity of the five physiological parameters strongly triggered for virus resistance

The triggered five parameters ; H_2O_2 (μmol/100 mg), untailed cells (%), tail length (μm), tail DNA (%) and tail moment units shown in Table 2 and Fig. 3B revealed 100% for both sensitivity and specificity by AUROC between asymptomatic and symptomatic groups. The suggested cutoff value of H_2O_2 to differentiate between the two groups was 1.095 μmol/100 mg shown in Fig. 3A C and V. In Table 2 numerical measurements by Comet Assay and Genotoxicity of (C) in asymptomatic and (V) symptomatic samples assessed the immune response to DNA using tail moment in Datura plants. Suggested cutoff values of the following parameters are: tailed cells, tail length, tail DNA and tail moment units that were 7.500%, 1.645 μm, 1.880% and 3.201 units respectively.

Table 1 The independent T-test between the asymptomatic and symptomatic groups in all studied parameters. There is significant difference between asymptomatic group and symptomatic group in all parameters where P value < 0.05.

Studied parameter	Group	Mean ± S.D.	% change	P value
Chlorophyll-a (mg/g)	Asymptomatic	2.73 ± 0.09	100.00	0.001
	Symptomatic	1.98 ± 0.16	72.43	
Chlorophyll-b (mg/g)	Asymptomatic	1.12 ± 0.08	100.00	0.007
	Symptomatic	0.88 ± 0.10	78.19	
Total protein (mg/g)	Asymptomatic	3.43 ± 0.08	100.00	0.003
	Symptomatic	3.07 ± 0.13	89.51	
H_2O_2 (μmol/100 mg)	Asymptomatic	0.65 ± 0.07	100.00	0.001
	Symptomatic	1.66 ± 0.18	255.12	
DNA (μg/100 mg)	Asymptomatic	8.41 ± 0.32	100.00	0.001
	Symptomatic	6.86 ± 0.33	81.53	
RNA (μg/100 mg)	Asymptomatic	99.28 ± 3.36	100.00	0.020
	Symptomatic	89.77 ± 4.87	90.42	
Salicylic Acid (μg/g)	Asymptomatic	4.69 ± 0.32	100.00	0.006
	Symptomatic	4.05 ± 0.18	86.42	
Untailed cells (%)	Asymptomatic	95.67 ± 2.08	100.00	0.005
	Symptomatic	86.50 ± 3.51	90.42	
Tailed cells (%)	Asymptomatic	4.33 ± 2.08	100.00	0.005
	Symptomatic	13.50 ± 3.51	311.54	
Tail length (μm)	Asymptomatic	1.06 ± 0.16	100.00	0.001
	Symptomatic	2.67 ± 0.45	251.89	
Tail DNA (%)	Asymptomatic	1.42 ± 0.11	100.00	0.001
	Symptomatic	2.87 ± 0.57	201.29	
Tail moment units	Asymptomatic	1.50 ± 0.21	100.00	0.002
	Symptomatic	7.85 ± 2.78	522.40	

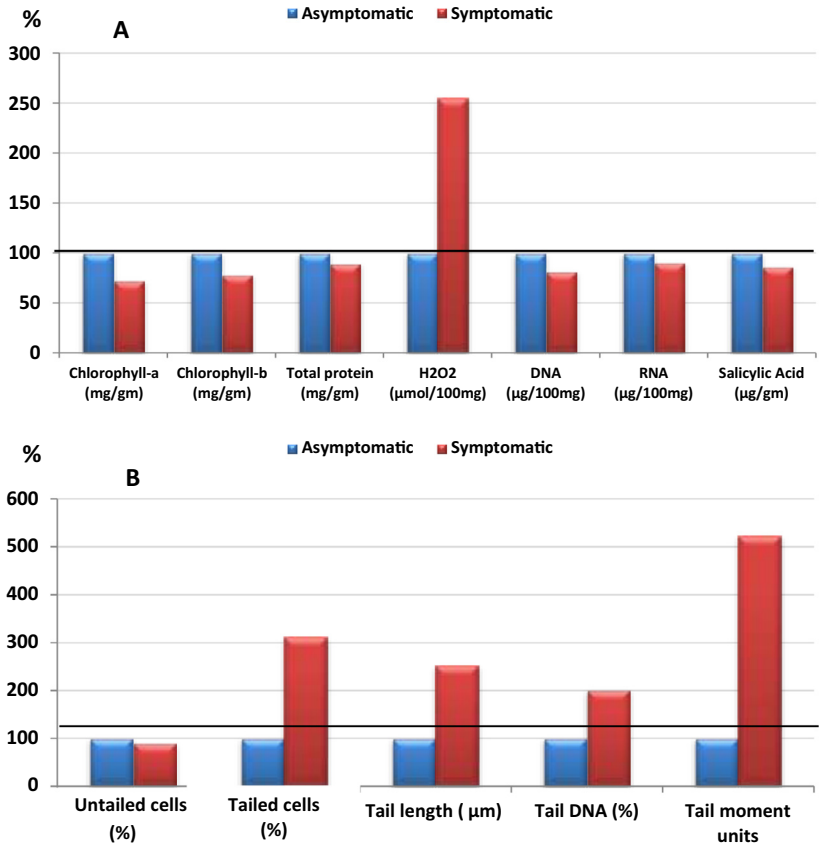

Figure 2 Percentage change of all studied parameters in the symptomatic group of *Datura metel* leaves infected with virus 3 days after infection.

