

## Original Article

# The Effect of High Temperature Treatment on Wheat Streak Mosaic Virus Resistance and Certain Resistance-Related Chemicals in Bread Wheat

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## Abstract

**Background and Aims:** To evaluate the effect of temperature on wheat streak mosaic virus (WSMV) resistance phenotype, through total protein, phenol, and peroxidase activity in bread wheat, a factorial experiment was conducted using Adl-Cross (resistant) and Marvdasht (susceptible) cultivars.

**Materials and Methods:** Results showed that incubation at 32°C changed the gene expression for resistance to WSMV and mosaic symptoms were observed in Adl-Cross. Total protein reduction in inoculated Adl-Cross was significant at 32°C. Results also indicated that high temperature either prevented expression of genes or degenerated available proteins involved in resistance mechanism. Total protein in infected Marvdasht was significantly reduced as compared with healthy control plants. Since electrophoretic pattern indicated reduction of ribulose 1, 5-bisphosphate carboxylase (RBPC) subunits in infected Marvdasht, reduction of protein may have probably been due to a decrease in the synthesis of RBPC. Mean of phenolic compounds content in Adl-Cross was higher as compared to Marvdasht in both infected and non-infected plants. Total phenol increased 2.8 and 4.06 percent in inoculated Marvdasht and Adl-Cross, respectively. The trend of increase in phenolic compounds indicated that their synthesis and accumulation was higher in Adl-Cross as compared to Marvdasht.

**Results:** These results indicated the role of phenolic compounds in prevention of viral movement and spread in resistant cultivar. Thin-layer chromatography (TLC) analysis showed that the intensity of a spot with Relative flow ( $R_f$ ) 0.622 increased at high temperature. Increase in total phenol at high temperature may have been due to increase in intensity of this spot. Spot concentration with  $R_f$  0.622 at high temperature was higher in infected samples as compared to uninfected samples.

**Conclusion:** This showed an interaction of virus and temperature. Also, a spot with the same  $R_f$  and different color was observed 8 days after inoculation at 25°C. The color change in this spot showed that high temperature might cause a decrease in concentration of phenolic compounds which are in turn effective in resistance to WSMV. Another possibility is that the compounds effective in resistance of Adl-Cross are changed to neutral forms at higher temperatures. There were no significant differences between genotypes for peroxidase activity in healthy plants at 25°C. Viral infection reduced the peroxidase activity in Marvdasht but, showed a significant increase in Adl-Cross. High temperature reduced peroxidase activity in both infected and uninfected plants. Peroxidase enzyme probably affects synthesis of compounds effective in resistance. Also, reduction in enzyme activity at high temperature increased reactive oxygen species (ROS) and this led to oxidative stress.

**Keywords:** Biochemical Changes; Bread Wheat; Resistance; Temperature; Wheat Streak Mosaic Virus

## Introduction

**W**heat streak mosaic virus (WSMV) is a major threat to winter wheat production worldwide, yet little is known about the genetic control of resistance. Plant resistance studies to date have indicated that no wheat cultivar is immune to WSMV. Although low level of resistance is available in commercial cultivars, they show significant losses under severe epiphytotics (20). High levels of WSMV resistance have been identified in *Agropyron elongatum*. Several wheat/*Agropyron* addition, substitution, or translocation lines and germplasms have been released that carry the resistance (14, 26). The resistance from *A. intermedium* has been shown to be tightly linked to the gene identified as *wsm1*. This source was demonstrated to be temperature-sensitive (22). This was one of the first known temperature-sensitive sources of resistance to WSMV. Seifers & Martin (21) also characterized a new source of temperature-sensitive resistance found in CO960293 wheat. A completely strain-specific resistant genotype "Adl-Cross" was introduced by Yassaie *et al.* (27) in Iran. Hassani and Assad (10) showed that resistance in "Adl-Cross" is apparently conditioned by a dominant gene.

