Available online at www.sciencedirect.com
ScienceDirect
Plant Physiology and Biochemistry 45 (2007) 480–489
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Research article

Physiological and metabolic changes of *Cucurbita pepo* leaves in response to zucchini yellow mosaic virus (ZYMV) infection and salicylic acid treatments

Deya Eldeen Mohammed Radwan [a,b], Khalaf Ali Fayez [b], Sabry Younis Mahmoud [c], Ahmed Hamad [d], Guoquan Lu [a,*]

[a] *College of Agriculture & Biotechnology, Zhejiang University, 268 Kaixuan Road, Hangzhou 310029, Zhejiang, China*
[b] *Botany Department, Faculty of Science, Sohag University, Sohag 82524, Egypt*
[c] *Virology Department, Faculty of Agriculture, Sohag University, Sohag 82786, Egypt*
[d] *Botany Department, Faculty of Science, Assiut University, Assiut, Egypt*

Received 26 July 2006; accepted 5 March 2007
Available online 12 March 2007

Abstract

The changes of some physiological and biochemical parameters in pumpkin (*Cucurbita pepo* cv Eskandarani) leaves associated with zucchini yellow mosaic virus (ZYMV) infection and the effect of exogenous application of salicylic acid (SA) were studied in this paper. In comparison to the untreated leaves, ZYMV infected leaves showed many symptoms, including severe mosaic, size reduction, stunting and deformation. Results from analysis of physiological parameters indicated that viral infection and SA treatments affected metabolism. Viral infection decreased pigment, protein and carbohydrate levels. But with all SA treatments, the protein and carbohydrate contents are noticeably increased. Moreover, the other biochemical parameters showed variable alterations. The peroxidase (POX, EC 1.11.1.7) activity and proline contents were induced by both viral infection and SA treatments. In addition, protein patterns represent some newly synthesized polypeptides which reflect formation of pathogenesis related proteins in all treatments. SA treatment increases the plant resistance against ZYMV. This can be noticed through reduction of percentage of the infected plants, decrease in disease severity and virus concentration of the plants treated with SA then inoculated with virus. All results show significant changes in metabolism affected by either viral infection or SA treatments and also indicate that exogenous SA plays an important role in induction of defense mechanism against ZYMV infection.

Keywords: Metabolism; Peroxidase; Pigments; Pumpkin; Salicylic acid; Systemic acquired resistance; Zucchini yellow mosaic virus

1. Introduction

Many types of environmental stresses both biotic and abiotic produce characteristic changes in physiology and metabolic processes of higher plants [26]. Among these stresses, attack by pathogens which cause many biochemical changes lead to harmful effects on plant health.

There are more than 20 viruses infecting cucurbit crops [8]. Zucchini yellow mosaic virus (ZYMV) is one of the most important viruses affect cucurbit production. It causes destructive diseases to a large variety of economically important cucurbit plants including zucchini squash (*Cucurbita pepo*) [44].

ZYMV was reported in many African countries including Algeria, Egypt, Madagascar, Mauritius, Morocco, Reunion, Swaziland and Tunisia [22]. Morphological symptoms

Abbreviations: Chl, chlorophyll; POX, peroxidase; SA, salicylic acid; SAR, systemic acquired resistance; SA + V, treated with salicylic acid then inoculated with virus; ZYMV, zucchini yellow mosaic virus.

* Corresponding author. Tel./fax: +86 571 8697 1117.
E-mail addresses: deya90@yahoo.com (D.E.M. Radwan), lugq10@zju.edu.cn (G. Lu).

0981-9428/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2007.03.002

observed on leaves are vein clearing, yellowing, mosaic, and deformations of leaves [8]. The disease can completely destroy the crop under favorable conditions leading to yield loss.

It was reported that plant viruses affect physiological processes such as photosynthesis [26] by decreasing the photosynthesis rate (depending on the infection stage), decreasing pigment contents [28], soluble sugar contents, reducing starch accumulation [35], and increasing the respiration rates [33]. Técsi et al. [35] reported changes in host metabolism in relation to virus replication in the infected marrow cotyledons. Some enzymes are widely influenced in response to pathogen attack [42]. Among these enzymes, Peroxidase is the first to show changes in its activity during viral infection stages. For instance, POX shows increase in its activity in *Cucurbita pepo* plants infected with viruses such as CMV [35].

SAR is considered a form of resistance induced in plants against subsequent infection and attack by a broad spectrum of organisms [1,11]. Several chemicals have been reported as SAR inducers in plants [36]. SA is considered one of the key components of defense signal transduction which induces the full set of SAR genes [23]. Also, SA is known as a regulator for physiological processes such as plant defence mechanisms against harmful microorganisms.

Application of SA led to H_2O_2 accumulation and may include the induction of at least one of cellular protective mechanisms that are concomitant with the accumulation of active oxygen species suggesting that SA application results in a state of oxidative stress [27]. Therefore, it can be predicted that SA may affect numerous metabolic processes in plants [25] and through the potentiation of oxidative burst; SA can control both biotic and abiotic defense programs [3]. For this, we found that it is important to study the effects of SA as well as SA followed by virus inoculation.

The term systemic acquired resistance refers to a change in the physiology of the plant [31]. There is a number of biochemical and physiological changes that have been established to be associated with SAR, which include cell death and oxidative burst [20], deposition of lignin [37], accumulation of proline [13] the synthesis of phytoalexins [4,29] accumulation of pathogenesis-related proteins [16] changes in pigments content, chlorophyll synthesis and POX activity [28].

In this paper, we investigate effects of ZYMV infection, SA and (SA + V) treatments on some physiological and biochemical parameters in pumpkin (*Cucurbita pepo*) leaves. Specifically, the analyses are of proteins, pigments and carbohydrate contents, POX activity and the protein profile by SDS-PAGE also indicate the induction of resistance by SA treatments against ZYMV infection.