Table 2 The area under the receiver-operating characteristic (ROC) for the symptomatic group increased in all five parameter levels versus asymptomatic: AUROC = 1.00 with 100% sensitivity 100% specificity.

Increased changes Parameters	Area under the curve	Cutoff value	Sensitivity %	Specificity %
H_2O_2 (µmol/100 mg)	1.000	1.095	100.0	100.0
Tailed cells (%)	1.000	7.500	100.0	100.0
Tail length (µm)	1.000	1.645	100.0	100.0
Tail DNA (%)	1.000	1.880	100.0	100.0
Tail moment units	1.000	3.201	100.0	100.0

3.3. Symptomatic group and the correlation between physiological studied parameters

As shown in Table 3, fifty six significant correlations were found between twelve physiological studied parameters at the 0.05 and 0.01 levels with *R* (Person correlation). 25 (+) positive correlations were found between all parameters in the symptomatic group, while 31 negative correlations (−) were found significant within the same group at 0.05 and 0.01 levels. The strongest significant positive correlations were illustrated in Figs. 4–6. There were significant positive correlations at 0.01 between Chl-a (mg/g) ~ total protein (mg/g) ($r = 0.927$, $P \leqslant 0.001$); Chl-a (mg/g) ~ salicylic acid (µg/g) ($r = 0.907$, $P \leqslant 0.001$); H_2O_2 (µmol/100mg) ~ tail length (µm) ($r = 0.946$, $P \leqslant 0.001$) and tail DNA (%) ~ tail moment units ($r = 0.994$, $P \leqslant 0.001$). The strongest significant negative correlations at 0.01 were Chl-a (mg/g) ~ H_2O_2 (µmol/100 mg) ($r = -0.955$, $P \leqslant 0.001$); Chl-a (mg/g) ~ tail length (µm) ($r = -0.966$, $P \leqslant 0.001$); Chlorophyll-a (mg/g) ~ tail DNA (%) ($r = -0.938$, $P \leqslant 0.001$); Chl-a (mg/g) ~ tail moment units($r = -0.930$, $P \leqslant 0.001$); total protein (mg/g) ~ tail length (µm) ($r = -0.969$, $P = 0.001$); total protein (mg/g) ~ tail moment units ($r = -0.956$, $P \leqslant 0.001$) and untailed cells (%) ~ tail DNA (%) ($r = -0.981$, $P \leqslant 0.001$) in Figs. 5 and 6.

3.4. Chlorophyll-a is a (+) predictor of H_2O_2 and tail DNA (%) is a (−) predictor of tail moment units in the symptomatic group

Multiple regression analysis was utilized to find the correlation between the selected parameters using *SPSS* program where the *P* value is 0.001 (Table 4). An R^2 of 0.900 indicated that 90% of the variation in the dependent variable (H_2O_2) was explained by the predictor variables (Chl-a). The β coefficients value (−1.256) had an adverse contribution of a variable about the chlorophyll variables in explaining the variation of the H_2O_2 variable as an immune resistant response. On the other

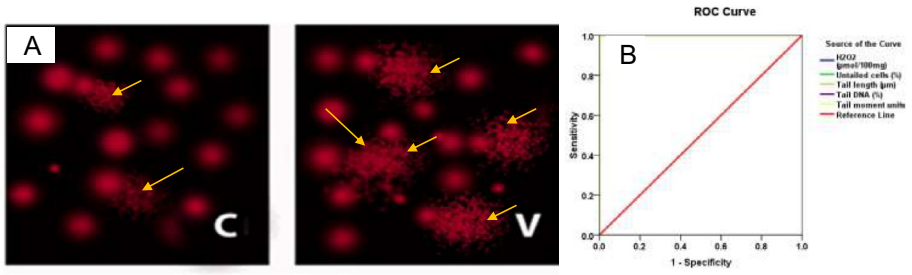

Figure 3 (A) C and V. numerical measurements by Comet Assay and Genotoxicity while C (asymptomatic) and V (symptomatic) samples meaning the damages in the DNA using tail moment in Datura plants. (B) The area under the receiver-operating characteristic (AUROC) for the symptomatic group increased in all five parameter levels versus asymptomatic: AUROC = 1.00 with 100% sensitivity 100% specificity.

hand, R^2 of 0.985 indicated that 98.5% of the variation in the tail moment unit's dependent variable is explained by tail DNA (%) as predictor variables. The β coefficient value (+4.508) had a positive contribution of variable about tail DNA (%) variables in explaining the variation in the tail moment units' variable in the symptomatic group.

