Evaluation of genotoxicity of the aqueous extracts from wild *Artemisia herba-alba* and *Jasmina montana* against *Zucchini yellow mosaic virus* (ZYMV) in *Cucurbita pepo* plants

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Accepted 16th October 2018

Abstract. Squash or cucurbit (*Cucurbita pepo*) is one of the famous and important vegetable plants in most world countries. The squash is infected by the critical *Zucchini yellow mosaic virus* (ZYMV) in Egypt. Though pesticides can protect the plants of pest infections, there are no effective compounds that can be applied as virucides. In this work, the effects of aqueous extracts from *Jasmina montana* and *Artemisia herba-alba* on ZYMV infection in the squash plants were examined. In addition, SDS-PAGE protein patterns and enzyme activities were evaluated and induction of resistance by plant elicitors against ZYMV was studied. The results observed that using of the aqueous extracts from *A. herba-alba* or *J. montana* extracts prior to ZYMV inoculation recorded 100% inhibition of virus infection. Also, complete inhibition was obtained by treatment of *A. herba-alba* extract with the virus inoculum led to destroy the virus particles due to presence of the effective compounds (terpens, phenolics and the essential oils) in the plant extract. SDS-PAGE protein profiles and enzyme activities were studied in treated and untreated plants. Genomic DNA variation was studied using random amplified polymorphic DNA (RAPD) and Inter-simple sequence repeat (ISSR) loci. The alterations in RAPD and ISSR assays of representative squash plants can be applied to comprehend of induced systemic resistance. These inducers stimulated resistance in the squash plants. Therefore, elicitors should be taken into consideration in the breeding programs for ZYMV control.

Keywords: antiviral activities, SDS-PAGE, isozyme markers, molecular markers

INTRODUCTION

Zucchini yellow mosaic virus (ZYMV) is an important virus belong to the genus *Potyvirus* under family *Potyviridae*, affecting cucurbits production globally (Zechmann *et al.*, 2003; Lecoq *et al.*, 2014). The virus can completely destroy the crop yield under favorable conditions. The resistance to ZYMV has not yet been established (Deya *et al.*, 2006).

The resulting elevated resistance due to an inducing agent upon infection by a pathogen is called systemic acquired resistance (SAR)

(Ramamoorthy *et al.*, 2001). A development of SAR is often associated with various cellular defense responses, such as synthesis of pathogen-related proteins, phytoalexins and accumulation of active oxygen species, rapid alterations in the cell wall and enhanced activity of various defense related enzymes (Thakur and Sohal, 2013).

Extracts of wild medicinal and aromatic plants have been applied as a natural product and as a safe method to control virus infection. However,

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this is only true when they are mixed with the virus prior to inoculation or when applied within a short period of time before or after inoculation (Ottai *et al.*, 2011). The effect of plant extracts was attributed to their chemical contents which induce the SAR to induce or create anti-phytoviral agents.

DNA based techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), and simple sequence repeat (SSR) are used to evaluate the variation at the DNA sequence level. RAPD can be used to detect genotoxicity and differences can be clearly shown when comparing DNA fingerprint from untreated and treated plants to genotoxic agents (Raj *et al.*, 2014).

This work aims to study the effects of *J. montana* and *A. herba-alba* extracts on ZYMV infection for the squash plants. The alterations of protein patterns by (SDS-PAGE), enzyme activity (Native-PAGE) and genetic diversity in genomic DNA (molecular markers) as indicators for the induced resistant form the objectives of this the work.

MATERIALS AND METHODS

Plant extracts. Leaves of two wild medicinal plants, *Artemisia herba-alba* and *Jasmina montana* (both belonging to family *Asteraceae*) were collected at the summer season of 2013 from Deserts of Ras Sader City, South of Sinai Governorate and identified in laboratory of Genetics and Breeding of Medicinal and Aromatic Plants, Genetic Engineering and Biotechnology Division, National Research Centre, Egypt. The plants were separately extracted by grinding dry leaves in a sterilized pestle and mortar with sterilized dsH₂O (1:5 w/v). The pulps were squeezed through two layers of cheesecloth and used as inhibitors (Mahdy *et al.*, 2010).