2. Materials and methods

2.1. Plant materials

Seeds of pumpkin (*C. pepo* cv Eskandarani) were sown in a mixture of sand and clay (1:2 v/v) in plastic pots (10 cm in diameter) in separated growth chambers with a photoperiod of 12 h. Day and night temperatures were 24 °C and 18 °C, respectively; the relative humidity was about 70%. The plants were kept at 100% relative water content.

2.2. SA treatments and virus inoculation

After 21 days of growth, plants were divided into eight groups. Each group consists of three replications. One group was left as a control without any treatment. The other groups were treated with 10, 50 and 100 μM of SA by spraying the leaves until run-off. The control was sprayed by water. To improve spread, two drops of Tween 80 were added. Inoculation of virus was performed 3 days after spraying. All leaves were mechanically inoculated. The inoculum was prepared from infected top leaves, ground in a mortar containing 0.1 M phosphate buffer pH 7.0 (1:2 w/v); the homogenate was filtrated through two layers of muslin, and the leaves of healthy plants were dusted with carborundum, and rubbed gently with cotton swab previously dipped into the suspension of virus inoculum. Three weeks after inoculation the percentage of infected plants and the severity of symptoms were examined using the following rating scale: 0 = no symptoms; 1 = chlorotic local lesions and mild mosaic; 2 = severe mosaic and 3 = blisters and malformation. Disease severity values were calculated using the following formula according to Yang et al. [43]:

$$\text{Disease severity (DS)} = \frac{\sum (\text{Disease grade} \times \text{number of plants each grade})}{(\text{Total number of plants} \times \text{highest disease grade})}$$

Three weeks after inoculation the youngest fully developed leaves from both control and treated plants were collected for analysis of biochemical changes.

2.3. ZYMV detection

2.3.1. DAS-ELISA

DAS-ELISA Technique was applied as described by Clarke and Adams [6] for ZYMV concentrations in the infected and (SA + V) treated leaves using an ELISA Kit (completely ready for use). Kits were supplied by SANOFI, Sante Animale, Paris, France.

2.3.2. Electron microscopy

Leaf pieces developing severe symptoms were ground in a drop of PBS pH 7 + 0.01% (w/v) sodium sulphite (Na_2SO_3). The leaf extracts were then transferred to carbon-coated Formvar grids for seven minutes. After washing with distilled water, the grids were negatively stained with 2% uranyl acetate and examined with a JEOL 1220 transmission electron microscope.

2.4. Determination of photosynthetic pigments

Contents of Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoids were spectrophotometrically determined according to Metzner et al. [24]. The pigment contents of those infected, treated with SA and inoculated after treatment

with SA were then compared to the control without any treatments. After 15 days of inoculation with virus, the leaves with similar age were taken to extract the pigments. The photosynthetic pigment contents were extracted from a known fresh weight of leaves in 85% (v/v) aqueous acetone. The extract was centrifuged at 4000 rpm for 10 min, the supernatant was then taken and diluted by 85% aqueous acetone to the suitable concentration for spectrophotmetric measurements. The extinction was measured against a blank of a pure 85% aqueous acetone at three wavelengths of 452.5, 644 and 663 nm. Using the following equations:

$$\text{chlorophyll } a = 10.3 * E_{663} - 0.98 * E_{644} = \mu g/ml$$

$$\text{chlorophyll } b = 19.7 * E_{644} - 3.87 * E_{663} = \mu g/ml$$

$$\text{Total carotenoids} = 4.2 * E_{452.5} - \{(0.0264 * \text{chl } a) + (0.426 * \text{chl } b)\} = \mu g/ml$$

Finally, these pigment fractions were calculated as mg/g fresh weight.

2.5. Determination of proline content

Proline content of leaves was determined according to Bates et al. [2]. A known dry weight (0.1 g) of leaves was extracted in 10 ml of aqueous 3% sulfosalicylic acid overnight. The extract was centrifuged at 1500 rpm for 10 min. A total of 2 ml of the supernatant were mixed with 2 ml of fresh acid ninhydrin solution for reaction and 2 ml glacial acetic acid in a test tube for 1 h at 100 °C. The reaction was terminated in an ice bath, and the mixture was extracted with 4 ml toluene. The extract was vigorously stirred for 20 s using a test tube stirrer. Therefore, the chromophore-containing toluene was aspirated from the aqueous phase, and its absorbence was measured at 520 nm. The proline content was determined from a standard curve and calculated on a dry weight basis.

2.6. Determination of carbohydrates and protein contents

Carbohydrate content was determined in the aqueous solution with anthrone sulfuric acid reagent according to Fales [10] and Schlegel [32], using glucose as a standard. To extract water-soluble carbohydrates, a known weight of tissue powder were boiled in distilled water in a water bath for 1 h and for extraction of total carbohydrates, 50 mg of dry tissue powder was boiled in 1 N HCl in water bath for 1.5 h. Then the extracts were cooled and filtrated through a centered glass funnel. A total of 0.5 ml of the extract was mixed with 4.5 ml of anthrone reagent (0.2 g anthrone, 8 ml absolute ethyl alcohol, 30 ml distilled water, and 100 ml concentrated sulfuric acid D = 1.84). The mixture was then boiled in water bath for 7 min. After cooling, the developed blue green colour was measured against blank by using Spekol Carl-Zeiss spectro-colorimeter at wavelength of 620 nm. Soluble and total carbohydrate contents were finally calculated as mg/g dw. The water insoluble carbohydrates were calculated as the difference between the amount of the total and water-soluble carbohydrates.