4. Discussion

The reversion of plants against virus infection is associated with triggering immune defense responses that slow down or hold infection at individual level stages of the host–virus interaction. Fig. 1 showed the infected detached leaves of Datura plants that have Necrotic Local Lesions (NLL) as a hypersensitive reaction to programed cell death. As the first line of defense, plants usually evolve various physical and chemical barriers. This inducible defense response could interfere with pathogen establishment (Jones and Dangl, 2006). In the present study, twelve biochemical alteration defense parameters within the symptomatic group triggered with significant quantitative values after three days of virus inoculation explain the degree of physiological reclamation and reactions of the plant in Table 1 and Fig. 2A and B. These results indicated the highest five biochemical alteration defense level parameters for adaptive mechanisms. Survival under these stressful conditions counts on the plant's ability to perceive the stimulus, produce and transport signals, and instigate biochemical changes that adjust the metabolism accordingly (Johnsi Rani and Merlin Rose, 2012).

The most important feature of plant resistance to pathogens is early and elevated levels of expression of various defense enzymes (Vanitha and Umesha 1, 2008). Accordingly, phenolic compounds, protein, proteases, play an active role in disease resistance against Cotton Leaf Curl Burewala Virus (CLCuBuV). These consequences could impulse signal defense

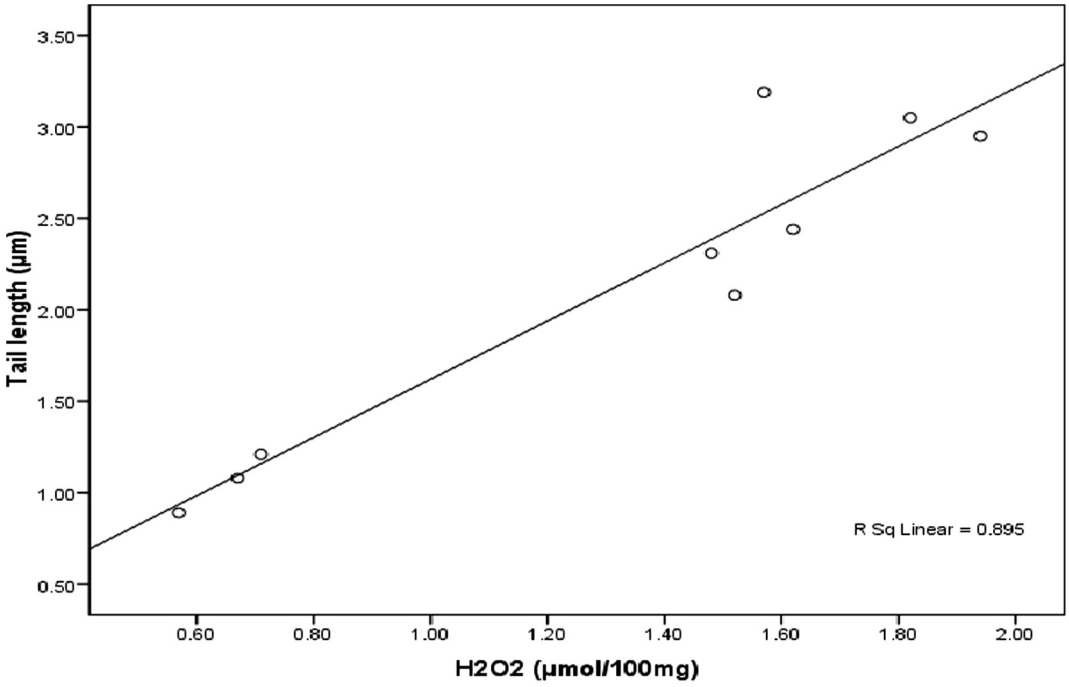

Figure 4 Strong positive correlations with tail length(highly significant (P value < 0.01)) are represented in the graphs (with best fit line curve) for the estimated H_2O_2 a in *Datura metel* infected with virus. Symptomatic leaves 3 days after infection.

Table 3 Statistical correlation at the P value $< 0.05^{*}$ and P value $< 0.01^{**}$ between all parameters in the symptomatic group.

