131
Arab Univ.
J. Agric. Sci.,
Ain Shams Univ.,
Cairo, 19(1), 131-140, 2011

EFFECT OF NEEM AND WILLOW AQUEOUS EXTRACTS ON FUSARIUM WILT DISEASE IN TOMATO SEEDLINGS: 1-INDUCTION OF ANTIOXIDANT DEFENSIVE ENZYMES

[8]

Farag Hanaa[1], R.M.; Zeinab A. Abdou[1]; Dawlat A. Salama[1]; Mervat A.R. Ibrahim[1] and H.A.M. Sror[1]

1- Agricultural Biochemistry Dept., Fac. Agric. Ain Shams Univ., Shoubra Elkhema, Cairo, Egypt

Keywords: Willow, Neem, Tomato seedlings, *Fusarium oxysporum*, Antioxidant enzymes

Abbreviations: POX, Peroxidase; CAT, Catalase; SOD, Superoxide dismutase; ROS, Reactive oxygen species; MDA, Malondialdhyde; EDTA, Ethylenediaminetetraacetic acid; PVP, Polyvinylpyrrolidone; TBA, Thiobarbituric; TCA, Trichloroacetic acid; NBT, Nitroblue tetrazolium.

ABSTRACT

Fusarium wilt disease is one of the major plant diseases that affect tomato production. The effects of neem (*Azadirachta indica*) and willow (*Salix babylonica*) aqueous extracts on fusarium wilt disease in tomato seedlings were investigated. Four weeks old tomato seedlings were treated with 10% of either neem and willow aqueous extracts and then infected with *Fusarium oxysporum* after 4 days of treatment. The results showed that the percentage of disease incidence was increased in non treated tomato seedlings in time dependent manner and reached the maximum level (65%) after 6 weeks of infection. Treatments of tomato plants with neem and willow aqueous extracts reduced the percentage of disease incidence to the level of 25.5% and 27.8% after 6 weeks of infection respectively. The results show that infection of tomato seedling with *Fusarium oxysporum* led to many morphological and biochemical changes including, reducing the growth of tomato shoot and root, increasing the level of lipid peroxidation and marked increase in the activities of antioxidant defensive enzyme i.e. POX, CAT, and SOD. Treatment with neem and willow aqueous extracts significantly exhibited a growth promotion of tomato shoot and root in infected or non infected seedling. Moreover, application of neem and willow aqueous extracts with fusarium, significantly reduce the level of lipid peroxidation and induce high activities of antioxidant defensive enzymes after 3 and 7 days of infection. Electrophoretic pattern of POX demonstrated that *Fusarium oxysporum* caused up regulation of several POX isoenzymes. It could be concluded that neem and willow aqueous extracts reduced the disease incidence of fusarium wilt in tomato seedlings by increasing the activities of antioxidant defensive enzymes and decreasing the level of lipid peroxidation.

INTRODUCTION

Fusarium wilt disease of tomato caused by (*Fusarium oxysporum* f.sp. *Lycopersici*) and causes several losses. Several effective synthetic fungicides are now available to control this disease. Recently, due to increasing information about the effects of synthetic pesticides on human health and environment, many research efforts have been carried out to find alternatives and environmentally safe methods can be used to control plant diseases (**Singh and Prithiviraj, 1997; Paul and Sharma, 2002; Agbenin *et al* 2004**). Using some plant products in plant disease control seems to be an effective method to control many plant diseases. Aqueous extracts of several parts of the neem plant neem showed inhibitory effects against fungal spore germination (**Singh *et al* 1980**). Aqueous leaf extract of neem exhibited considerable control of *Fusarium oxysporum* disease development in banana (**Singh *et al* 1993**). Also, many researches showed that the application of some plant extracts can induce systemic resistance in many plants

(Received December 26, 2010)
(Accepted January 3, 2011)

through accumulations of pathogenesis related proteins (PR- proteins) (**Sateesh *et al* 2004**). