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INDUCED RESISTANCE IN ONION PLANTS TO WHITE ROT BY CERTAIN CHEMICALS.*

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Abstracts: Treating Giza 6 onion transplants before transplanting by dipping in Salicylic acid (SA, 100 ppm), Acetic acid (AA, 50 ppm) and Bion (ASM, 50 ppm) or by twice sprays on grown plants (15 and 30 days after transplanting date) or by combined treatment (dipping + spraying) induced the resistance of onion plants to white rot, since, percentages of infection was significantly decreased in treated plants compared with untreated ones. In general, lowest disease incidence in different treatments of the tested chemicals was achieved by combined treatments followed by spray treatments and finally dipping treatments. Treating Giza 6 onion plants with different resistance inducers and different methods of applications increased accumulation of total phenols and free salicylic acid (SA) and levels of peroxidase (PO) and polyphenoloxidase (PPO) in treated plants compared with healthy or diseased untreated plants. In general, the combined treatments of the tested chemicals (dipping + spraying) caused the highest increase in levels of total phenols, free SA and the tested oxidative enzymes followed by spraying treatments and finally dipping treatments. Results indicated also that untreated diseased onion plants had higher levels of phenolic compounds and PO and PPO enzymes than that of untreated healthy ones.

Key wards: Induced resistance, onion white rot, *Sclerotium cepivorum*, salicylic acid, acetic acid, bion.

Introduction

White rot of onion incited by the soil inhabiting fungus *Sclerotium cepivorum* Berk. causes a greet loss in the main production areas of winter onion (*Allium cepa* L.) in Upper Egypt (Abd El-Razik *et al.*, 1973) and around the world (Littley and Rahe, 1987; Peŕez *et al.* 1994 and Tyson *et al.*, 2000).

One of the potential methods of reducing the severity and incidence of plant diseases is by induction of resistance against the pathogens. Certain chemicals, such as salicylic acid and 2.6-dichloroisonictinc acid, potassium salts, Bion and amino butyric acid were reported to induce systemic acquired resistance (SAR) in plants (Oostendorp *et al.*, 2001).

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Bion has been developed as a potent of SAR which does not have antimicrobial properties, but instead it increases crop resistance to diseases by activating the SAR signal transduction pathway in several plant species. Induction of SAR by Bion was reported in several host plants against a broad spectrum of fungal, viral and bacterial pathogens (Ishii *et al.*, 1999; Anfoka, 2000, Brisset *et al.*, 2000, Abo-Elyousr *et al.*, 2005 and Hukkanen *et al.* 2007). The development of SAR is associated with various cellular defense responses. These include synthesis of pathogensis-related (PR) proteins and phytoalexins, accumulation of reactive oxygen species, rapid alterations in cell walls and enhanced activity of various defense-related enzymes (Conrath *et al.*, 2001). Peroxidases have been implicated in a variety of defense-related processes, including the hypersensitive response, lignification, cross-linking of phenolics and glycoproteins, suberization and phytoalexin production (Wojtaszek, 1997).

In the present study, the induction of resistance in Giza 6 onion to white rot by Salicylic acid, Acetic acid and Bion was investigated as well as its relations to potential changes in peroxidase, polyphenoloxidase and phenol contents as biochemical markers for SAR against infection by *Sclerotium cepivorum* Berk.

Materials and Methods

Source of *Sclerotium cepivorum* isolates:

Two isolates of *S. cepivorum* Berk., the incitant of white rot disease of onion, used in the current study were isolated from naturally infected onion plants (cultivar, Giza 6) showing typical symptoms of the disease and collected from winter onion fields located in Assiut Governorate. Fungal isolates were identified by using the morphological features of mycelia and sclerotia as described by Clements and Shear (1957) and Domsch *et al.* (1980). Pathogenicity of the tested isolates was previousely proved by the authors (Sahar, Abd El-Razik *et al.*, 2007)

Toxicity of certain chemical inducers on growth of *S. cepivorum* in *vitro*:

The toxicity of the chemical inducers namely Salicylic acid (SA), Acetic acid (AA) and Acibenzolar-S-Methyl (ASM), (known under the commercial name Bion) on growth of *S. cepivorum* isolates No. I and II in liquid medium was studied. Equal disks (5 mm in diameter) were taken from 6-day old cultures of the tested isolates grown on PDA medium at 20°C and used for inoculation of 100 ml conical flasks containing

sterilized Potato-Dextrose Broth (PDB) medium amended with tested concentrations of tested chemicals. Solutions of the tested chemicals in sterile distilled water were prepared and aliquots were pipetted to PDB medium to give final concentrations of 250, 500, 750 and 1000 ppm. For control treatment, same amounts of sterile distilled water were added. The final volume of the medium of the different treatments was adjusted to 20 ml/flask. Each treatment consisted of 5 replications. All treatments were incubated at 20°C for 10 days. Mycelial dry weight (MDW) of different treatments was recorded after separation of fungal growth by filtration through Whatman No. 1 filter paper and oven dray at 60°C for 48 hrs. the following equation was used to calculate percentage inhibition of fungal growth:

$$\% \text{ inhibition of fungal growth} = \frac{\text{MDW of the control} - \text{MDW of the treatment}}{\text{MDW of the control}} \times 100$$

Effect of certain chemical inducers on incidence of onion white rot and accumulation of phenolics and certain oxidative enzymes in treated plants:

In the greenhouse, effect of salicylic acid (SA), Acetic acid (AA) and Acibenzalor-S-methyl (ASM) on incidence of white rot on Giza 6 onion cultivar was tested in 2006/2007 growing season.

Autoclaved pots (25 cm in diameter) filled with autoclaved clay soil were infested by the *S. cepivorum* isolates No. I and II (3 sclerotia/g soil) before transplanting with 45-day old seedlings of Giza 6 onion cultivar (6 transplants/pot). Five pots were used as a replicate for each tested treatment.

Application of the tested chemical inducers was carried out by dipping onion transplants in the tested concentration of AA, SA and ASM at 50, 100 and 50 ppm respectively before transplanting, or by spray the tested chemicals on the growing plants, twice (after 15 and 30 days from transplanting date), or by dipping onion transplants in the tested chemical followed by twice spraying with the same chemicals (after 15 and 30 days from transplanting date). Untreated pots with the tested chemicals and none infested pots with the pathogen were used as control.

Disease incidence:

Percentage of infection was recorded after 4 months from transplanting date. The following equation was used to calculate

percentage of infection for each tested isolate.

$$\text{Infection}\% = \frac{\text{No. of infected plants}}{\text{Total No. of plants}} \times 100$$

Chemical analysis:

For determination of phenolics and salicylic acid and activity of peroxidase and polyphenoloxidase enzymes in treated and untreated plants, three plant samples were randomly selected from different pots of each treatment 20 days after last chemical treatments. The hole plants of each sample were pooled to make one sample, cut into small pieces, mixed together and 3 subsamples (one gram each) were taken from each sample and used for each assay.

Extraction for phenols and SA determinations:

One gram of freshly harvested plant sub-samples was immersed in liquid N_2, macerated in a clean pastel-mortar, and finally, crushed samples were suspended in 5 ml of ethanol-water (80-20 v/v). Samples were collected in screw-capped tubes, and the suspension was subjected to ultra sonication (Sonicator Model, Julabo, USR3, USA) for 15 minutes at 4°C followed by centrifugation at 7500 g for 15 min. The clear supernatant was subjected to charcoal treatment to remove pigments from each sample and was then transferred to glass tubes after filtering through Whatman filter paper (No. 1). The residue was reextracted twice and the supernatant was pooled before evaporation under vacuum. (Julkunen-Titto, 1985)

Dried samples were resuspended in 1 ml high performance liquid chromatography (HPLC) grade methanol by vortexing and were filtered through Miliporne filter membrane (pore size 0.45 µm) and stored at 4°C until analysed by HPLC for SA content or by Spectrophotometer for total phenolics. (Singh *et al.*, 2002)

Quantification of free SA:

Onion plant samples prepared for SA determination were analyzed through HPLC according to Singh *et al.* (2002) with an High performance liquid chromatography (HPLC) system (Hitachi, Ltd. Tokyo, Japan; Model 655A-11) equipped with two reciprocating pumps, a variable wavelength UV monitor (Model 655A-22) and Chromato-Integrator (Model, D2000). Running conditions included mobile phase methanol: 0.4% acetic acid (80:20 vol/vol), flow rate 1.0 ml/min, injection volume 10 µL, and detection at 254 nm. Samples (10 µL each) were injected by sample injector (Model 655A) one time in the sample loop, and the peak area of individual compound was taken for quantification. Salicylic acid was used as internal and external standards. SA present in the samples was identified by comparing reaction time (Rt) of standard and well as by conjunction. Concentrations were

calculated by comparing peak area of reference compound with those in the samples run under the same elution conditions.

Quantification of total phenols:

A total phenol of the extracts was determinate according to the Folin-Ciocalteu colorimetric method as described by Julkunen-Titto (1985). The reaction mixture consisted of 100 µL of test sample, 5 ml of distilled water and 500 µL of Folin-Ciocalteu color reagent (Fluka, Buchs, Switzerland) and vigorously shaken. After 3 min, 1 ml of a saturated solution of sodium carbonate was added and the sample made up with distilled water to a volume of 10 ml. Absorbance was measured after 60 min at 767 nm using Jenway 6305 Spectrophotometer. Plant extract was replaced with ethanol in blank and standard curve was made by gallic acid. Total phenol content was expressed as mg gallic acid per gram plant material.

Determination of enzymes activities:

One gram of freshly harvested plant subsamples was immersed in liquid N_2, homogenized with Na-acetate buffer pH 5.2 (10 ml) and centrifuged at 10,000 rpm. for 30 min at 4°C. Total protein content and enzymes activity were determined in the supernatants. Three replicates were used for each test. The method described by Bradford (1976) was adopted in this study using Bradford-reagent prepared as follows:

The reaction mixture consisted of 100 µL of sample extract and 1.5 ml Bradford-reagent. They mixed gently and incubated for 15 min at room temperature. Protein content was assayed spectrophotometrically using Jenway 6305 spectrophotometer at 595 nm with bovine serum albumin (0-5.0 g/ml) as standard. Plant extract was replaced with Na-acetate buffer (pH 5.2) in blank.

1-Peroxidase (PO) activity:

Activity of PO was determined spectrophotometerically using guaiacol as a substrate as described by Putter (1974). The reaction mixture consists of 0.2 ml supernatant, 1 ml of 0.1 M Na-acetate buffer (pH 5.2), 0.2 ml guaiacol (1%) and 0.2 ml H_2O_2 (1%). The mixture was incubated at 25°C for 5 min and then measured at 436 nm. Plant extract was replaced with Na-acetate buffer pH 5.2 in blank. Enzyme activity was calculated according to change in absorbance and was expressed as enzyme unit/mg protein as the following equation: PO activity units = OD 436 nm/mg protein

2-Polyphenoloxidase (PPO) activity:

Activity of PPO was determined using the method of Batra and Kuhn (1975). The reaction mixture was as follows: 0.5 ml supernatant, 2 ml phosphate buffer (pH 6.5) containing EDTA (0.372 g) and 0.5

ml Brenzcatechin substrate (10 mM). The mixture was incubated at 37°C for 2 hr. and measured at 410 nm optical density. Plant extract was replaced with Na-acetate buffer pH 5.2 as a blank. PPO activity was determined according to the following equation: PPO units = OD 410 nm/mg protein

Statistical analysis:

Data were subjected to statistical analysis using analysis of variance and means were compared using L.S.D. test as described by Gomez and Gomez (1984).

Results

***In vitro*, toxicity of certain chemical inducers on growth of *S. cepivorum*:**

Data in Figure (1) indicate that the lowest inhibition % of fungal growth was achieved by 250 ppm conc. of SA (5.1 and 28.0%) followed by 500 ppm (35.7 and 52.8%) then 750 ppm (90.6 and 80.0%) and finally 1000 ppm (100 and 91.2%) for isolates No. I and No. II, respectively. The inhibition % of fungal growth of the tested isolates was increased with increasing AA concentration. AA at conc. 250 ppm caused the least inhibition % of fungal growth (38.8 and 23.2%) followed by conc. 500 ppm (39.6 and 34.4%) then 750 ppm conc. (50.6 and 73.6%) and finally 1000 ppm conc. (90.2 and 96.0%) for isolates No. I and No. II, respectively.

Also, data indicate that the tested of ASM at conc. 250 ppm caused the least inhibition % of fungal growth (72.5 and 23.2%) followed by 500 ppm conc. (90.6 and 34.4%) then 750 ppm conc. (97.6 and 73.6) and finally 1000 ppm (100%) for growth of isolates No. I and No. II, respectively.