The studied parameters Positive and negative correlations	R (Person correlation)	Sig. at 0.05 and 0.01
1. Chlorophyll-a (mg/g) ~ Chlorophyll-b (mg/g)	0.684^{*}	0.042
2. Chlorophyll-a (mg/g) ~ total protein (mg/g)	0.927^{**}	0.001
3. Chlorophyll-a (mg/g) ~ DNA (μg/100 mg)	0.844^{**}	0.004
4. Chlorophyll-a (mg/g) ~ RNA (μg/100 mg)	0.816^{**}	0.007
5. Chlorophyll-a (mg/g) ~ salicylic Acid (μg/g)	0.907^{**}	0.001
6. Chlorophyll-a (mg/g) ~ untailed cells (%)	0.893^{**}	0.001
7. Total protein (mg/g) ~ DNA (μg/100 mg)	0.802^{**}	0.009
8. Total protein (mg/g) ~ RNA (μg/100 mg)	0.747^{*}	0.021
9. Total protein (mg/g) ~ salicylic acid (μg/g)	0.760^{*}	0.017
10. Total protein (mg/g) ~ untailed cells (%)	0.934^{**}	0.001
11. H_2O_2 (μmol/100 mg) ~ tailed cells (%)	0.873^{**}	0.002
12. H_2O_2 (μmol/100 mg) ~ tail length (μm)	0.946^{**}	0.001
13. H_2O_2 (μmol/100 mg) ~ tailed DNA (%)	0.895^{**}	0.001
14. H_2O_2 (μmol/100 mg) ~ tail moment units	0.882^{**}	0.002
15. DNA (μg/100 mg) ~ Salicylic Acid (μg/g)	0.756^{*}	0.018
16. DNA (μg/100 mg) ~ untailed cells (%)	0.732^{*}	0.025
17. RNA (μg/100 mg) ~ salicylic acid (μg/g)	0.686^{*}	0.041
18. RNA (μg/100 mg) ~ untailed cells (%)	0.756^{*}	0.018
19. Salicylic Acid (μg/g) ~ untailed cells (%)	0.756^{*}	0.019
20. Tailed cells (%) ~ tail length (μm)	0.959^{**}	0.001
21. Tailed cells (%) ~ tail DNA (%)	0.981^{**}	0.001
22. Tailed cells (%) ~ tail moment units	0.973^{**}	0.001
23. Tail length (μm) ~ tail DNA (%)	0.971^{**}	0.001
24. Tail length (μm) ~ tail moment units	0.976^{**}	0.001
25. Tail DNA (%) ~ tail moment units	0.994^{**}	0.001
1. Chlorophyll-a (mg/g) ~ H_2O_2 (μmol/100 mg)	-0.955^{**}	0.001
2. Chlorophyll-a (mg/g) ~ tailed cells (%)	-0.893^{**}	0.001
3. Chlorophyll-a (mg/g) ~ tail length (μm)	-0.966^{**}	0.001
4. Chlorophyll-a (mg/g) ~ tail DNA (%)	-0.938^{**}	0.001
5. Chlorophyll-a (mg/g) ~ tail moment units	-0.930^{**}	0.001
6. Chlorophyll-b (mg/g) ~ H_2O_2 (μmol/100 mg)	-0.737^{*}	0.024
7. Total protein (mg/g) ~ H_2O_2 (μmol/100 mg)	-0.905^{**}	0.001
8. Total protein (mg/g) ~ tailed cells (%)	-0.934^{**}	0.001
9. Total protein (mg/g) ~ tail length (μm)	-0.969^{**}	0.001
10. Total protein (mg/g) ~ tail DNA (%)	-0.947^{**}	0.001
11. Total protein (mg/g) ~ tail moment units	-0.956^{**}	0.001
12. H_2O_2 (μmol/100 mg) ~ DNA (μg/100 mg)	-0.842^{**}	0.004
13. H_2O_2 (μmol/100 mg) ~ RNA (μg/100 mg)	-0.887^{**}	0.001
14. H_2O_2 (μmol/100 mg) ~ salicylic acid (μg/g)	-0.842^{**}	0.004
15. H_2O_2 (μmol/100 mg) ~ untailed cells (%)	-0.873^{**}	0.002
16. DNA (μg/100 mg) ~ untailed cells (%)	-0.732^{*}	0.025
17. DNA (μg/100 mg) ~ tail length (μm)	-0.828^{**}	0.006
18. DNA (μg/100 mg) ~ tail DNA (%)	-0.740^{*}	0.023
19. DNA (μg/100 mg) ~ tail moment units	-0.733^{*}	0.025
20. RNA (μg/100 mg) ~ tailed cells (%)	-0.756^{*}	0.018
21. RNA (μg/100 mg) ~ tail length (μm)	-0.826^{**}	0.006
22. RNA (μg/100 mg) ~ tail DNA (%)	-0.793^{*}	0.011
23. RNA (μg/100 mg) ~ tail moment units	-0.792^{*}	0.011
24. Salicylic Acid (μg/g) ~ tailed cells (%)	-0.756^{*}	0.019
25. Salicylic Acid (μg/g) ~ tail length (μm)	-0.827^{**}	0.006
26. Salicylic Acid (μg/g) ~ tail DNA (%)	-0.834^{**}	0.005
27. Salicylic Acid (μg/g) ~ tail moment units	-0.818^{**}	0.007
28. Untailed cells (%) ~ tailed cells (%)	-1.000^{**}	0.001
29. Untailed cells (%) ~ tail length (μm)	-0.959^{**}	0.001
30. Untailed cells (%) ~ tail DNA (%)	-0.981^{**}	0.001
31. Untailed cells (%) ~ tail moment units	-0.973^{**}	0.001

alarm survival of the infected plant for the incidence of H_2O_2. Accordingly, data obtained in Table 1 and Fig. 2A and B showed five quantitative parameters as percentage changes that are very convincing and responsive. About the greatest

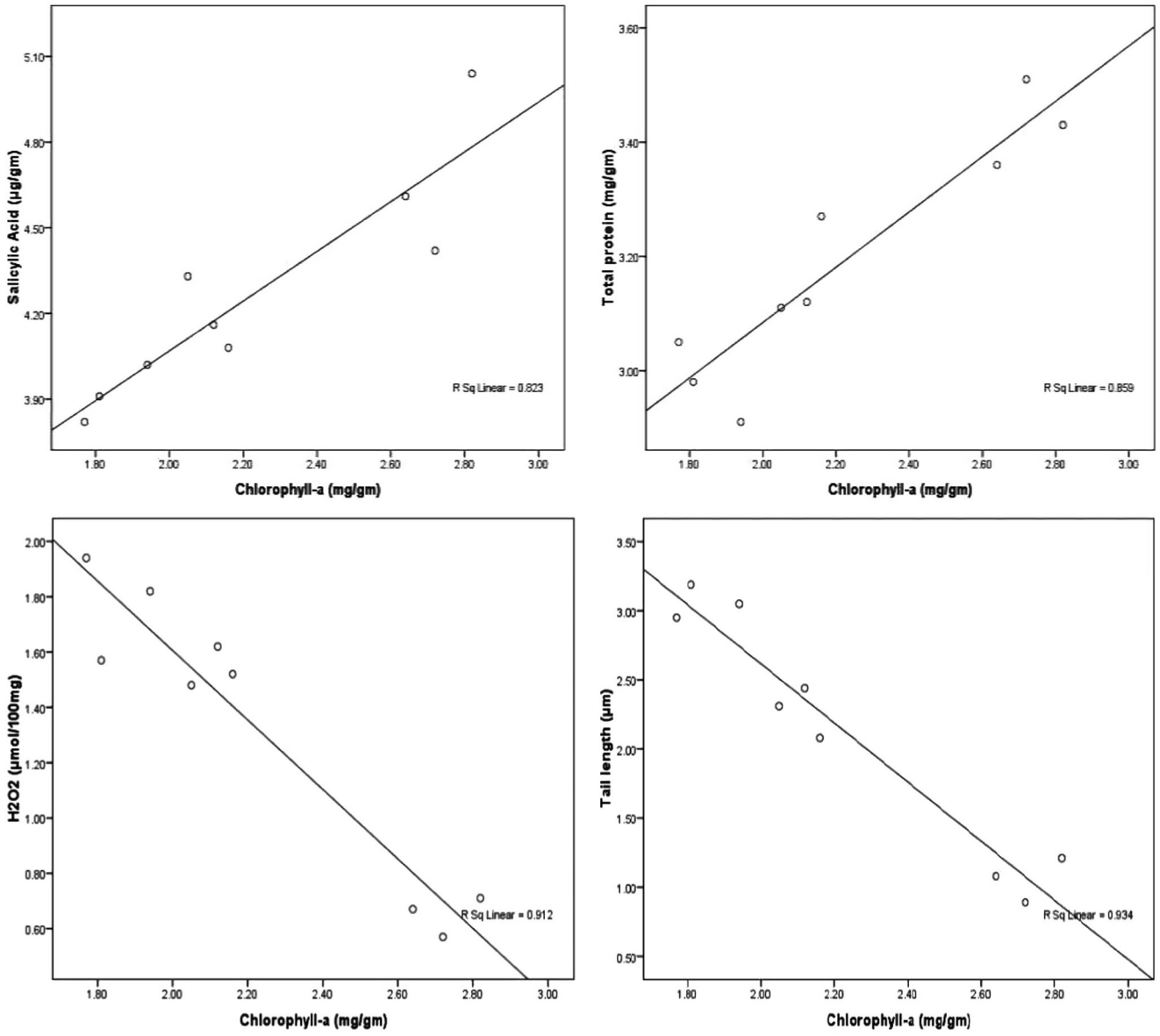

Figure 5 All positive and negative strongest correlations highly significant at P value < 0.01 that are represented in the graphs (with best fit line curve)for the estimated Chlorophyll a in *Datura metel* infected with virus in symptomatic group 3 days after infection.

values; tail moment units were 522.40%, tailed cells (%) were 311.54%, H_2O_2 (µmol/100 mg) was 255.12%, tail length (µm) was 251.89%, and tail DNA (%) was 201.29%.

Siddique et al. (2014) showed markedly higher concentrations of total phenols and crucial activity of protease, malondialdehyde (MDA), superoxide dismutase (SOD), and polyphenol oxidase (PPO) in resistant genotype after infection with Cotton Leaf Curl Burewala virus (CLCuBuV).They proposed a kind of correlation between constitutively induced levels of these enzymes and plant defense that could be contemplated as biochemical parameters for studying plant-virus compatible and incompatible interactions.

Comet Assay showed numerical measurements of the DNA damages by calculating the length of the tail and percentage of total DNA in the tail. The "tail moment unites" (TM) is the most significant and informative feature of the comet image that has 522.40% in the infected group (Table 1 and Fig. 2B). The value resembles the amount of DNA in the tail and the mean distance of migration in the tail. The higher tail moments indicate greater DNA damages. Accordingly, "tail moment" (TM) was capable, with 100% sensitivity and specificity (Cut-off value = 3.201) as reported in Tables 2 and 5. It measures and detects single- and double-strand breaks in genomic DNA. As Accordingly, our findings (Tables 1, 2 and 5, Figs. 2B, 3A and B and 4) could explain and evaluate the high genotoxic response toward TMV infection in *D. metel* due to the rapid and transient production of the oxidative burst, with AOS. The Asymptomatic value was 1.50 units while the TMV-infected cells had 7.85 units, i.e. DNA triggering response for damage to be considered equal to that value or more. The control value of the "tail moment"(TM) was 2.181 units while the TMV-infected cells had 18.870 units, i.e.

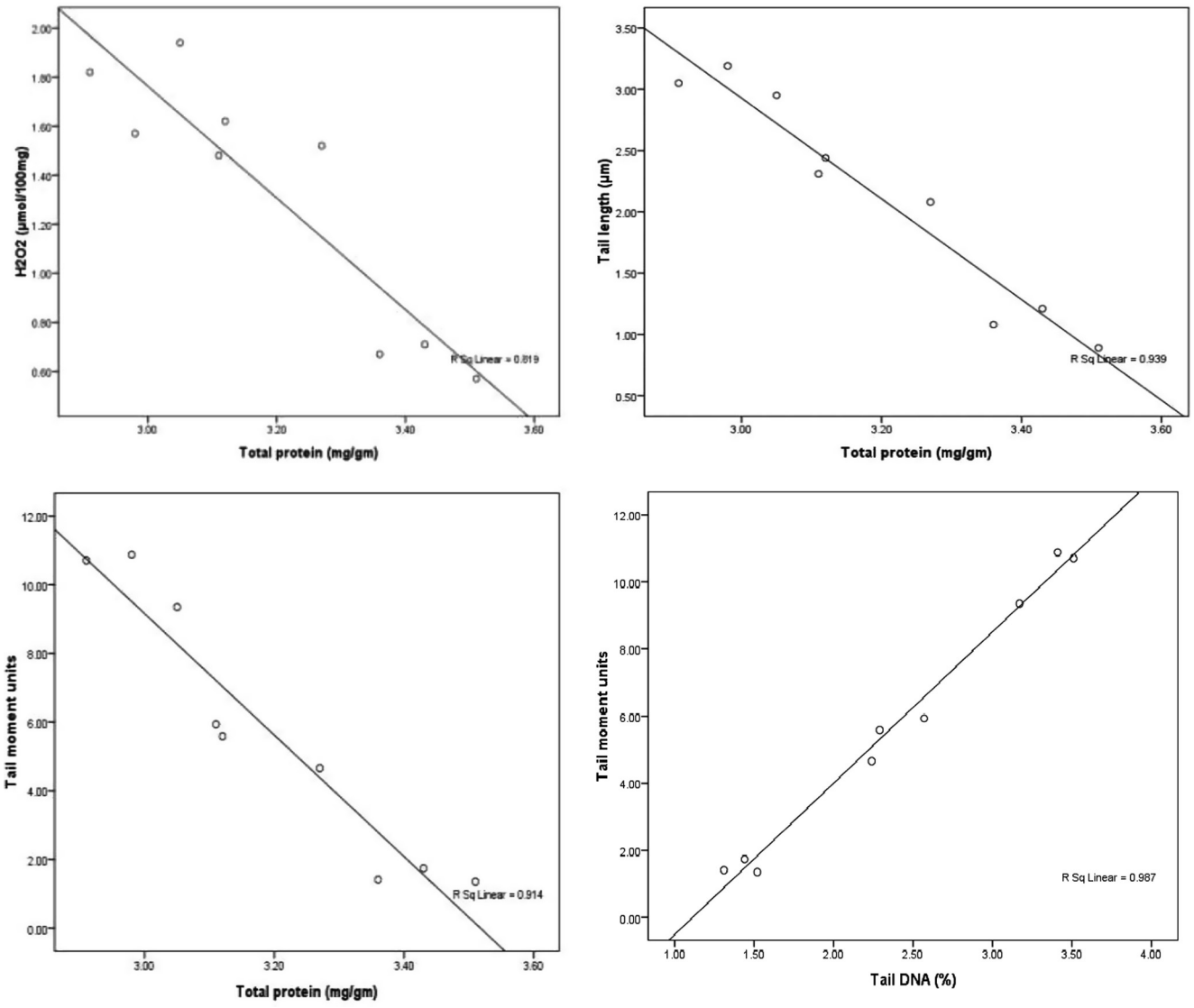

Figure 6 All positive and negative strongest correlations highly significant at P value < 0.01 that are represented in the graphs (with best fit line curve) for the estimated protein in *Datura metel* infected with virus in the symptomatic group 3 days after infection.

Table 4 Multiple regression using stepwise method for H_2O_2 (µmol/100 mg) as a dependent variable.

Predictor variable	Beta	P value	Adjusted R square	Model	
				F value	P value
Chlorophyll-a (mg/g)	−1.256	0.001	0.900	72.823	0.001

Table 5 Multiple regression using stepwise method for tail moment units as a dependent variable.

Predictor variable	Beta	P value	Adjusted R square	Model	
				F value	P value
Tail DNA (%)	4.508	0.001	0.985	539.801	0.001

ten times DNA damage after one week from infection (Al-Huqail et al., 2014). Fig. 4 showed a high correlation between H_2O_2 and tail length of DNA in the symptomatic studied group with $R^2 = 0.895$.

Concerning chlorophyll immune response, Chl-a is a crucial predictor variable parameter for the plant resistance as illustrated in Table 4. Using Multiple Regression Analysis, an R^2 of 0.90 and (β) coefficient = -1.256 indicate that Chl-a explains 90% of the variation in the dependent (H_2O_2) parameter in a negative direction. Moreover, Chl-a (+) strongly correlated with protein and salicylic acid. Contrarily, had (−) strong association with DNA damage parameters in addition to H_2O_2 (Tables 3 and 4, Figs. 5 and 6). The SA level usually interconnected with the increase in plant tolerance to environmental stress (Noreen and Ashraf, 2008). Thus, in conditions where plants are at a danger of elevating ROS-dependent;

signaling systems and metabolic processes need to be initiated depending on photosynthesis reaction. Failure to keep redox balance results in growth defects or the begining of cell death (Mateo et al., 2004). Acclimation of plant leaves to excess excitation energy (EEE) is controlled, partially by a particular alteration in the redox level of the plastoquinone (PQ) pool. It proved that the plant's response to EEE could be regulated by multiple hormonal/ROS signaling pathways, and EEE stimulates systemic acquired resistance as well as basal defenses to virulent biotrophic bacteria (Kruk and Karpinski, 2006). Chl-b acts indirectly in photosynthesis by transferring light absorbed to Chl-a and conceded an accessory pigment (Mateo et al., 2004).

Results of the present study proved a high correlation between Chlorophyll-a as a predictor of variant and redox changes of H_2O_2. 90% of the dependent variable (H_2O_2) could be explained by the predictor variables of Chlorophyll-a while the β coefficients value (−1.256) showed a negative contribution (Table 4).

It also proved that local and systemic acclimatization in *Arabidopsis thaliana* leaves in swift response to EEE, connected with cell death and managed by specific redox changes of the plastoquinone (PQ) pool. This redox alteration caused a rapid reduction of stomatal conductance, global induction of ASCORBATE PEROXIDASE2 and PATHOGEN RESISTANCE1, and elevation production of ROS and ethylene that signals through ETHYLENE INSENSITIVE2 (EIN2) (Mühlenbock et al., 2008).

Moreover, cutoff quantitative potential physiological parameters of H_2O_2, tailed cells, tail length, tail DNA and tail moment units were obtained and measured in the infected TMV plants. These were as follows: 1.095 µmol/100 mg 7.500%, 1.645 µm, 1.880% and 3.201 units respectively as defensive plant response to virus infection.

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References

Al-Huqail, A.A., Khan, F., Al-Zahim, N., Aref, N.M., 2014. Olive leaf extract trigger defense physiological markers in datura metel against tobacco mosaic virus. J. Pure Appl. Microbiol. 8 (1), 825–842, Retrieved from: <http://www.scirp.org/journal/papercitationdetails.aspx?PaperID=2135&JournalID=65#.VkdoC3YrLIU>.

Ara, I., Bukhari, N.A., Aref, N., Shinwari, M.M., Bakir, M., 2014. Antiviral activities of streptomycetes against tobacco mosaic virus (TMV) in Datura plant: evaluation of different organic compounds in their metabolites. Afr. J. Biotechnol. 11 (8), 2130–2138, Retrieved from: <http://www.ajol.info/index.php/ajb/article/viewFile/100388/89638>.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24 (1), 1.

Baker, C.J., Orlandi, E.W., 1995. Active oxygen in plant pathogenesis. Ann. Rev. Phytopathol. 33 (1), 299–321.

Bechtold, W.A., Patterson, P.L., 2005. The Enhanced Forest Inventory and Analysis Program: National Sampling Design and Estimation Procedures. US Department of Agriculture Forest Service, Southern Research Station Asheville, North Carolina.

Bewick, V., Cheek, L., Ball, J., 2004. Statistics review 13: receiver operating characteristic curves. Crit. Care 8 (6), 508.

Bolwell, G.P., Davies, D.R., Gerrish, C., Auh, C.-K., Murphy, T.M., 1998. Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. Plant Physiol. 116 (4), 1379–1385.

Clarke, P.J., 2002. Habitat islands in fire-prone vegetation: do landscape features influence community composition? J. Biogeogr. 29 (5–6), 677–684.

Dicenta, F., Martínez-Gómez, P., Burgos, L., Egea, J., 2000. Inheritance of resistance to plum pox potyvirus (PPV) in apricot, *Prunus armeniaca*. Plant Breed. 119 (2), 161–164, Retrieved from: <http://jxb.oxfordjournals.org/content/59/8/2147.full.pdf>.

Enyedi, A.J., Yalpani, N., Silverman, P., Raskin, I., 1992. Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc. Natl. Acad. Sci. 89 (6), 2480–2484.

Fetoni, A.R., Ferraresi, A., La Greca, C., Rizzo, D., Sergi, B., Tringali, G., Troiani, D., 2008. Antioxidant protection against acoustic trauma by coadministration of idebenone and vitamin E. NeuroReport 19 (3), 277–281.