Protein contents were determined according to Lowry et al. [21] using Bovine serum albumin as a standard. Tissue samples (0.1 g dry weight) were extracted in 10 ml distilled water for 2 h at 90 °C for analysis of soluble protein and for total protein, 50 mg were extracted in 10 ml NaOH (0.1 N) for 2 h at 90 °C. The extracts were centrifuged and the supernatants were collected. One millimeter of extract was added to 5 ml of alkaline reagent (50 ml 2% Na_2CO_3 prepared in 0.1 N NaOH +1 ml 0.5% $CuSO_4.5H_2O$ prepared in 1% Sod. Pot. Tartarate) and mixed thoroughly then allowed to stand for 10 min. A total of 0.5 ml of Folin phenol reagent diluted 1:1 (v/v) was then added and mixed immediately. After 30 min, the extinction against appropriate blank was measured at 700 nm. Results were expressed as mg/g dry weight. Insoluble proteins were calculated as the difference between the amounts of total and water-soluble proteins.

2.7. Determination of relative POX activity

POX activity was carried out by grinding one gram of the fresh leaves material at 4 °C in a mortar in 1 ml extraction buffer at 0.1 M Tris–HCl, pH 7.0. The homogenate was centrifuged at 15 000 rpm at 10 °C for 15 min. Supernatants were collected for measuring POX activity. Fifty micronliter of extracted samples in 10 ml of assay mixture were spectrophotometrically measured at 470 nm. The assay mixture for POX activity contained 40 mM potassium phosphate pH 7.2, 0.1 mM EDTA, 5 mM guaiacol, 0.3 mM hydrogen peroxide. Then, the relative POX activity was determined (O.D./g fresh weight/h) as described previously [17,38]. POX activity of grown under non-stress conditions was considered as 100% and a control treatment.

2.8. SDS PAGE analyses

SDS PAGE was performed using 10% acrylamide gels according to the procedure of Laemmli [19]. Protein samples (40 μg each) were mixed with an equal volume of buffer containing 0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glyerol, and 10% 2-mercaptoethanol and bromophenol blue as tracking dye. The prepared mixture was heated in a water bath for 3 min at 96 °C and loaded onto gel for 6 h at 10 °C in run buffer containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Protein bands were visualized using Commassie Brilliant blue dye.

2.9. Statistics

All data were compared according to the analysis of variance (ANOVA) and Least significant difference (LSD) tests at 0.05 probability level.

Fig. 1. Effect of ZYMV infection, SA and SA + V treatments on leaf morphology of pumpkin (*Cucurbita pepo* cv Eskandarani). (A) Control, (B) ZYMV infected showing severe symptoms in the form of mosaic and blisters, (C, E, G) sprayed with 10, 50 and 100 μM SA respectively, (D, F, H) sprayed with 10, 50, 100 μM (SA + V) causes decrease in disease severity and the symptoms totally disappeared in the 100 μM (SA + V) level.

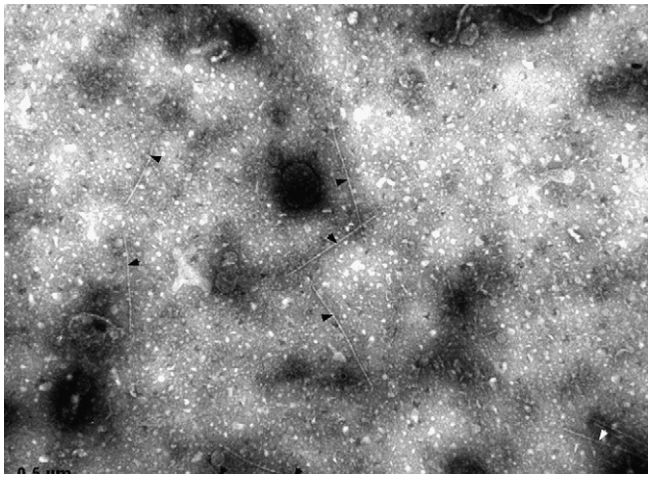

Fig. 2. Particles of ZYMV in the sap of infected pumpkin (*Cucurbita pepo* cv Eskandarani) leaves. ZYMV occurs as flexuous filamentous particles, which are 750 nm long and approximately 10 nm in width.

3. Results

3.1. Changes in virus infection

Virus infected leaves of pumpkin (*C. pepo* Eskandarani) showed severe symptoms on the new leaves 2 weeks post inoculation. These symptoms include severe mosaic, green blisters, size reduction and deformation (Fig. 1). The observations using electron microscopy revealed the presence of flexuous filamentous particles about 750 nm long (Fig. 2). Treatment with 10 and 50 μM SA delayed the appearance of symptoms for 4 days while 100 μM SA caused more than 7 days delay in comparison with others infected but untreated with SA. Also, SA reduced the percentage of infection (Table 1) and the severity of symptoms which were examined 15 days after inoculation. Ten, 50 μM SA treated plants showed mild symptoms, but 100 μM SA sprayed leaves showed no symptoms in comparison to virus inoculated only leaves.

Table 1
Effect of ZYMV infection, treatment with SA and SA + V on virus concentration, percentage of infection (%) and disease severity of pumpkin (*C. pepo* cv Eskandarani) leaves

Treatments	Virus concentration	Percentage of infection (%)	Disease severity (%)
Infected	1.642 ± 0.137	95.24 ± 2.25	66.2
10 μM SA + Virus	0.899 ± 0.075	53.33 ± 1.55*	13.3
50 μM SA + Virus	0.406 ± 0.090	22.86 ± 0.95*	2.6
100 μM SA + Virus	0.174 ± 0.025	2.14 ± 0.08*	0.21

A decrease in virus concentration, percentage of infection and in disease severity noticed with the increase of SA level. The values are means of three replicates ± standard deviation. *: The values are significantly different in comparison with the control using LSD test at 0.05 level. In ELISA test for virus concentration, the positive and negative controls are 1.530 and 0.122 respectively; positive control means infected leaves showed symptoms typically. And negative control means infected leaves showed no symptoms.

According to the values of disease severity calculated using the formula of Yong et al. (1996). SA could reduce the disease severity where the concentration of 100 μM SA was the most effective one in reduction of disease severity.

From virus concentrations analysis by using DAS-ELISA we can conclude that the infected and SA treated leaves gave positive reactions which reflect the appearance of symptoms on plant leaves with different degrees according to the concentration of virus, whereas, the concentration decreased gradually with the increase of SA sprayed dose. The lowest value of virus concentration is 0.174 was determined in case of 100 μM SA treatment (Table 1).

3.2. Photosynthetic pigment content

The photosynthetic pigment contents (Chl *a*, Chl *b* and carotenoids) of leaves were significantly decreased in response to both ZYMV infection and (SA + V) while the SA treated leaves without inoculation appeared to be unaffected. From the data shown in Table 2, we can conclude that ZYMV infection reduces the Chl *a*, Chl *b* and carotenoid contents to about 48%, 53% and 52% to those of control, respectively. Also ZYMV infection causes a decrease in total pigment content by 48% in comparison to the control. The *a*/*b* ratio for ZYMV infected leaves is higher than control.

Both 50 and 100 μM (SA + V) caused a slight decrease in Chl *b* and carotenoid contents and also caused a 27% and 25% reduction of total pigment contents when compared with the healthy leaves. On the contrary, 50 and 100 μM SA without inoculation showed a slight increase. In all SA treatments, the *a*/*b* ratio was noticed to be higher than control. The increase in the *a*/*b* ratio was due to the decrease in Chl *b* content than Chl *a* content. So, Chl *b* is more sensitive to ZYMV infection and SA treatments.

3.3. Protein and carbohydrate content

Leaf protein and carbohydrate contents of control, virus infected, and SA treated leaves are shown in Table 3 and Fig. 3 respectively. In response to viral inoculation, the soluble, insoluble and total protein contents increased significantly in comparison to uninoculated control.

Except for 10 μM SA treatment, a significant increase in protein levels was noticed with all SA treatments with or without virus inoculation (Table 3). This means the accumulation of all protein fractions as a response to both viral infection and SA treatments. Spraying of leaves with 100 μM SA without inoculation increased the soluble and total proteins to percentages of 153.28% and 125.30% respectively. On the other hand, 100 μM (SA + V) caused an increase of 156.61% and 127.04% for soluble and total protein contents with respect to the corresponding controls.

From the results of carbohydrate content analysis, 50 and 100 μM SA treatments with or without virus inoculation caused significant increase in comparison to control (Fig. 3). SA seems to be the factor causing increase of soluble and total

Table 2
Effect of ZYMV infection, treatment with SA and (SA + V) on pigments content (mg g^{-1} fw) of pumpkin (*C. pepo* cv Eskandarani) leaves

Treatments	Chl *a*	Chl *b*	Carotenoid	*a*/*b*	Total	%
Control	0.43 ± 0.04	0.19 ± 0.02	0.25 ± 0.03	2.30	0.87	100.00
Infected	0.25 ± 0.09*	0.09 ± 0.03*	0.12 ± 0.04*	2.75	0.46	52.50
10 μM SA	0.48 ± 0.01	0.18 ± 0.00	0.25 ± 0.01	2.64	0.91	104.15
10 μM SA + Virus	0.27 ± 0.04*	0.11 ± 0.01*	0.18 ± 0.02	2.52	0.56	64.36
50 μM SA	0.44 ± 0.03	0.16 ± 0.01	0.24 ± 0.01	2.68	0.85	97.36
50 μM SA + Virus	0.38 ± 0.01	0.10 ± 0.00*	0.16 ± 0.01	3.88	0.64	73.69
100 μM SA	0.43 ± 0.05	0.15 ± 0.02	0.22 ± 0.03	2.79	0.81	92.89
100 μM SA + Virus	0.34 ± 0.12	0.11 ± 0.04*	0.20 ± 0.07	3.00	0.66	75.78

ZYMV infection causes significant decrease in the all pigment fractions. Chl *b* is more sensitive to (SA + V) treatments, but spraying SA without followed virus inoculation has no significant effect. The total pigment content decrease and the *a*/*b* ratios increase with increasing SA level. The values are means of three replicates ± standard deviation. *: The values are significantly different in comparison with the control using LSD test at 0.05 level.

carbohydrates. Furthermore, virus infected leaves showed a slight increase in soluble carbohydrate. Interestingly, the total carbohydrate content seems to be decreased in viral infected leaves. Among the treatments, the 100 μM (SA + V) caused the highest increase, 346.66% and 149.35% for soluble and total carbohydrates contents respectively.

3.4. Proline content

Proline contents of treated and untreated leaves were also determined. The most obvious results shown in Table 4 are the significant increase in proline contents in all infected and SA-treated leaves. Spraying SA to the leaves caused proline accumulation. Moreover, Proline content of leaves treated with 100 μM SA and virus was increased by 1.5 fold of the control.

3.5. POX activity

The effect of viral infection and treatment with SA on the activity of POX enzyme is shown in Fig. 4. The activity of POX enzyme varied significantly among the groups. In virus inoculated leaves, the activity increases significantly to be about 3 folds when compared to that of the control. Also, the increase reached the highest value at a treatment with 10 μM (SA + V) and then the activity declined with the increase of SA concentration. The POX activity for leaves treated with 10, 50, and 100 μM (SA + V) were 485.7%, 387.6% and 345%, respectively. On the other hand, spraying of SA without following inoculation caused significant increase in POX activity.

3.6. Protein patterns

The changes in protein patterns of pumpkin (*C. pepo* cv Eskandarani) infected and SA treated leaves were analysed by SDS-PAGE. The results shown in Fig. 5 indicated that all treatments varied greatly when compared to the control. Inoculation with virus caused appearance of two new polypeptides of about 78 and 73 kDa. In SA treatments, many alterations were observed, these alterations involving the appearance of newly synthesized polypeptides of about 258, 233, 145, 136, 109, 78, 73 and 31 kDa when spraying with 100 μM (SA + V), while the polypeptide of 84 kDa showed a high intensity accumulation in 50 and 100 μM SA and virus treated leaves. Valuable changes were observed for a treatment with 50 μM SA and virus where the polypeptides of 182, 78, 73, 31, and 28 kDa were induced. Moreover, the polypeptides of about 29, 27 kDa disappeared and the band of about 84 kDa was accumulated, in comparison with the control bands intensity. In response to 10 μM (SA + V) and 100 μM SA treatments, two newly synthesized bands of about 78 and 73 kDa were detected. Furthermore, it was noticed that all lanes concerning SA treatments showed a higher amount of protein in comparison with control and virus infected lanes.

Table 3
Effect of ZYMV infection, treatment with SA and SA + V on protein contents (mg g^{-1} dw) of pumpkin (*C. pepo* cv Eskandarani) leaves

Treatments	Soluble proteins	Insoluble proteins	Total proteins
Control	45.93 ± 3.14	161.67 ± 11.24	207.60 ± 8.12
Infected	54.80 ± 1.56*	210.40 ± 11.76*	265.20 ± 13.31*
10 μM SA	46.13 ± 2.32	157.34 ± 20.68	203.47 ± 23.00
10 μM SA + Virus	59.53 ± 4.82*	193.00 ± 11.81*	252.53 ± 10.88*
50 μM SA	62.53 ± 0.61*	183.33 ± 1.67*	245.87 ± 1.51*
50 μM SA + Virus	62.40 ± 2.27*	188.67 ± 20.31*	251.07 ± 21.59*
100 μM SA	70.40 ± 3.65*	189.73 ± 6.62*	260.13 ± 9.93*
100 μM SA + Virus	71.93 ± 1.62*	191.80 ± 16.63*	263.73 ± 16.81*

An increase in protein levels occurs in response to all treatments. The values are means of three replicates ± standard deviation. *: The values are significantly different in comparison with the control using LSD test at 0.05 level.

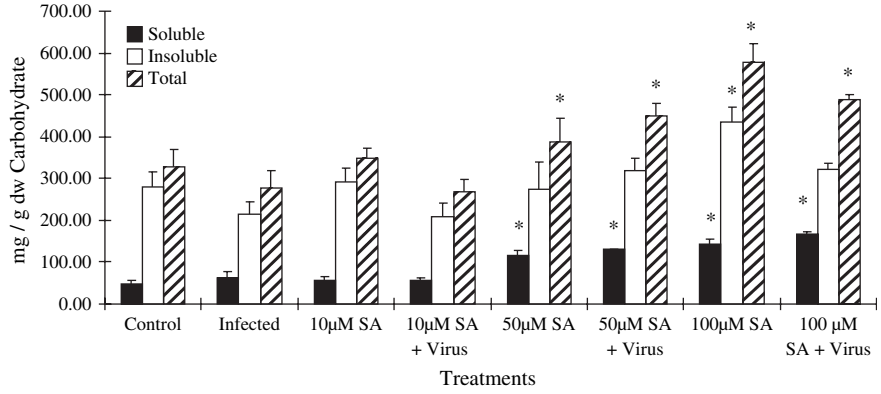

Fig. 3. Effect of ZYMV infection and SA treatment on leaf carbohydrate content of pumpkin (*C. pepo* cv. Eskandarani). The values are means of three replicates ± standard deviation. The carbohydrate content is lowered by ZYMV infection and induced by SA treatments. *: The values are significantly different in comparison with the control using LSD test at 0.05 level.

4. Discussion

Virus infection results in many alterations of physiological, biochemical, and metabolic processes within the plant [12]. These alterations which occur during viral infection development lead to the appearance of disease symptoms [35]. To our knowledge, very little is known for plant-pathogen interactions characterized by systemic infection, such as ZYMV infection. In our work, many changes were observed in the morphology and metabolic processes of pumpkin leaves associated with ZYMV infection, SA and (SA + V) treatments. In comparison to the control, severe symptoms appeared on virus-infected leaves in a form of mosaic and, the new leaves showed deformation of leaf morphology. They take a tendrile like appearance leading to foliage growth reduction and yield loss. In the analysis of pigments, Chl *a*, *b* and carotenoid contents, we found that virus infection as well as SA treatments cause a significant decrease associated with ZYMV infection and (SA + V) treatments. Similar results were obtained by Técsi et al. [35]. They reported that appearance of systemic chlorosis due to virus infection is accompanied with a decrease in photosynthesis. It is known that plant viruses, which cause systemic infections, may be particularly important as inhibitors of chlorophyll synthesis, since they spread continuously during plant growth and development [34]. In addition, Wood [41] had investigated that a decrease in net photosynthetic rate often accompanied with a decrease in chlorophyll contents in virus infected plants. From the values of *a*/*b* ratio, we can deduce that Chl *b* is more sensitive to viral infection than Chl *a*. Moreover, the total values of pigment contents seem to be unaffected by SA treatments, however, the *a*/*b* ratio is higher than control in all SA levels. To support these results, the severity of symptoms index showed that SA treatment caused reduction in severity of symptoms especially with 100 μM SA which caused no apparent symptoms on the leaves after virus inoculation. Moreover, the effect of virus infection on carotenoid contents is not clear, as their levels are unaffected or reduced after infection [15,34]. According to our results, the carotenoid content is reduced in case of ZYMV infected leaves while with SA treatments the level remain unchanged in comparison to the control. SA may lead to improve the carotenoid molecules from degradation to prevent breakdown of other pigment constituents. As a result of chlorophyll content decreased in response to ZYMV infection, the insoluble and total carbohydrate contents are decreased significantly with ZYMV infection. Infected leaves are usually characterized by a decrease in the concentration of soluble sugars and starch accumulation [12,14]. SA treatments cause a noticeable increase in all carbohydrate fractions the increase reaches up to 2 fold in some cases. It seems that SA enhances the synthesis of sugar even if applied followed by viral infection and the sugar content increases with the increase of SA concentration.