Virus isolate and treatments. *Zucchini yellow mosaic virus* (ZYMV) isolate was obtained from the Virology Laboratory, Department of Agriculture Microbiology, Faculty of Agriculture, University of Ain Shams. ZYMV isolate was maintained in

squash plants *Curcubita pepo* cv. Eskandarani. Where the seeds of squash were obtained from The Egyptian Seeds Oil and Chemical Company, Cairo, Egypt and sown in a sandy clay land (1:2, v/v) in plastic pots (10 cm in diameter) in the greenhouse under a photoperiod of 12 hours. Day and night temperatures were 24 and 18°C, respectively. The relative humidity was 70±5 RH%. Systemically infected leaves were used as sources of inoculum in all experiments. The healthy leaves of squash were rubbed gently with a cotton swab dipped into the suspension of the virus inoculums (Ling and Levi, 2007).

After 21 days of growth, squash plants were divided into ten groups. Each group comprised of eight replicates:

The first group: The plants were inoculated with the virus and sprayed with *J. montana* extract after 24 hours from inoculation (pre-treatment); the second group: the plants were inoculated with the virus and sprayed with *A. herba-alba* extract after 24 h from inoculation (pre-treatment); the third group: the plants were inoculated with mixture of virus inoculum and *J. montana* plants extract (1:1) (with-treatment); The fourth group: the plants were inoculated with mixture of virus inoculum and *A. herba-alba* plants extract (1:1) (with-treatment); The fifth group: the plant leaves were sprayed with *J. montana* extract until run-off and inoculated by virus after 24 h (post-treatment); the sixth group: the plant leaves were sprayed with *A. herba-alba* extract until run-off and inoculated by virus after 24 h (post-treatment); the seventh group: the plant leaves were sprayed with *J. montana* extract without inoculation (aqueous extract control); the eight group: the plant leaves were sprayed with *A. herba-alba* extract without inoculation (aqueous extract control); the ninth group: the plants were inoculated with ZYMV virus only (viral control); the tenth group: was considered as a control without treatment (healthy control).

All samples were tested for the presence of ZYMV by the double antibody sandwich enzyme linked immune sorbent assay (DAS-ELISA) as described by Clark and Adams, (1977). The percentage of viral inhibition was estimated. Three weeks after inoculation, the youngest fully developed leaves from both the control and treated plants were collected for analysis of biochemical and molecular changes.

Electrophoretic analysis of protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done according to (Laemmli, 1970) as modified by (Studier, 1973).

Polyphenol oxidase (PPO) and peroxidase (POX) isoforms. For analysis of PPO and POX isozymes by Native-PAGE, 500 mg fresh treated and untreated leaves of *C. pepo* L. cv. Eskandarani were homogenized in liquid nitrogen and 100 µl of 0.2 M phosphate buffer was added (pH 7.0 was adjusted by potassium phosphate, monobasic) and 10 µl of 2-mercaptoethanol before centrifugation at 14,000 rpm for 15 minutes at 4°C. The supernatant was stored at a temperature of -20°C until isozyme analysis (Stagemann *et al.*, 1985). PPO and POX isozymes were detected according to (Baaziz *et al.*, 1994; Brown, 1978).

Genomic DNA extraction. Young plant leaves of *C. pepo* L. cv. Eskandarani were soaked in liquid nitrogen for DNA extraction using 2% cetyl trimethylammonium bromide (CTAB) (Brown and Myers, 1998).

RAPD analysis. A total of four primers were used to amplify DNA (Mahfouze *et al.*, 2012a) (manufactured by Bioneer, New technology certification from ATS Korea). The total reaction mixture was 25 µl contained 10X PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs mixed, 10 pmol primer, 1.25 U *Taq* polymerase and about 150 ng genomic DNA. RAPD-PCR amplification was performed in thermal cycler (Biometra Inc., Germany). The temperature profile was as follows: an initial denaturation at 94°C for 3 minutes; followed by 35 cycles of denaturation temperature 94°C for 5 minutes; annealing temperature 37°C for 1 minute; and extension temperature 72°C for 1 minute, and final extension at 72°C for 5 minutes.

ISSR profiles. A total of four anchored ISSR primers were used to amplify DNA (Life Technologies, Gaithersburg, Md.). Each 25 µl amplification reaction consisted of 2.5 µl 10X PCR buffer, 2.5 µl 25 mM MgCl₂; 0.5 µl 40 mM dNTPs; 1 µl *Taq* DNA polymerase (1 unit/µl); 2 µl 0.4 µM primer. Amplification was carried out in DNA thermocycler (Biometra, Germany) under

the following conditions: one cycle of 3 minute at 94°C, followed by 28 cycles of denaturation temperature 94°C for 45 seconds, annealing temperature 52°C for 30 seconds, and extension temperature 72°C for 2 minutes at; a final extension 72°C for 6 minutes.

Amplification products were separated on a 1.5% agarose gel containing 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) and 0.5 µg/ml ethidium bromide at 90 V. Gels were analyzed by UVI Geltec version 12.4, 1999-2005 (USA).

Data analysis. A matrix for SDS-PAGE, POX, PPO, RAPD and ISSR combined was generated by scoring reproducible bands as 1 for their presence and as 0 for their absence across the treatments. Genetic similarity coefficients were computed according to Nei and Li (1979). The data were subsequently used to construct a dendrogram using the un-weighted pair group method of arithmetic averages (UPGMA) (Sneath and Sokal, 1973) employing sequential, agglomerative hierarchic and non-overlapping clustering (SAHN). All the computations were carried out using the software NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.1 (Rohlf, 2000). Correlation coefficients were calculated using similarity coefficients obtained from combined SDS-PAGE, POX, PPO, RAPD and ISSR analysis.

RESULTS

Efficacy of inducers on squash plants against ZYMV. The study showed effects of plant inducers on inhibition of ZYMV virus in the squash plants (Figures 1 and 2). Data in Table 1 showed that post-treatment with separate *A. herba-alba* and *J. montana* extracts gave complete inhibition (100%) of the ZYMV. Application of *A. herba-alba* extract with virus inoculum (with treatment) appeared also with maximum inhibition of 100%. Meanwhile, *A. herba-alba* (pre-treatment) and *J. montana* (with treatment) extracts showed 75% inhibitions against ZYMV infection. On the other hand, the pre-treatment of *J. montana* extract had no effect 0% inhibition (Table 1 and

Figure 1). These results were confirmed by DAS-ELISA.

Protein patterns. Protein patterns of treated and untreated squash plants were analyzed by SDS-PAGE. SDS-PAGE analysis revealed 12 protein bands with different molecular weights (MWs) ranged from 10 to 245 kDa as shown in Figure 3 and Table 2. The nine bands were monomorphic (75%) and three bands were polymorphic (25%). It was observed that, the highest number of protein bands appeared in ZYMV infected plants and treated *J. montana* extract (pre-treatment) (12 polypeptides). However, the lowest number of polypeptides showed in the viral control, *J. montana* aqueous extract control and ZYMV resistant plants and treated *A. herba-alba* extract (pre-treatment) (nine polypeptides). On the other hand, one polypeptide with MW 56 KDa showed in the healthy control and ZYMV resistant squash plants and treated *J. montana* and *A. herba-alba* extracts (with treatment) and ZYMV infected plants and sprayed *J. montana* extract (pre-treatment) and disappeared in all treatments. In contrast, the polypeptide of 27.5 KDa disappeared in the viral control; *J. montana* aqueous extract control, ZYMV resistant plants and treated *A. herba-alba* extract (pre-treatment) and revealed in all treatments (Figure 3 and Table 2). Furthermore, one new polypeptide of about 15.6 KDa induced ZYMV infected squash plants and treated *J. montana* extract (with and pre-treatments) and ZYMV resistant plant and treated *A. herba-alba* extract (post-treatment).

Figure 1. Effect of treatment of *J. montana* extract on squash plants. 1-Healthy control; 2,3-viral control; 4-*J. montana* aqueous extract control; 5-TYLCV infected squash plants (pre-treatment); 6-TYLCV resistant squash plants (with treatment); 7-TYLCV diseased squash plants (with treatment); 8-TYLCV resistant squash plants (post-treatment).

Figure 2. Effect of treatment *A. herba-alba* extract on squash plants. 1-Healthy control; 2,3-Viral control; 4-*A. herba-alba* aqueous extract control; 5-TYLCV resistant squash plants (pre-treatment); 6-TYLCV infected squash plants (pre-treatment); 7-TYLCV resistant squash plants (with treatment); 8-TYLCV resistant squash plants (post-treatment).

Table 1. Effect of ZYMV infection and plant inducers treated on squash plants.

Treatment		Symptoms	% Inhibition
1.	Virus + <i>J. montana</i> extract (pre-treatment)	SM, D	0
2.	Virus + <i>A. herba-alba</i> extract (pre-treatment)	M, B, D	75
3.	Virus + <i>J. montana</i> extract (with treatment)	SM	75
4.	Virus + <i>A. herba-alba</i> extract (with treatment)	NS	100
5.	<i>J. montana</i> extract + virus (post-treatment)	NS	100
6.	<i>A. herba-alba</i> extract + virus (post-treatment)	NS	100
7.	<i>J. montana</i> aqueous extract control	NS	-
8.	<i>A. herba-alba</i> aqueous extract control	NS	-
9.	Viral control	SM, TLL, D	0
10.	The healthy control	NS	-

B = blisters; D = deformation; M = mosaic; NS = no symptoms; SM = severe mosaic; TLL = thread-like leaf

Figure 3. SDS-PAGE binding patterns of plant inducers treated squash plants. Lane M = protein ladder ranged from 11 to 240 KDa, lane C = healthy control, lane V = viral control, lane J = *J. montana* aqueous extract control, and lane A = *A. herba-alba* aqueous extract control; I = infected, R = resistant.

Table 2. SDS-PAGE protein patterns of inducers treated squash plants and untreated ones.

No.	MW (kDa)	C	V	J	A	<i>J. montana</i> extract				<i>A. herba-alba</i> extract			
						pre (I)	with (I)	with (R)	post (R)	pre (I)	pre (R)	with (R)	post (R)
1	245	1	1	1	1	1	1	1	1	1	1	1	1
2	168	1	1	1	1	1	1	1	1	1	1	1	1
3	127	1	1	1	1	1	1	1	1	1	1	1	1
4	56	1	0	0	0	1	0	1	0	0	0	1	0
5	38	1	1	1	1	1	1	1	1	1	1	1	1
6	31.5	1	1	1	1	1	1	1	1	1	1	1	1
7	27.5	1	0	0	1	1	1	1	1	1	0	1	1
8	25	1	1	1	1	1	1	1	1	1	1	1	1
9	23	1	1	1	1	1	1	1	1	1	1	1	1
10	15.6	0	0	0	0	1	1	0	0	0	0	0	1
11	13	1	1	1	1	1	1	1	1	1	1	1	1
12	10	1	1	1	1	1	1	1	1	1	1	1	1
Total		11	9	9	10	12	11	11	10	10	9	11	11

0 = absence of band; 1 = presence of band; C = the healthy control; V = viral control; J = *J. montana* aqueous extract control; A = *A. herba-alba* aqueous extract control; I = infected; R = resistant.

POX and PPO isozymes. The effects of plant extracts application and virus infection on activities of POX and PPO isozymes are shown in Figure 4. Isoenzyme patterns of the POX and PPO exhibited a range of five and six isoforms with the R_f value of (0.089 to 1.000) and (0.468 to 0.921), respectively. The most differences in the activities of POX and PPO were detected in ZYMV infected squash plants and treated *J. montana* extract (with and pre-treatments) (three isozyme markers), followed by ZYMV diseased and resistant plants and treated *A. herba-alba* extract (pre-treatment) (two isoforms). However,

lowest differences in POX and PPO isozymes scored in the ZYMV resistance plants and treated *A. herba-alba* extract (with-treatment) and *A. herba-alba* aqueous extract control (one marker) (Figure 4 and Table 3). On the other hands, no changes in the POX and PPO activities in all remaining treatments compared with the healthy control ones.

RAPD analysis. RAPD profiles by four primers differed between the healthy control and treated plants with *J. montana* and *A. herba-alba* extracts with visible changes in the number and size of

amplified DNA fragments (Table 4). The results showed polymorphic numbers of the genetic bands, which were the electrophoretic products of PCR for treated squash plants comparing with untreated ones. The maximum number of bands (56 bands) appeared in ZYMV resistance squash plants and treated with *J. montana* extract (post-treatment). However, the minimum number of bands (41 bands) appeared in the viral control. A total of 65 RAPD bands were obtained by using four primers, and 26 (40%) of them showed polymorphism (Table 5). Furthermore, all bands generated were ranged between 150 and 3500 bp (Figure 5). The change in RAPD profiles of the healthy control plants and treatments was discernible from appearance/disappearance of some bands (Tables 4 and 5). The highest number of polymorphic bands generated with the primers RAPD-1 (64.71%) while primer RAPD-3 gave the lowest number of polymorphic (26.32%). The number of amplification products per RAPD primer varied from 12 for primer RAPD-2 to 19 for primer RAPD-3 (Table 5). All treatments induced an increase in number of bands except viral control, compared with the healthy control plants. Besides, ZYMV resistance squash plants and treated *A. herba-alba* (post-treatment) scored the highest molecular markers (three bands) with molecular sizes of 3500, 570 and 210 bp. On the

contrary, squash plants diseased and treated *J. montana* extract (with and pre-treatment) appeared one specific band of 1250 and 460 bp, respectively (Figure 5 and Table 5).

Figure 4. PPO and POX isozyme profiles of plant inducers treated squash plants. Lane C = healthy control; lane V = viral control; lane J = *J. montana* aqueous extract control; lane A = *A. herba-alba* aqueous extract control; I = infected; R = resistant.

Table 3. POX and PPO isozyme markers of inducers treated squash plants and the untreated ones.

No.	Rf	C	V	J	A	<i>J. montana</i> extract				<i>A. herba-alba</i> extract			
						pre	with	with	post	pre	pre	with	post
						(I)	(I)	(R)	(R)	(I)	(R)	(R)	(R)
POX													
1	0.089				1	1				1	1	1	
2	0.918					1	1						
Total = 2		0	0	0	1	2	2	0	0	1	1	1	0
PPO													
1	0.667					1	1			1	1		
Total = 1		0	0	0	0	1	1	0	0	1	1	0	0
Total of POX and PPO bands = 3		0	0	0	1	3	3	0	0	2	2	1	0

0 = absence of band; 1 = presence of band; C = the healthy control; V = viral control; J = *J. montana* aqueous extract control; A = *A. herba-alba* aqueous extract control; I = infected; R = resistant.

Table 4. Polymorphism of the RAPD marker of plant inducers treated squash plants compared with the healthy control.

Primer No.	C	V	J	A	<i>J. montana</i> extract				<i>A. herba-alba</i> extract			
					pre (I)	with (I)	with (R)	post (R)	pre (I)	pre (R)	with (R)	post (R)
1	10	6	11	10	11	8	12	14	11	11	11	14
2	9	8	8	11	9	9	9	10	9	9	10	10
3	15	16	16	15	15	17	17	17	17	16	16	16
4	12	11	15	12	14	13	14	15	14	15	15	14
Total	46	41	50	48	49	47	52	56	51	51	52	54

C = the healthy control; V = viral control; J = *J. montana* aqueous extract control; A = *A. herba-alba* aqueous extract control; I = infected; R = resistant.

Table 5. RAPD amplified bands, polymorphic bands and unique markers for inducers treated squash plants using four primers.

Primer Code No.	Primer sequences	Size range of the scorable bands (bp)	Total bands	No. of monomorphic bands	No. of polymorphic bands	% Polymorphism	Unique bands
RAPD-1	GTTTCGCTCC	165-1250	17	6	11	64.71	1250, 570, 210
RAPD-2	AACGCGCAAC	150-3500	12	8	4	33.33	3500
RAPD-3	GGACGGCGTT	160-1500	19	14	5	26.32	460
RAPD-4	AAGCCCGAGG	175-1420	17	11	6	35.29	0
Total		150-3500	65	39	26	40%	7.69%

RAPD-1

RAPD-2

RAPD-3

RAPD-4

Figure 5. RAPD profiles of genomic DNA extracted of squash plants treated with elicitors using four primers. Lane M = 100 bp DNA ladder; lane C = healthy control; lane V = viral control; lane J = *J. montana* aqueous extract control; lane A = *A. herba-alba* aqueous extract control; I = infected; R = resistant.