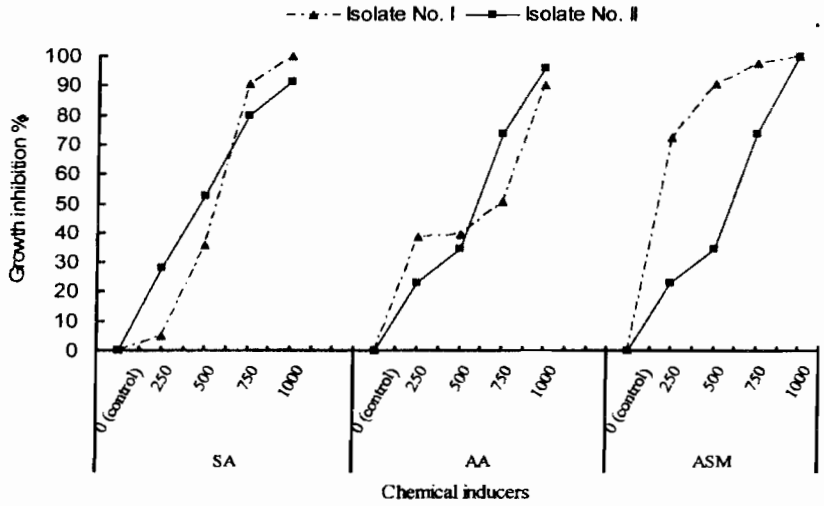

Figure (1): Toxicity of Salicylic acid (SA), acid Acetic Acid (AA) and Bion (ASM) on growth of *S. cepivorum* isolates (No. I and No. II) on PDB medium.

Effect of SA, AA and ASM on incidence of white rot on Giza 6 onion cultivar:

Data in Table (1) indicate that in general, applied SA to onion plants by different methods of application significantly decreased percentage of infection with white rot from 89.2 in untreated plants to 40.6-71.3% in treated ones, respectively. The least percentage of infection with white rot was achieved by combined treatment with SA (dipping treatments + spraying treatment) followed by spraying treatment then dipping treatment. Data also indicate that, the pathogen isolate No. I caused the highest % of infection compared with that caused by isolate No. II. Data also, indicate that, in general, application of AA to onion plants significantly decreased percentage of infection with white rot from 85.8% in untreated plants to 53%in treated ones. Combined dipping with spraying treatments caused the least percentages of infection followed by spraying treatments and finally dipping treatments. In case of infection % no differences were found within spraying twice and dipping treatment followed by spraying. Isolates No. I of the pathogen caused the highest % of infection with the disease compared with isolate No. II.

Data also, indicate that, in general, applied ASM to onion plants by different methods of applications (spraying, dipping and spraying + dipping) significantly decreased percentage of infection with white rot from 89.2% in untreated plants to 15.2 % in treated ones. In almost all tested treatments combined treatments include spraying and dipping in ASM, caused the lowest disease incidence followed by spraying or dipping treatments. Data also indicate that the pathogenic capability of the tested isolates of *S. cepivorum* was similar.

Effect of certain chemical inducers on accumulation of phenols, salicylic acid and activities of certain oxidative enzymes in onion plants:

1-Effect on total phenols and free SA:

Data in Table (2) indicate that all onion plants treated with SA, AA and ASM by dipping or spraying or by combined treatment (dipping + spraying) showed higher contents of total phenols and free SA compared with control plants (healthy and diseased). The highest amounts of total phenols and free-SA were found in plants infected by the pathogen isolates No.I and No. II, respectively and treated with SA by combined treatment (dipping + spraying) followed by spraying treatment then dipping treatment.

Data also indicate that slight differences were found in levels of total phenols and free SA in plants infected by the pathogen isolates No. I and No. II which treated or not treated with SA, AA and ASM.

Table(1): Effect of Salicylic acid (SA), Acetic acid (AA) and Acibenzolar-S-Methyl (ASM) on incidence of onion white rot.

Treatments		Isolates No.		
		No. I	No. II	$\overline{X}$
SA (100 ppm)	Dipping before transplanting	79.1*	63.4	71.3
	Spraying twice (15 and 30 days) after transplanting	52.3	44.6	48.5
	Dipping treatment followed by spraying treatment	48.0	33.3	40.6
	Untreated (control)	86.9	91.4	89.2
$\overline{X}$		66.58	58.18	-
AA (50 ppm)	Dipping before transplanting	88.8	79.8	84.3
	Spraying twice (15 and 30 days) after transplanting	79.7	56.3	68.0
	Dipping treatment followed by spraying treatment	57.1	48.8	53.0
	Untreated (control)	86.7	84.8	85.8
$\overline{X}$		78.08	64.43	-
ASM (50 ppm)	Dipping before transplanting	57.1	46.7	51.9
	Spraying twice (15 and 30 days) after transplanting	25.0	34.2	11.8
	Dipping treatment followed by spraying treatment	9.1	21.2	15.2
	Untreated (control)	86.9	91.4	89.2
$\overline{X}$		44.53	48.38	-

* percentage of infection

	SA		AA		ASM	
L.S.D. at	**5%**	**1%**	**5%**	**1%**	**5%**	**1%**
Pathogen isolates (A)	8.4	11.1	8.1	10.9	6.2	8.1
Treatments (B)	15.6	20.8	15.2	20.3	11.2	15.0
A x B	22.1	29.5	21.6	28.7	16.3	22.2

Table(2): Accumulation of total phenols and free salicylic acid in Giza 6 onion plants grown in soil infested and non-infested with *S. cepivorum* isolates and treated with SA, AA and ASM.

Treatments		Isolates No.			
		No. I		No. II	
		Total phenols mg/g DW	Free SA µg/g DW	Total phenols mg/g DW	Free SA µg/g DW
SA (100 ppm)	Dipping before transplanting	8.24	0.94	8.39	1.01
	Spraying twice (15 and 30 days) after transplanting	8.39	0.95	8.80	1.04
	Dipping treatment followed by spraying treatment	9.49	1.02	9.06	1.09
AA (50 ppm)	Dipping before transplanting	7.09	0.99	7.84	0.64
	Spraying twice (15 and 30 days) after transplanting	8.57	0.91	7.57	0.93
	Dipping treatment followed by spraying treatment	9.08	1.03	9.54	1.01
ASM (50 ppm)	Dipping before transplanting	9.21	1.03	9.13	1.19
	Spraying twice (15 and 30 days) after transplanting	9.03	1.11	9.16	1.6
	Dipping treatment followed by spraying treatment	10.57	2.1	10.40	1.49
Untreated (control)	Healthy	1.02	0.86	1.02	0.86
	Diseased	6.55	0.93	6.60	0.98

DW = Dry weight.

2-Effect on activity of peroxidase and polyphenoloxidase:

Activity of peroxidase (PO) and polyphenoloxidase (PPO) enzymes was determined in tissues of onion plants 20 days after last application of SA, AA and ASM. Determination of enzymes activity was also done in diseased and healthy untreated onion plants for comparison. The levels of activities of PO and PPO were expressed as enzyme unites per mg protein. Data in Table (3) indicate that the PO and PPO levels in untreated healthy and diseased onion plants varied between 3.24-3.41 PO units/mg protein and 0.89-1.09 PPO units/mg protein, respectively. Levels of both enzymes in untreated diseased

plants were higher than that in untreated healthy ones. Application of SA, AA and ASM to onion plants by different treatments (dipping or spraying or dipping + spraying) highly increased PO and PPO activities in infected plants by the tested isolates of *S. cepivorum* compared with untreated healthy or diseased plants. In general, dipping + spraying method of SA, AA and ASM application caused the highest levels of PO and PPO followed by spraying method then dipping method. Isolate No. I of the pathogen showed higher amount of PPO than isolate No. II in all tested treatments, however, the opposite was almost true in case of PO enzyme.

Table(3): Activity of peroxidase (PO) and polyphenoloxidase (PPO) in Giza 6 plants grown in soil infested and non-infested with *S. cepivorum* isolates and treated with SA, AA and ASM.

Treatments		Isolates No.			
		No. I		No. II	
		PO unites/mg protein	PPO unites/mg protein	PO unites/mg protein	PPO unites/mg protein
SA (100 ppm)	Dipping before transplanting	5.1	2.83	8.75	1.25
	Spraying twice (15 and 30 days) after transplanting	6.24	4.07	8.83	1.50
	Dipping treatment followed by spraying treatment	7.91	6.10	12.89	2.42
AA (50 ppm)	Dipping before transplanting	7.27	2.59	11.40	1.69
	Spraying twice (15 and 30 days) after transplanting	8.45	1.65	12.5	1.83
	Dipping treatment followed by spraying treatment	9.40	4.12	13.54	2.05
ASM (50 ppm)	Dipping before transplanting	5.85	3.13	7.37	2.00
	Spraying twice (15 and 30 days) after transplanting	6.39	3.74	11.70	3.29
	Dipping treatment followed by spraying treatment	7.05	5.49	16.47	5.42
Untreated (control)	Healthy	3.24	0.89	3.24	0.89
	Diseased	3.41	1.09	3.41	1.09