Gibbons, J.D., Chakraborti, S., 2011. Nonparametric Statistical Inference. Springer.

Gullner, G., Tóbiás, I., Fodor, J., Kömives, T., 1999. Elevation of glutathione level and activation of glutathione-related enzymes affect virus infection in tobacco. Free Radical Res. 31, S155–161.

Guzmán-Téllez, E., Montenegro, D.D., Benavides-Mendoza, A., 2014. Concentration of salicylic acid in tomato leaves after foliar aspersions of this compound. Am. J. Plant Sci., Retrieved from: <http://filescirp.org/Html/27-2601457_47341.htm>.

Halliwell, B., Gutteridge, J.M., 1999. Free radicals in Biology and Medicine, 3. Oxford University Press, Oxford.

Hernandez, J., Jimenez, A., Mullineaux, P., Sevilia, F., 2000. Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ. 23 (8), 853–862.

Hernández, J.A., Rubio, M., Olmos, E., Ros-Barceló, A., Martínez-Gómez, P., 2004. Oxidative stress induced by long-term plum pox virus infection in peach (*Prunus persica*). Physiol. Plant. 122 (4), 486–495.

Jabs, T., Dietrich, R.A., Dangl, J.L., 1996. Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide. Science 273 (5283), 1853–1856.

Johnsi Rani, R., Merlin Rose, C., 2012. Salt stress tolerance and stress proteins in wheat (*Tritium aestivum* L.). Int. Res. J. Pharm. 3 (3), 143–146, Retrieved from: <www.irjponline.com>.

Jones, J.D., Dangl, J.L., 2006. The plant immune system. Nature 444 (7117), 323–329.

Juchimiuk, J., Hering, B., Maluszynska, J., 2006. FISH for micronuclei analysis in barley cells. Toxicol. Lett. 164, S57–S58.

Kruk, J., Karpinski, S., 2006. An HPLC-based method of estimation of the total redox state of plastoquinone in chloroplasts, the size of the photochemically active plastoquinone-pool and its redox state in thylakoids of Arabidopsis. Biochim. Biophys. Acta (BBA)-Bioenerg. 1757 (12), 1669–1675.

Lowry, O., Farr, A., Rosebrough, N., Randall, R.J., 1951. Protein measurement of emulsifying capacity by electrical resistance. J. Food Sci. 193, 265–275.

Martínez-Gómez, P., Dicenta, F., Audergon, J., 2000. Behaviour of apricot (*Prunus armeniaca* L.) cultivars in the presence of sharka (plum pox potyvirus): a review. Agronomie 20 (4), 407–422.

Mateo, Y., Budygin, E.A., John, C.E., Banks, M.L., Jones, S.R., 2004. Voltammetric assessment of dopamine clearance in the absence of the dopamine transporter: no contribution of other transporters in core or shell of nucleus accumbens. J. Neurosci. Methods 140 (1), 183–187.

Mühlenbock, P., Szechyńska-Hebda, M., Płaszczyca, M., Baudo, M., Mateo, A., Mullineaux, P.M., Karpiński, S., 2008. Chloroplast signaling and LESION SIMULATING DISEASE1 regulate crosstalk between light acclimation and immunity in Arabidopsis. Plant Cell 20 (9), 2339–2356.

Noreen, S., Ashraf, M., 2008. Alleviation of adverse effects of salt stress on sunflower (*Helianthus annuus* L.) by exogenous application of salicylic acid: growth and photosynthesis. Pak. J. Bot. 40 (4), 1657–1663.

Ogur, M., Rosen, G., 1950. The nucleic acids of plant tissues; the extraction and estimation of deoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem. 25 (2), 262–276.

Overmyer, K., Brosché, M., Kangasjärvi, J., 2003. Reactive oxygen species and hormonal control of cell death. Trends Plant Sci. 8 (7), 335–342.

Purcărea, C., Cachiţă-Cosma, D., 2010. Studies regarding the effects of salicylic acid on maize (*Zea mays* L.) seedling under salt stress. Studia Universitatis Vasile Goldis Seria Stiintele Vietii (Life Sciences Series) 20 (1).

Ruxton, G.D., 2006. The unequal variance *t*-test is an underused alternative to student's *t*-test and the Mann–Whitney *U* test. Behav. Ecol. 17 (4), 688–690.

Sapan, C.V., Lundblad, R.L., Price, N.C., 1999. Colorimetric protein assay techniques. Biotechnol. Appl. Biochem. 29 (2), 99–108.

Siddique, Z., Akhtar, K.P., Hameed, A., Sarwar, N., Imran-Ul-Haq, Khan, S.A., 2014. Biochemical alterations in leaves of resistant and susceptible cotton genotypes infected systemically by cotton leaf curl Burewala virus. J. Plant Interact. 9 (1), 702–711.

StatSoft, I., 2001. STATISTICA (data analysis software system), version 6, p. 150 (Tulsa, USA).

Vanitha, S., Umesha 1, S., 2008. Variations in defense related enzyme activities in tomato during the infection with bacterial wilt pathogen. J. Plant Interact. 3 (4), 245–253.