Plants respond to pathogen attack or elicitor treatments by activating a wide variety of protective mechanisms designed to prevent pathogen replication and spreading (16). The defense mechanisms include the fast production of reactive oxygen species (ROS) (8), alterations in the cell wall constitution, accumulation of antimicrobial secondary metabolites known as phytoalexins (11), and activation and/or synthesis of defense peptides and proteins (6). The analysis of chemical

changes in plant constituents when resistance is broken as a result of exposure to high temperature may lead to further understanding of resistance mechanism. The objective of this investigation was to use both symptom expression and ELISA to determine the temperature stability of resistance to WSMV in the variety "Adl-Cross" and demonstrate the biochemical changes induced in resistant and susceptible cultivars in various temperature treatments.

## Methods

### Plant materials and pathogen

Seeds of resistant (Adl-Cross) and susceptible (Marvdasht) genotypes were obtained from Agriculture and Natural Resources Research Center, Shiraz, Iran. Marvdasht has been cultivated in a major wheat growing region in Fars province. The WSMV isolate was collected from this area and maintained on the susceptible genotype Marvdasht. Seeds of resistant and susceptible genotypes were sown separately in 15-cm diameter pots and placed in growth chamber. Ten days after seed emergence, seedlings of each genotype were divided into two groups. Seedlings in one group were inoculated with WSMV, and seedlings in the other group served as control. Seedlings in each group and each genotype were divided into four groups for four temperature treatments (a-b).

In treatment (a) seedlings were kept at 25°C before and after inoculation from sowing up to 12 days after inoculation. Other treatments were similar to treatment (a) except in treatment (b) two days before inoculation, in treatment (c) two days after inoculation and in treatment (d) two days before and two days after inoculation the temperature was raised to 32 °C.

The factorial combination of two genotypes-infected and not infected- and four temperature levels was used in a completely random design with three replications. Leaves from all 16

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experimental units were harvested on five different times, e.g., one hour, 1 day, 2 days, 4 days and 8 days after inoculation. Sample leaves were weighted and stored in -70°C for later protein, free phenolic extraction, peroxidase and other analysis. Twelve days following inoculation, all experimental units except susceptible ones were harvested to test the infection to WSMV by indirect ELISA (7) using a WSMV antiserum and clarified leaf extracts obtained by 30% chloroform treatment followed by a low speed centrifugation.

### Biochemical analysis

Leaves were used to prepare tissue extracts. The leaf tissue (0.2 gr fresh weights) was homogenized in 1 ml of 50mM Tris-HCl, pH 8.0 at 25°C, 10mM MgCl<sub>2</sub>, 2.5mM dithioerythritol and 10% glycerol (v/v). The homogenates were clarified by centrifugation at 10000g for 5 min. The resultant extracts were used for enzyme assay and measuring protein content.

### Total protein electrophoresis

Total soluble protein concentration was measured by the method of Bradford (4). The protein profiles were characterized and identified by using one-dimensional SDS-PAGE according to the method described by Laemmli (13).

### Peroxidase assay

Peroxidase activity was determined with guaiacol as substrate, using a modified procedure of Maehly and Chance (15). Enzyme extracts (50µl) were mixed with 3 ml of phosphate buffer (0.1M, pH 5.8, 30 °C), containing guaiacol (18 mM). After equilibration at 30 °C for 1 min the enzyme

reaction was started by adding 50µl of an H<sub>2</sub>O<sub>2</sub>-solution (250µl H<sub>2</sub>O<sub>2</sub>/10ml distilled water) and the absorbance at 470 nm was recorded for 2 min.

### Extraction and analysis of phenolic compounds

Free phenolics were extracted essentially according to Campbell and Ellis (5). Half a gram samples were homogenized in two volumes of 50% methanol for 1.5 h at 80 °C. The extract was centrifuged for five minutes at 3000 g and the supernatant was used for the Folin- Ciocalteu assays, and thin-layer chromatographic (TLC) analyses.

### Folin-Ciocalteu assay

The method of Julkunen-Tiitto (12) was used to determine phenolic content of the methanol extracts described above. Fifty micro liters of extract were diluted to 1 ml with water and mixed with 0.5ml of 2M Folin-Ciocalteu reagent and 2.5 ml of 20% Na<sub>2</sub>CO<sub>3</sub>. The absorbance of the samples was measured at 725 nm at room temperature after 20 min. Phenolic content was determined from a standard curve prepared with caffeic acid.

### Chromatographic analysis of phenolic extract

Methanol extracts (50 µl aliquots) were separated on silica gel-G TLC plates using n-butanol: acetic acid: water (5: 1: 2) as solvent system. Phenolic compounds were visualized with long (320 nm) and short (254 nm) UV light (5).

### Statistical analysis

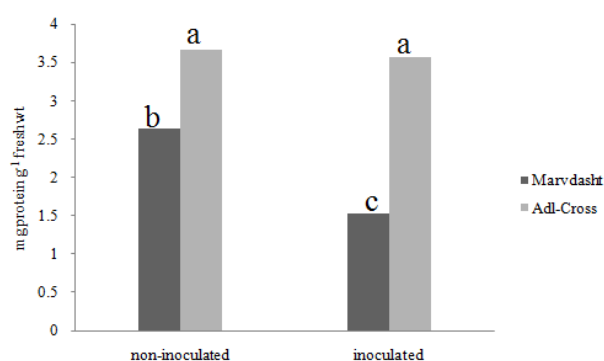
SAS general linear model (SAS Institute, Carry, NC, U.S.A., Version 6.06) was employed for a completely randomized design

**Table 1.** Mean ELISA values of Adl-Cross samples in various temperature treatments.

Treatment <sup>†</sup>	Blank	Contro									
		Health	l +	a-N	a-I	b-N	b-I	c-N	c-I	d-N	d-I
		y									
Mean	0.071*	0.095	0.901	0.094	0.308	0.147	0.987	0.149	0.973	0.112	1.005
Absorbance	f	ef	b	ef	c	de	a	d	a	def	a

<sup>†</sup>N: non inoculated; I: Inoculated; a-d refer to temperature treatments. a, all seedlings were kept at 25°C from sowing up to 12 days after inoculation; b-d, seedlings kept at 25°C for the whole period except for 2 days before inoculation (b), 2 days after inoculation (c), 2 days before and 2 days after inoculation (d) in which the temperature was raised to 32°C.

- Values marked with the same letters are not significant.

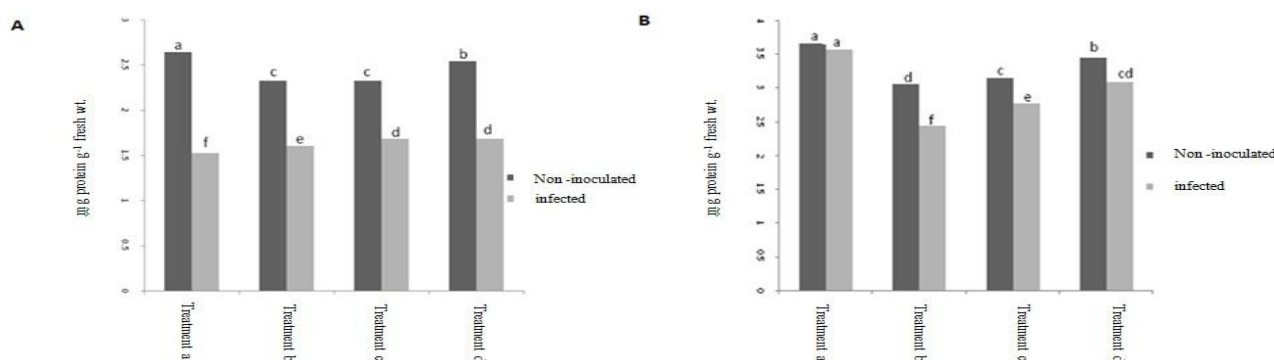


**Fig. 1.** Mean protein changes in Adl-Cross and Marvdasht in response to wheat streak mosaic virus infection at 25°C.

(inoculated or non-inoculated) and temperature. The means were compared by Duncan's test.

## Results and Discussion

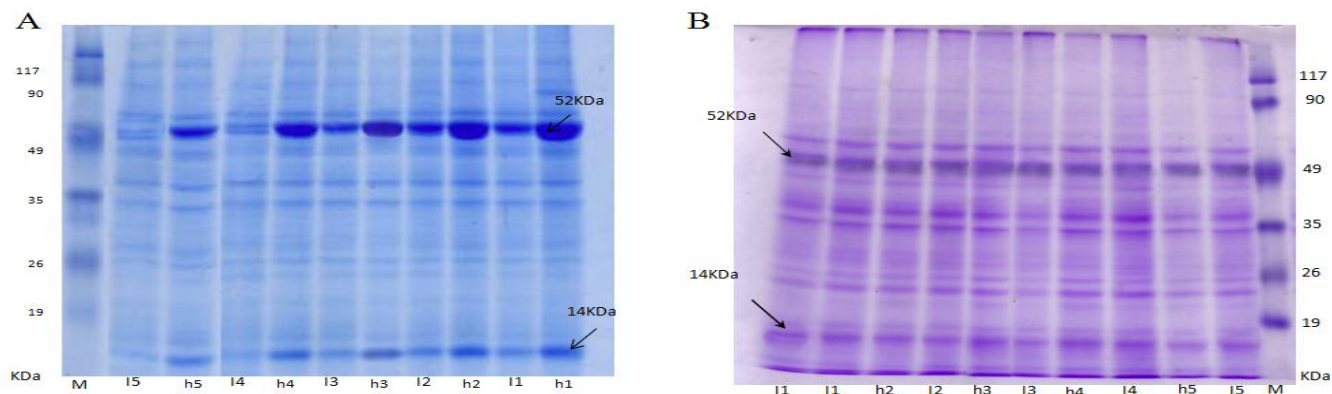
The resistant Adl-Cross remained symptomless as previously reported (27), but mean ELISA absorbance was significant at 25 °C (Table 1). The resistant genotype developed typical mosaic symptoms and tested positive in ELISA at 32 °C. Apparently temperature at 32 °C changed the gene expression for WSMV resistance. Such breaking of resistance to



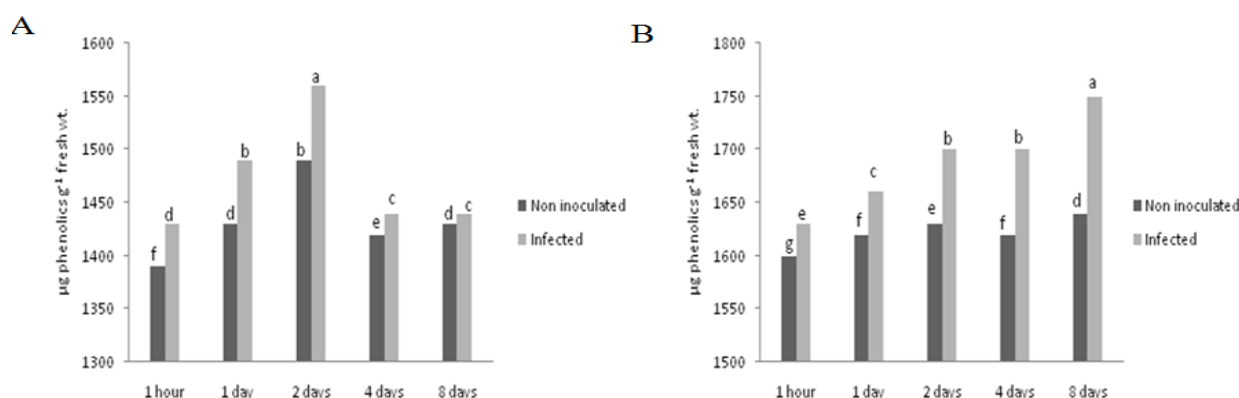
**Fig. 2.** Total protein changes (mg g<sup>-1</sup> fresh wt.) in different temperature treatments (a-d) in Marvdasht (A) and Adl – Cross (B) in response to infection by WSMV.

- (a) Seedlings were grown at 25 °C from sowing to 12 days after inoculation
- (b) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except for two days before inoculation when the temperature was raised to 32 °C.
- (c) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except for two days after inoculation when the temperature was raised to 32 °C.

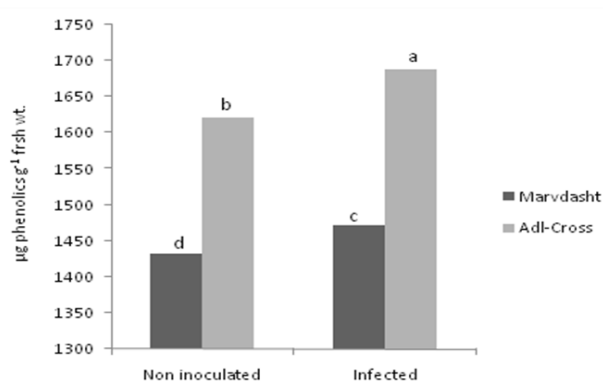
Seedlings were grown at 25 °C from sowing to 12 days after inoculation except for two days before and two days after inoculation when the temperature was raised to 32 °C.



**Fig. 3.** Protein-banding pattern of uninfected and infected leaves of Marvdasht (A) and Adl-Cross (B) at 25°C. Lane M: Protein markers; h: healthy leaves; I: Infected sample. 1, one hour after inoculation; 2-5 refer to 1, 2, 4 and 8 days after inoculation.



**Fig. 4.** The effect of WSMV infection on total methanol extractable phenolics in Marvdasht (A) and Adl-Cross (B), sampled at different intervals at 25°C.



**Fig. 5.** The effect of WSMV infection on total methanol extractable phenolics in Adl-Cross and Marvdasht at 25°C.

WSMV at elevated temperatures has been reported previously (22). There has also been a positive correlation between exposure duration to high temperature and percentage of infected plants due to resistance breaking (18, 24).

#### Changes in Protein

The amount of total protein in Adl-Cross was higher as compared to Marvdasht in both inoculated and non-inoculated conditions at 25°C (Fig. 1).

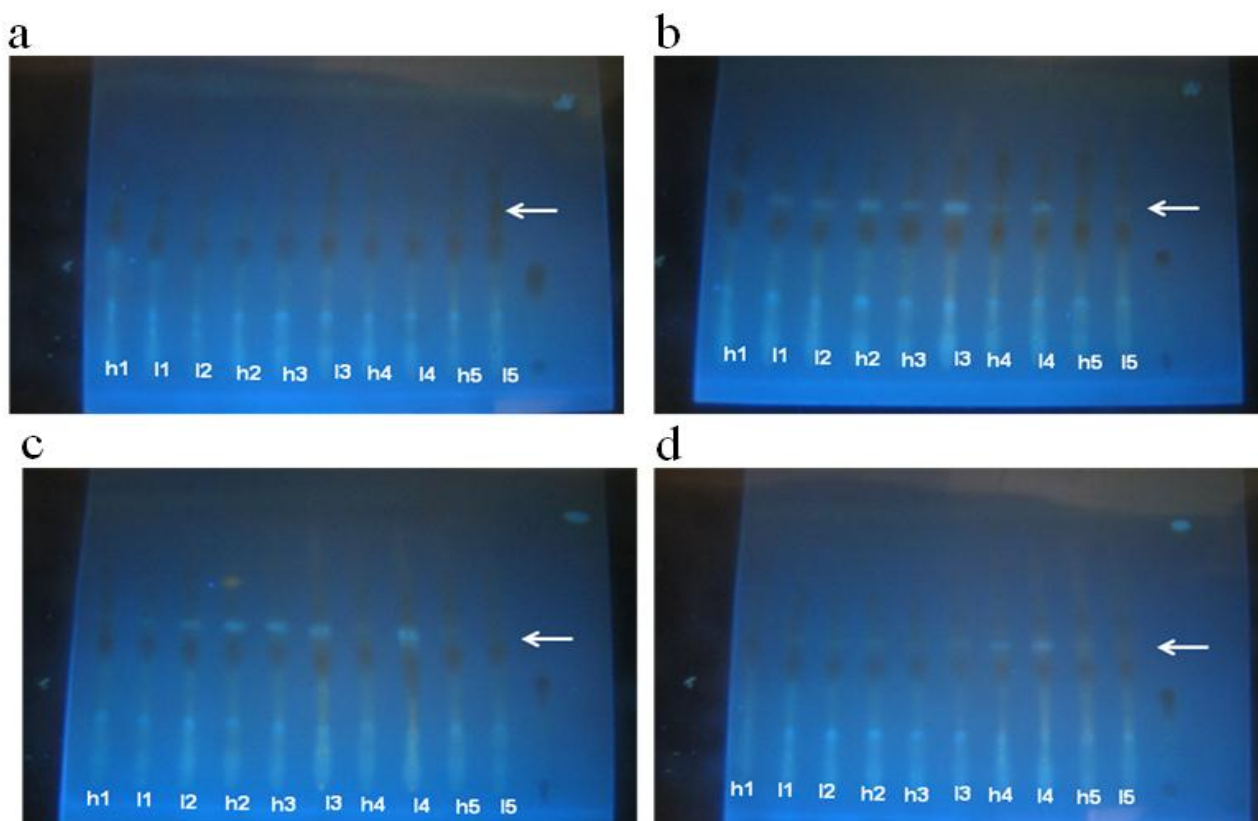
In general, WSMV infection decreased the total protein in both genotypes. Total protein reduction in susceptible Marvdasht was significant at low and high temperature (Fig. 2A). However, the amount of total protein reduction in inoculated Adl-Cross was not significant at 25°C. Raising the temperature to 32°C resulted in both breaking of resistance and decreasing of total protein (Fig. 2B). High temperature probably prevented expression of

genes involved in resistance mechanism or degenerated available proteins in the resistant genotype. The loss of soluble protein in virus infected leaves has been attributed in part to the damage to chloroplasts, or inhibition of protein synthesis (2).

The SDS-PAGE electrophoresis pattern of proteins extracted from leaves of Marvdasht and Adl-Cross at 25°C (Fig. 3) showed a reduction in the amount of two polypeptides of *Mr* 14000 and 52000 in infected Marvdasht, while, there was no significant changes in Adl-Cross at 25°C. The polypeptide of *Mr* 52000 had been reported to be ribulose-1,5-bisphosphate carboxylase (RuBPCase), the predominant protein of green leaves (9). The small subunit of RuBPCase is the *Mr* 14000 polypeptide coded by nuclear DNA and synthesized in the cytoplasm. The *Mr* 14000 reduced upon WSMV infection may be identified as the small subunit of RuBPCase (1). Therefore reduction of protein may have probably been due to a decrease in the synthesis of RuBPCase. The loss of large subunit and small sub-unit polypeptides was probably responsible for the loss of RuBPCase activity in infected Marvdasht. Reductions in the activity of RuBPCase in mildewed and rusted plants have been reported previously (25, 19).

#### Changes in Phenolic Compounds

Accumulation of phenolic compounds at 25°C in inoculated resistant and susceptible cultivars was more than in uninfected plants. The



**Fig. 6.** Analysis of methanol extracted compounds by TLC in Adl-Cross variety at different temperature treatments (a-d).

(a) Seedlings were grown at 25 °C from sowing to 12 days after inoculation

(b) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days before inoculation when the temperature was raised to 32 °C.

(c) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days after inoculation when the temperature was raised to 32 °C.

(d) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days before and two days after inoculation when the temperature was raised to 32 °C.

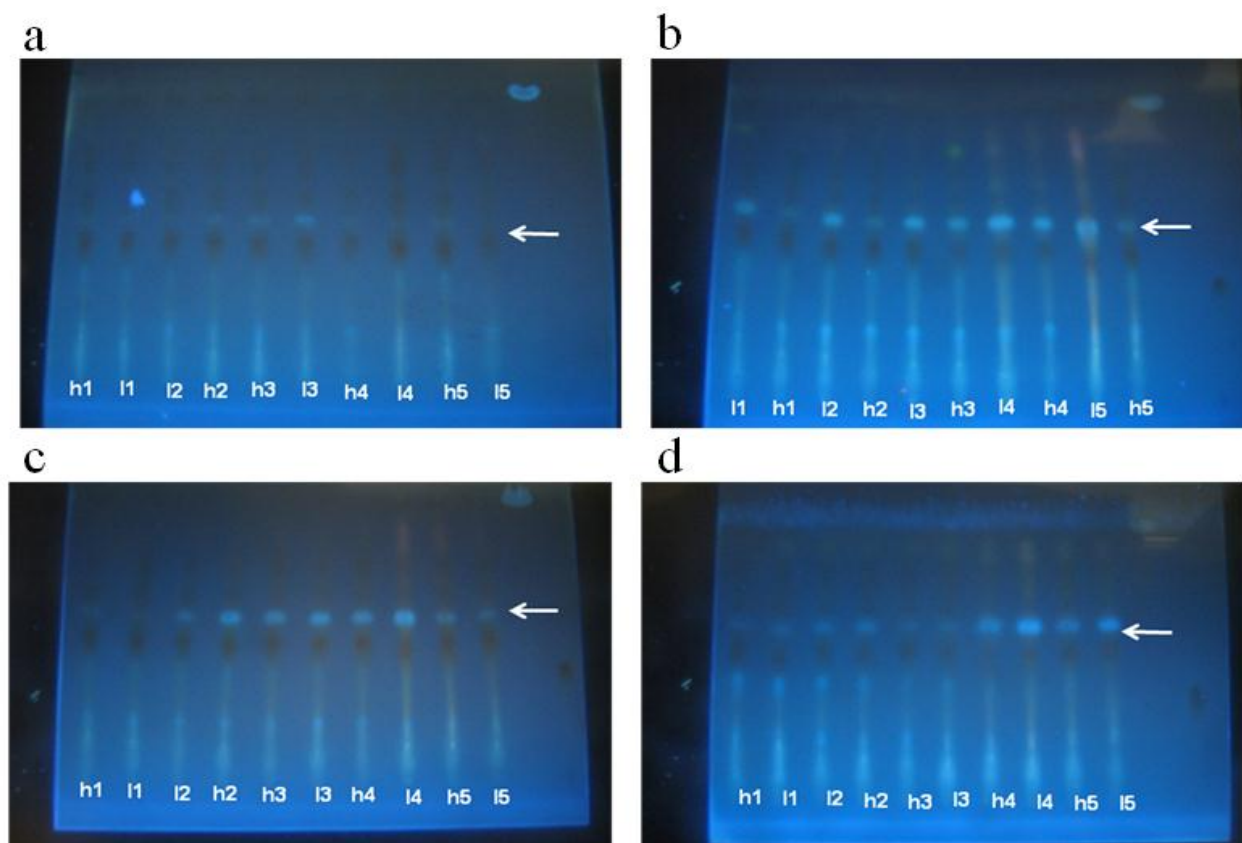
Numbers 1,2,3,4 and 5 indicate 1 hour, 1 day, 2 days, 4 days and 8 days after inoculation; h: healthy; i: infected.

highest value was recorded at the second day after inoculation in Marvdasht but declined thereafter (Fig. 4A). The results showed that the amount of phenolic compounds markedly increased in WSMV infected wheat leaves of resistant Adl-Cross over their uninfected counterparts from one hour to eight days after inoculation (Fig. 4B). Formation and accumulation of phenolic compounds was higher in Adl-Cross compared to Marvdasht. Figure 5 shows total phenol in Adl-Cross and Marvdasht in both inoculation and non-inoculation conditions. Total phenol increased 2.8 and 4.06 percent in inoculated Marvdasht and Adl-Cross, respectively, as compared to their non- infected counterparts. The higher

content of phenolic compounds in Adl-Cross may be correlated with its resistance to WSMV.

Thin layer chromatography (TLC) analysis confirmed that there were specific changes in the array of phenolics present over the course of infection and various temperature treatments. All described spots appeared dark grey-blue upon spraying with Folin-Ciocalteu. Our results indicated that all detected spots in the methanol extracts were phenolic in nature. When plates were viewed under short- and long-wave UV, there were eight distinct spots in both uninfected and infected samples. Results showed that the spot with Relative flow 0.622 was observed eight days after





**Fig. 7.** Analysis of methanol extracted compounds by TLC in Marvdasht variety at different temperature treatments (a-d).

(a) Seedlings were grown at 25 °C from sowing to 12 days after inoculation

(b) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days before inoculation when the temperature was raised to 32 °C.

(c) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days after inoculation when the temperature was raised to 32 °C.

(d) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days before and two days after inoculation when the temperature was raised to 32 °C.