Table 4
Effect of ZYMV infection, treatment with SA and SA + V on proline contents (mg g^{-1} dw) of pumpkin (*C. pepo* cv Eskandarani) leaves

Treatments	Proline content (%)
Control	2.46 ± 0.23
Infected	3.38 ± 0.16*
10 μM SA	2.85 ± 0.35
10 μM SA + Virus	3.64 ± 0.11*
50 μM SA	3.49 ± 0.26*
50 μM SA + Virus	3.79 ± 0.25*
100 μM SA	3.41 ± 0.02*
100 μM SA + Virus	3.86 ± 0.27*

Proline content increases gradually with the increase of SA levels. The values are means of three replicates ± standard deviation. *: The values are significantly different in comparison with the control using LSD test at 0.05 level.

Environmental stresses trigger the accumulation of proline in a wide variety of species [7,9]. Proline accumulation occurred in plants under the environmental stresses that lead to limit plant growth such as pathogen stress. In our work, the virus stressed plants showed higher levels of proline. Proline accumulation, a typical plant osmotic stress response, also occurs in response to biotic stress. Free proline content increases in Arabidopsis leaf tissues that activate a hypersensitive response toward microorganisms [24].

SA and (SA + V) treatments cause proline accumulation. SA without viral infection seems to enhance proline accumulation. These results are in accordance with the results obtained by Wei et al. [39], with the tobacco leaves treated

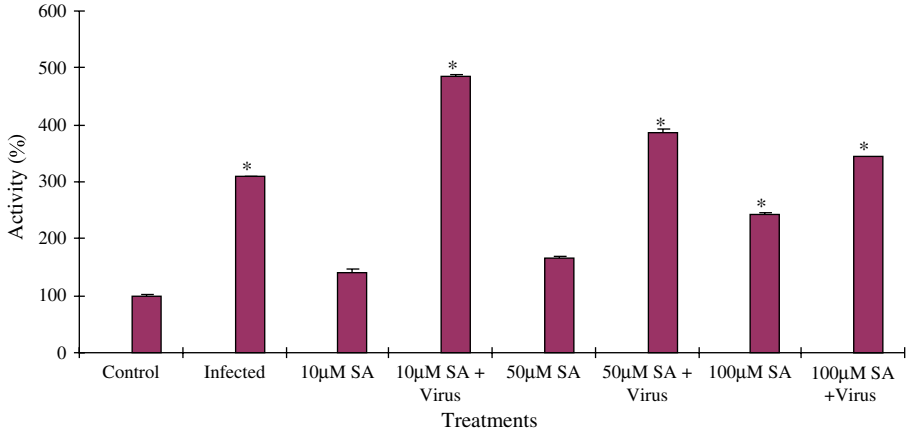

Fig. 4. Effect of ZYMV infection and SA treatments on peroxidase (POX) activity of pumpkin (*C. pepo* cv Eskandarani). The values are means of three replicates ± standard deviation. An increase in POX activity is noticed in response to ZYMV infection and SA treatments without inoculation but with SA + V treatments the activity decrease with the increase of SA level. *: The values are significantly different in comparison with the control using LSD test at 0.05 level.

with NO, SA and H_2O_2, he reported increase in proline, free amino acids and soluble proteins content with low concentrations of these signal molecules. In addition, the increase of proline with the combined treatment of SA with virus may be caused by the double factor effect (SA and virus stress).

POX is considered to be one of the antioxidant enzymes that are involved in the plant defense response to pathogen attack; it is often the first enzyme to show changes in its activity under stress [28]. We found a significant increase in POX activity in all treatments in comparison to the control. For example, the infected leaves show an increase in POX activity up to 3 fold. This result is in accordance with the results of Mojca et al. [28] who reported an increase in POX activity of potato leaves infected with potato virus Y. Virus infection appears to stimulate POX activity in all hosts in which necrotic or chlorotic symptoms are induced, the degree of stimulation correlating with severity of symptoms [41]. In our work, the results of severity of symptoms index are in accordance with the POX activity data, where the higher the severity of symptoms, the higher the POX activity.

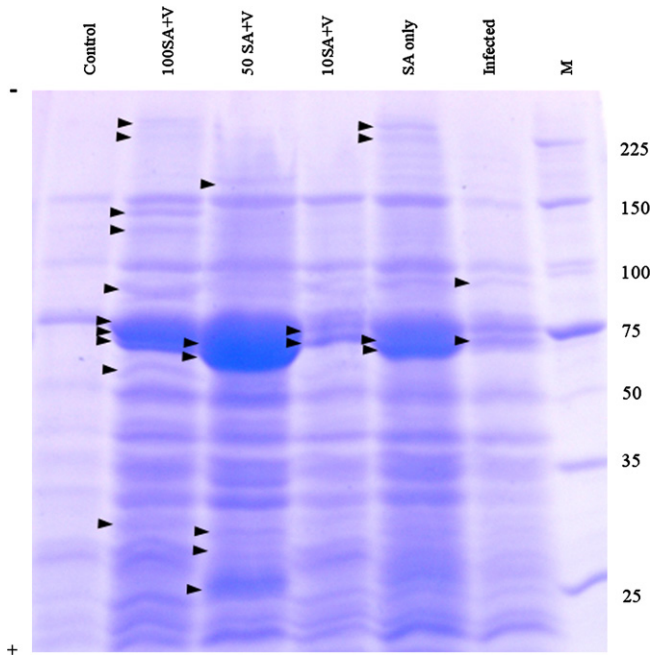

Fig. 5. SDS PAGE showing the changes in protein patterns of pumpkin leaves (*C. pepo* cv Eskadarani) infected with ZYMV and treated with 10, 50 and 100 µM (SA + V). Lane (M) is the protein marker. Arrows pointing to the polypeptides newly created under ZYMV, SA or (SA + V) treatments. They are considered as pathogenesis related proteins.

On the other hand, treatment with SA only causes a gradual increase in POX activity when compared to the control, but in case of (SA + V), we found all values of POX activity are higher than that of control. For the treatment with (SA + V), the trend of values declined by increasing of SA concentration. This indicates that SA affects the induction of at least one of the antioxidant system mechanisms. For this POX activity level can be used as an indicator for biotic stress and SAR development.

There is no clear relationship between POX activity and the chlorophyll contents because the site of peroxidase action is within the apoplast. But we found the chlorophyll content decreases while the POX activity increases for the same treatment level. POX is known to be involved in the active oxygen species (AOS) mechanisms. AOX accumulation causes oxidative damage through actions such as lipid peroxidation and membrane destruction. AOX is proposed to be responsible for chlorophyll degradation and POX levels increase during senescence [18]. So, there is a correlation between the decrease in chlorophyll content and the increase in POX activity in the green leaves infected with virus [28]. Also, POX is known to catalyze the final polymerization step of lignin synthesis and is directly associated with the increased ability of systemically protected tissues to lignify [5]. Therefore, this is a way to interpret the higher activity of POX during virus infection and SA treatments that lignifications process is considered as a pathogen resistance mechanism.

Soluble, insoluble and total protein contents as well as protein patterns were found to be affected by ZYMV infection

and SA treatments of pumpkin leaves. A significant increase in soluble, insoluble and total protein levels has noticed in response to ZYMV infection. Mojca et al. [28], reported significant decreases of soluble and ionically-bound proteins in green leaves of potato with local lesions.

In addition, 10 μM SA treatment causes no significant increase in soluble or total proteins. But higher concentrations, 50 and 100 μM SA, show a noticeable increase in all fractions. Popova et al. [30], found that no significant change in protein levels of barely seedlings treated with low SA concentrations. In case of spraying with 100 μM SA or 100 μM (SA + V), the protein level reaches the highest increase. This indicates that application of high SA concentrations to pumpkin leaves induces protein synthesis whatever the plant is attacked by pathogen or not.

Our results concerning the protein content are parallel with those describing protein patterns investigation by SDS PAGE. We found some new protein bands appeared and a high increase in the density of other protein bands. The newly synthesized polypeptides appeared in ZYMV infected sample are thought to be pathogenesis related proteins. Moreover, all lanes concerning SA treatments showed a higher amount of protein indicating that SA induced more defense proteins. Formation of new proteins and protein accumulation is considered a way and an indicator of resistance towards ZYMV infection. It is reported that the exogenous application of SA induces both resistance to TMV and the accumulation of PR proteins in tobacco plants [40]. In our experiment, SA treatments induce the formation of new proteins and cause increase in band density especially in case of (SA + V). This indicates that SA plays an important role in induction of resistance in pumpkin against ZYMV through formation of the new polypeptides or pathogenesis related proteins (PRs).

To conclude, all biochemical and physiological parameters analyzed in pumpkin leaves show significant changes in response to ZYMV infection and SA treatments. Also, from above information we can conclude that SA plays an important role in induction of SAR by decreasing the virus concentration, induction of peroxidase activity and pathogenesis related proteins.

References

[1] K. Audenaert, T. Pattery, P. Cornelis, M. Höfte, Induction of systemic resistance to *Botrytis cinerea* in tomato by *Psedomonas aeroginosa* 7NSK2: role of salicylic acid pyochelin, and pyocyanin, Mol. Plant-Microbe 15 (2002) 1147–1156.

[2] L.S. Bates, R.P. Waldern, I.D. Teare, Rapid determination of free proline for water stress studies, Plant Soil 39 (1973) 205–207.

[3] O. Borsani, V. Valpuesta, M.A. Botella, Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings, Plant Physiol 126 (2001) 1024–1030.

[4] D.J. Bowles, Defense related proteins in higher plants, Annu. Rev. Biochem. 59 (1990) 873–907.

[5] J.M. Chittoor, J.E. Leach, F.F. White, Induction of peroxidase during defense against pathogens, in: S.K. Datta, S. Muthukrishmnan (Eds.), Pathogenesis Related Proteins in Plants, CRC Press, Boca Raton, FL, 1999, pp. 171–193.

[6] M.E. Clark, A.N. Adams, Characteristics of microplate methods of Enzyme Linked Immunosorbent Assay for detection of plant viruses, J. Gen. Virol. 34 (1977) 475–483.

[7] L.N. Csonka, A.D. Hanson, Prokaryotic osmoregulation: genetics and physiology, Annul. Rev. Microbiol. 45 (1991) 569–606.

[8] C. Desbiez, H. Lecoq, Zucchini yellow mosaic virus, Plant Pathol. 46 (1997) 809–829.

[9] K. Dörffling, S. Schulenburg, G. Lesselih, H. Dörffling, Abscisic acid and proline levels in cold hardened winter wheat leaves in relation to variety – specific differences in freezing resistance, J. Agro. Crop Sci. 165 (1990) 230–239.

[10] F.W. Fales, The assimilation and degradation of carbohydrates by yeast cells, J. Biol. Chem. 193 (1951) 113.

[11] S. Ferrari, J.M. Plotnikova, G. De Lorenzo, F.M. Ausubel, Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires ED54 and PAD2, but not SID, EDS5 pr PAD4, Plant J. 35 (2003) 193–205.

[12] R.S.S. Fraser, Biochemistry of Virus Infected Plants, Research Studies Press, Letchworth/John Wiley and Sons, New York, USA, 1987.

[13] G. Fabro, I. Kovács, V. Pavet, L. Szabados, M.E. Alvarez, Proline accumulation and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions in *Arabidopsis*, Mol. Plant Microbe Interact. 17 (4) (2004) 343–350.

[14] R.N. Goodman, Z. Kiraly, K.R. Wood, The Biochemistry and Physiology of Plant Disease, University of Missouri Press, Columbia, 1986.

[15] T.W. Goodwin, G. Britton, Distribution and analysis of carotenoids, in: T.W. Goodwin (Ed.), Plant Pigments, Academic Press, London, 1988, pp. 61–132.

[16] Y.C. Jeun, Immunolocalization of PR-protein P14 in leaves of tomato plants exhibiting systemic acquired resistance against *Phytophthoria infestans* induced by pre-treatment with 3-aminobutyric acid and preinoculation with *Tobacco necrosis virus*, J. Plant Dis. Protect. 107 (2000) 352–367.

[17] M. Kar, D. Mishra, Catalase, peroxidase and polyphenol oxidase activities during rice leaf senescence, Plant Physiol. 57 (1976) 315–320.

[18] M. Kuroda, T. Ozawa, H. Imagava, Changes in chloroplast peroxidase activities in relation to chlorophyll loss in barley leaf segments, Plant Physiol. 80 (1990) 555–560.

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

[20] P.S. Low, J.R. Merida, The oxidative burst in plant defense function and signal transduction, Physiol. Plant. 96 (1996) 533–542.

[21] O.H. Lowery, N.J. Rosebrough, A.I. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 291–297.

[22] H.A. Mahgoub, C. Desbiez, C. Wipf-Scheibel, G. Dafalla, H. Lecoq, Characterization and occurrence of zucchini yellow mosaic virus in Sudan, Plant Pathol. 46 (1997) 800–805.

[23] K. Maleck, A. Levine, T. Eulgem, A. Morgan, J. Schmid, K. Lawton, J.L. Dangl, R.A. Dietrich, The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance, Nat. Genet. 26 (2000) 403–409.

[24] H. Metzner, H. Rau, H. Senger, Untersuchungen zur Synchronisierbakeit einzelner Pigmentmangel-Mutanten von *Chlorella*, Planta 65 (1965) 186–194.

[25] W.L. Min, H.M. Bakker, T.S. Lee, Screening of salicylic acid producing Rhizobacteria isolated from plant roots and rhizosphere, Korean J. Plant Pathol. 14 (6) (1998) 598–602.

[26] E. Miteva, D. Hristova, V. Nenova, S. Maneva, Arsenic as a factor affecting virus infection in tomato plants: changes in plant growth, peroxidase activity and chloroplast pigments, Sci. Hortic. 105 (2005) 343–358.

[27] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (2002) 405–410.

[28] M. Milavec, M. Ravnikar, M. Kovac, Peroxidases and photosynthetic pigments in susceptible potato infected with potato virus YNTN, Plant Physiol. Biochem. 39 (2001) 891–898.

[29] U. Neuenschwader, K. Lawton, J. Ryals, in: G. Stacey, N.T. Keep (Eds.), Systemic acquired resistance in plant-microbe interact, vol. 1, Chapman and Hall, New York, 1996, pp. 81–108.

[30] L. Popova, E. Ananieva, V. Hristova, K. Christov, K. Georgieva, V. Alexieva, Zh, Stoinova, Salicylic acid-and methyl jasmonate-induced protection on photosynthesis to paraquat oxidative stress, Bulg. J. Plant Physiol. (2003) 133–152 Special Issue.
[31] A.F. Ross, Systemic acquired resistance induced by localized virus infection in plants, Virology 14 (1961) 340–358.
[32] H.G. Schlegel, Die verwertung organischer sauren durch Chlorella ir lichet, Planta 47 (1956) 510.
[33] D. Shalitin, S. Wolf, Cucumber mosaic virus infection affects sugar transport in melon plants, Physiol. Plant. 123 (2000) 597–604.
[34] D.D. Sutic, J.B. Sinclair, Physiology of diseased plants, in: D.D. Sutic, J.B. Sinclair (Eds.), Anatomy and Physiology of Diseased Plants, 2, CRC Press, Boca Raton, 1990, pp. 157–221.
[35] L. Técsi, A.M. Smith, A.J. Maule, C. Richard, A spatial analysis of physiological changes associated with infection of cotyledons of marrow plants with cucumber mosaic virus, Plant Physiol. 111 (1996) 975–985.
[36] U. Rad, M.J. Mueller, J. Durner, Evaluation of natural and synthetic stimulants of plant immunity by microarray technology, New Phytol. 165 (2005) 191–202.
[37] C.P. Vance, T.K. Krik, R.T. Sherwood, Lignification as a mechanism of disease resistance, Annu. Rev. Phytopathol. 18 (1980) 259–288.
[38] K. Wakamatsu, U. Takahama, Changes in peroxidase activity and in peroxidase isozymes in carrot cells, Physiol. Plant. 88 (1993) 167–171.
[39] X. Wei, L.M. Wang, R.J. Long, G.X. Wang, Effects of exogenous nitric oxide, salicylic acid and hydrogen peroxide on free amino acid and soluble protein contents in tobacco leaves, Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao 32 (2) (2006) 257–260.
[40] R.F. White, Acetylsalicylic (aspirin) induces resistance to tobacco mosaic virus in tobacco, Virology 99 (1979) 410–412.
[41] K.R. Wood, Pathophysiological alternations, in: C.L. Mandahar (Ed.), Plant Viruses, Pathology, 2, CRC Press, Boca Raton, 1990, pp. 30–52.
[42] X.S. Ye, S.Q. Pan, J. Kuc, Activity, isozyme pattern, and cellular localization of peroxidase as related to systemic resistance of tobacco to blue mould (*Peronospora tabacina*) and tobacco mosaic virus, Phytopathology 80 (1990) 1295–1299.
[43] X. Yang, K. Liangyi, P. Tien, Resistance of tomato infected with cucumber mosaic virus satellite RNA to potato spindle tuber viroid, Ann. Appl. Biol. 129 (1996) 543–551.
[44] B. Zechmann, M. Muller, G. Zellnig, Cytological modifications in zucchini yellow mosaic virus (ZYMV)-infected Styrian pumpkin plants, Arch. Virol. 148 (2003) 1119–1133.