DISCUSSION

Results obtained in the present study indicated that SA, AA, and ASM proved to be toxic to growth of *S. cepivorium* isolates No. I and No. II on PDB medium at concentrations 250-1000 ppm. In general, the least inhibition % in fungal growth was achieved by 250 ppm con. According to the obtained results, 100 ppm conc of SA and 50 ppm con of AA and ASM were chosen for testing the possibility of induce resistance in onion to white rot disease. Dipping Giza 6 onion transplants in the tested chemicals before transplants, twice sprays on grown onion plants (15 and 30 days after transplanting date) and combined treatment includes dipping + spraying treatments with the tested chemicals induced resistance in onion plants to white rot, since, percentages of infection was significantly decreased in treated plants compared with untreated ones. In general, the lowest disease incidence in different treatments was achieved by the tested combined treatments followed by spraying treatments then dipping treatments. The role of SA, AA and ASM in induction of ISR against certain plant pathogens was widely accepted by Spletzer and Enyedi (1999) against *Alternaria solani* on tomato; Romero and Ritchie (2004) against *Xanthomonas axonopoldes* pv. *vesicatoria*; Carl *et al.* (2005) against CMV on cucumber, peper, squash and tobacco and Coram and Pang (2007) against *Ascochyta rabiei* on chickpea, however, there is no published data cover the role of the tested chemicals in activation of the resistance mechanisms in onion plants to white rot.

In the present work, the tested treatments of SA, AA and ASM resistance inducers increased accumulation of total phenols and free SA and levels of PO and PPO in treated onion plants compared with healthy and diseased untreated ones. In general, the combined treatments included dipping + spraying with the tested resistance inducers caused the highest increase in levels of phenolic compounds and the tested oxidative enzymes followed by spraying treatments and finally dipping treatments.

In all tested treatments, isolate No. I of the pathogen caused the higher levels of PPO and the lower level of PO than isolate No. II. Increase in levels of phenols, SA, PO and PPO following treatments of onion plants with the SA and ASM reported herin was previously suggested to be involved in resistant mechanisms of plants to other plants pathogens (Spletzer and Enyedi, 1999; Sekine *et al.*, 2004; Abo-Elyosur *et al.*, 2005 and Coram and Pang, 2007). Expression of resistance in onion plants to white rot following

treatments with the tested chemical inducers confirms that suggestion.

Testing effectiveness of the tested chemical resistance inducers against onion white rot under field conditions are needed before reach a final recommendation for commercial use of the tested chemical inducers.

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أستحثاث المقاومة فى البصل لمرض العفن الأبيض بواسطة بعض المواد الكيماوية.*

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وجد ان معاملة شتلات البصل (جيزة 6) قبل الزراعة وذلك عن طريق غمر الشتلات فى محلول حمض السلسليك وحمض الخليك وكذلك البيون أو بالرش على النباتات 15 و 30 يوم من الزراعة وايضا بالغمر والرش معا يؤدى الى حث النباتات ضد مرض العفن الابيض فى البصل حيث وجد ان نسبة المرض تقل وذلك بالمقارنة بالنباتات الغير معاملة. وعموماً وجد ان أقل نسبة اصابة تكون عند معاملة النباتات بواسطة الرش وغمر النباتات معا فى المواد المستخدمة السابقة.

وقد وجد ان معاملة النباتات بواسطة المواد الكيماوية السابقة تؤدى الى زيادة تراكم المواد الفينولية وكذلك المحتوى الكلى من حمض السلسليك وايضاً انزيم البيروكسيديز والبولفينول اكسيديز وذلك فى النباتات المعاملة بالمقارنة بالنباتات السليمة والمريضة ايضا. وعموماً وجد أن المعاملة بالرش والغمر معا تؤدى الى اكبر زيادة فى المكونات السابقة يليها رش النباتات واخيراً المعاملة بالغمر.

* بحث مقدم إلى المؤتمر العلمي الثاني لشباب الباحثين بكلية الزراعة جامعة أسيوط ، 6 مايو 2008.