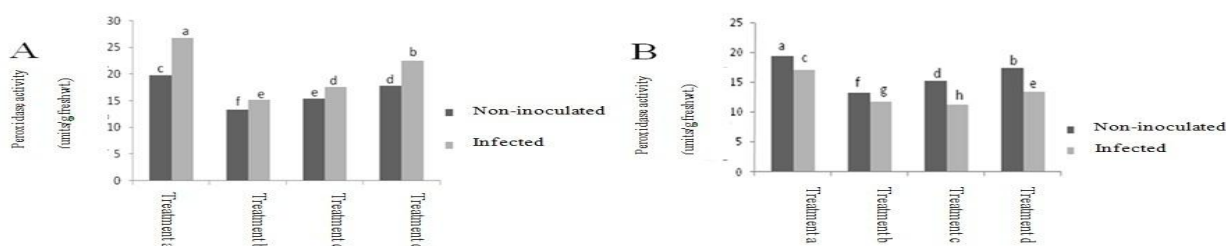
Numbers 1,2,3,4 and 5 indicate 1 hour, 1 day, 2 days, 4 days and 8 days after inoculation; h: healthy; i: infected.

inoculation in inoculated Adl-Cross at 25°C only (Fig. 6a). A spot with the same relative flow but different color was also observed at 32°C and increased in intensity in inoculated samples (Figs. 6b, 6c, 6d, 7b, 7c, and 7d). The color change in this spot ( $R_f$  0.622) at 32°C showed that high temperature might have caused a decrease in concentration of phenol compounds which is in turn effective in conditioning resistance in Adl-Cross. Another possibility is that compounds responsible for resistance in Adl-Cross have been changed to neutral form at the higher temperature.

These results showed that apparently the amount of total protein and total phenol in a genotype could be adopted as selective criteria in preliminary stages of selection for resistance to the virus

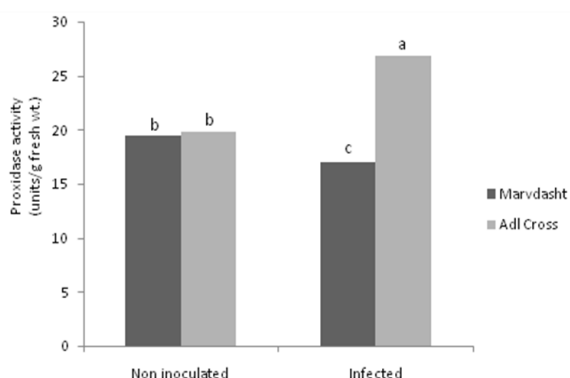
#### **Changes in the activity of peroxidase**

When the resistant genotype Adl-Cross was infected by WSMV a significant increase in peroxidase activity was observed in leaves of Adl-Cross at all temperature treatments as compared with non-infected control (Fig. 8A). However, infection significantly decreased peroxidase activity in leaves of Marvdasht (Fig. 8B) as compared with non-infected



**Fig. 8.** Peroxidase activity (units/g fresh wt.) at different temperature treatments (a-d) in Adl – Cross (A) and Marvdasht (B) varieties of wheat.

- (a) Seedlings were grown at 25 °C from sowing to 12 days after inoculation
- (b) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days before inoculation when the temperature was raised to 32 °C.
- (c) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days after inoculation when the temperature was raised to 32 °C.
- (d) Seedlings were grown at 25 °C from sowing to 12 days after inoculation except two days before and two days after inoculation when the temperature was raised to 32 °C



**Fig. 9.** The effect of WSMV infection on peroxidase activity in Adl-Cross and Marvdasht at 25°C.

control at all temperature treatments. Uninfected genotypes showed no significant differences for peroxidase activity at 25°C, but the difference was significant in inoculated conditions (Fig. 9). Increase in temperature reduced peroxidase activity in both infected and healthy plants. In parallel to the induction of phenolic compounds in Adl-Cross at 25°C, results also showed that activities of peroxidase significantly increased in response to WSMV. Peroxidase is important in the defense mechanism against pathogens, through its role in the oxidation of phenolic compounds to quinones, causing increased antimicrobial activity. It is believed that peroxidase may be

directly involved in stopping pathogen development (17, 23), accelerating the cellular death of cells close to the infection site, preventing the advance of infection and/or by generating a toxic environment which will inhibit the growth of the pathogen inside the cells (3). Peroxidase enzyme probably affects synthesis of compounds effective on resistance. Therefore, reduction of enzyme activity may reduce these compounds as well as quinone production. Quinones are more poisonous to pathogens than phenolic compounds. Also, reduction in enzyme activity caused by high temperature increases reactive oxygen species (ROS) and this leads to oxidative stress that is harmful to plant.

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