ISSR profiles. Genomic DNA was extracted from inducers treated squash plants (pre-, with, and post-) and the control and assayed by ISSR-PCR (Table 6). All treatments appeared a decrease in number of amplified fragments, except *A. herba-alba* extract treated resistant plants (pre-treatment) scored an increase in number of amplicons (62) comparing with the healthy control plants (56 loci) (Table 6). A total number of 72 reproducible and scorable ranging from 90 to 4000 bp were scored using four primers (Figure 6 and Table 7). Thirty-three out of 72 (45.83%) bands were polymorphic loci, and the remaining 39 (54.17%) were monomorphic. ISSR profiles showed substantial differences between the healthy control and treated squash plants with apparent changes (disappearance and/or appearance) in the number and size of amplified DNA fragments for different primers. The percentage of polymorphism ranged from 31.58 (ISSR-1) to 62.50% (ISSR-2). Amplicon numbers per primer ranged from 16 (ISSR-2 and ISSR-4) to 21 (ISSR-3). On the other hands, ZYMV resistance squash plants and treated *A. herba-alba*

extract (pre-treatment) and (with treatment) revealed two specific bands of (680 and 400 bp) and (1210 and 1100 bp), respectively. In contrast, the squash plants infected and treated *A. herba-alba* extract (pre-treatment) exhibited one unique band of 2000 bp (Figure 6 and Table 7).

Cluster analysis was done to estimate the level of polymorphism between the control plants and treatments. The resulted dendrogram from the combination among the banding patterns of POX, PPO, SDS-PAGE, RAPD and ISSR was shown in (Figure 7 and Table 8). The control and treated samples were clustered in three groups: The first group I: (similarity range 0.76 to 0.85) contained the pre and with treatments of the infected plants and treated with *J. montana* extract. The second group II: (similarity range 0.74 to 0.84) comprised of the control without treatment, viral control, *A. herba-alba* aqueous extract control and ZYMV resistance squash plants and treated *A. herba-alba* extract (pre and post-treatments). The third group III: (similarity range 0.71 to 0.91) consisted of all the remaining treatment.

Table 6. A number of the fragments amplified of each primer by ISSR in the treated and untreated squash plants.

Primer No.	C	V	J	A	<i>J. montana</i> extract				<i>A. herba-alba</i> extract			
					pre (I)	with (I)	with (R)	post (R)	pre (I)	pre (R)	with (R)	post (R)
ISSR-1	16	13	13	13	15	15	15	16	13	18	15	14
ISSR-2	13	12	8	11	13	7	8	7	7	14	10	9
ISSR-3	17	16	13	14	11	11	14	19	15	16	16	17
ISSR-4	10	11	10	11	11	14	11	13	13	14	13	15
Total	56	52	44	49	50	47	48	55	48	62	54	55

C = the healthy control; V = viral control; J = *J. montana* aqueous extract control; A = *A. herba-alba* aqueous extract control; I = infected; R = resistant.

Table 7. ISSR amplified bands, polymorphic bands and unique markers for inducers treated squash plants using four primers.

Primer Code No.	Primer sequences	Size range of the scorable bands (bp)	Total bands	No. of monomorphic bands	No. of polymorphic bands	% Polymorphism	Unique bands
ISSR-1	(GA)8 4C	140-4000	19	13	6	31.58	680, 400
ISSR-2	(CA)8 GC	190-2500	16	6	10	62.50	1210, 1100
ISSR-3	(CAC)3 GC	90-1235	21	11	10	47.62	0
ISSR-4	CGT (CA)8	100-330	16	9	7	43.75	2000
Total		90-4000	72	39	33	45.83%	6.94%

Figure 6. ISSR profiles of genomic DNA extracted of squash plants treated with elicitors using four primers. Lane M = 100 bp DNA ladder; lane C = healthy control; lane V = viral control; lane J = *J. montana* aqueous extract control; lane A = *A. herba-alba* aqueous extract control; I = infected; R = resistant.

Figure 7. Dendrogram of inducers treated squash plants and control ones based on POX, PPO, SDS-PAGE, RAPD and ISSR combination by UPGMA algorithm using Jaccard's similarity coefficient. C = healthy control; V = viral control; J = *J. montana* aqueous extract control; A = *A. herba-alba* aqueous extract control; Jm, Ah (pre) = mechanically inoculated squash plants with the virus and was treated with *J. montana* or *A. herba-alba* extracts, respectively (pre-treatment); Jm, Ah (with) = squash plants was inoculated with mixture of virus inoculum and *J. montana* or *A. herba-alba* extracts, respectively (with-treatment); Jm, Ah (post) = squash plants was treated with *A. herba-alba* and *J. montana* extracts, respectively then were inoculated by virus (post-treatment); I = infected; R = resistant.

Table 8. Similarity index between the inducers treated squash plants and the control as estimated by combined POX, PPO, SDS-PAGE, RAPD and ISSR.

Combined PCR, FPG, SDS PAGE, RFLP and RSK.													
Treatment		C	V	J	A	<i>J. montana</i> extract				<i>A. herba-alba</i> extract			
						pre (I)	with (I)	with (R)	post (R)	pre (I)	pre (R)	with (R)	post (R)
C		1.00											
V		0.82	1.00										
J		0.84	0.78	1.00									
A		0.84	0.82	0.84	1.00								
<i>J. montana</i> extract	pre (I)	0.79	0.79	0.77	0.82	1.00							
	with (I)	0.76	0.77	0.79	0.80	0.85	1.00						
	with (R)	0.84	0.74	0.91	0.81	0.77	0.80	1.00					
	post (R)	0.78	0.71	0.82	0.75	0.72	0.77	0.86	1.00				
<i>A. herba-alba</i> extract	pre (I)	0.75	0.76	0.85	0.78	0.73	0.78	0.88	0.84	1.00			
	pre (R)	0.81	0.79	0.81	0.81	0.77	0.76	0.81	0.80	0.80	1.00		
	with (R)	0.78	0.72	0.79	0.75	0.76	0.81	0.81	0.82	0.81	0.76	1.00	
	post (R)	0.81	0.77	0.80	0.80	0.75	0.76	0.79	0.81	0.78	0.82	0.74	1.00

C = healthy control; V = viral control; J = *J. montana* extract aqueous control; A = *A. herba-alba* aqueous extract control; I = infected; R = resistant.

DISCUSSION

ZYMV is considered a serious problem for the *Cucurbita pepo* in all producing countries including Egypt. It causes yield loss of up to 95%. The resistance to ZYMV has not yet been established (Deya *et al.*, 2006). In this study, we observed that post-treatment with separate *A. herba-alba* and *J. montana* extracts completely inhibited (100%) of the ZYMV. Consequently, elicitors play a main role in inducing the resistance against ZYMV

infecting in the squash plants. However, application of *A. herba-alba* extract with virus inoculum (with treatment) appeared 100% inhibition. This extract contains some effective compounds such as terpens, phenolic compounds addition to the essential oils which can be destroyed the virus particles. This conclusion was in agreement with Teixeira *et al.* (1997) and Othman and Shoman (2004), obtained 100% inhibition in the *Tobacco Necrosis Virus* when applied the extract of *Plectranthus tenuiflorus* plant with the virus inoculum. Abdel-Shafi (2013)

observed that aqueous decoction and infusion of *Nigella sativa* L inhibited the production of ZYMV symptoms on the squash plants by 85% and 80%, respectively. Percival (2001) mentioned that *Artemisia* spp., *Jasminum* spp., Rosa hybrids, *Salix* spp (Willow), sunflower and petunia plants induce SAR. Verma *et al.* (1998) reported that antiviral resistance by plant extracts could be due to one of the following mechanisms i.e., *de novo* synthesis of antiviral substances, production of virus inhibiting agents and production of mobile inducing signal that bind to the host plant surface, which produces virus inhibiting agents. Also, Madhusudhan *et al.* (2005) reduced the tobacco viruses by spray treating neem oil (5%).

This study observed that the maximum number of polypeptides showed in ZYMV infected plants and treated *J. montana* extract (pre-treatment) (12 polypeptides). However, minimum number of polypeptides scored in the viral control, *J. montana* aqueous extract control and ZYMV resistance plants and treated *A. herba-alba* extract (pre-treatment) (nine polypeptides). Similar results were noticed by Madhusudhan *et al.* (2005), that a wide variety of factors induced the pathogen-related proteins (PR); pathogen (virus, fungi, bacteria...etc.) infection and various chemical treatments.

These results showed that POX and PPO isozyme activities were changed as a result of virus infection and plant extracts treatment. Our results are in agreement with those reported by Milavec *et al.* (2008); Mahfouze *et al.* (2012b); Abdel-Shafi (2013), indicating that the increase in phenolic compounds, since the oxidative enzymes play an important role in oxidation of free phenols, which are accumulated as a result of viral infection and these newly synthesized polyphenols and their oxidation products may limit the viral activity in resistance tissues (Quiroga *et al.*, 2000). Several studies have demonstrated the role of peroxidase in the expression of genes related to defense, cell wall plasticity, and plant cell elongation. Besides the degradation of reactive oxygen species (ROS), peroxidases can also generate reactive oxygen species (H_2O_2 , OH^- , O_2^-), which are toxic to pathogens (Riedle-Bauer, 2000; Vieira *et al.*, 2010). Milavec *et al.* (2008) and Mahfouze *et al.* (2012b) stated that there are no changes in activity of POXs isoenzymes in the potato plants infected by PVY^{NTN}.

Our results indicated that, both ZYMV infected (without-treatment) and treated plants effluent induced DNA change in plants. However, the number of disappeared bands was higher in the inoculated plants by virus only comparing with the treatments, which revealed that DNA change was more severe in plants infected by ZYMV. The changes in the genomic DNA amplicon pattern can be interpreted as direct DNA alteration, such as recombination or mutagenesis. The resulting changes in genomic DNA were visualized on an agarose gel after RAPD and ISSR techniques, where they were indicators of genetic dissimilarities between the treatment and control squash plants. These results were agreement with Atienzar *et al.* (2000); Wolf *et al.* (2004) and Raj *et al.* (2014), who found that the disappearance of bands may be related to the DNA change (e.g., single- and double-strand breaks, modified bases, abasic sites, oxidized bases, bulky adduct, and DNA-protein cross links), point mutations, and/or complex chromosomal rearrangements induced by elicitors. Atienzar *et al.* (2002) reported that mutation can only be responsible for the appearance of new bands if they occur at the same locus in a sufficient number of cells (a minimum of 10% of mutations may be required to get new PCR product visible in agarose) to be amplified by PCR. Briefly, new bands could be attributed to mutation, while the bands which disappeared could be attributed to DNA change (Atienzar and Jha, 2006). In the present study, cluster analysis was done to estimate the level of polymorphism between the control plants and treatments. Swaileh *et al.* (2008) and Raj *et al.* (2014) mentioned that the cluster analysis method is considered one of the most effective methods in numerical analysis regarding band scoring and analysis of RAPD finger printing.

Our finding on comparison between 'untreated' and 'treated' squash genomes showed that SDS-PAGE, POX, PPO, and ISSR analysis can be used for evaluation of DNA change on squash plants caused by inducers. On the basis of these considerations, we could suggest that biochemical and molecular markers are powerful tools for measuring qualitative and quantitative changes produced by inducers.

CONCLUSION

This study has shown that the aqueous extract of the medicinal plants *J. montana* and *A. herba-alba* proved to have antiviral activities against ZYMV. The treatment with plant inducers led to protein content, antioxidant enzymes and DNA change in the squash plants. The obtained unique bands clearly differentiated the samples treated with inducers compared with untreated, which occurred in RAPD and ISSR profiles of the squash plants, can be used successfully in induced systemic resistance understanding.

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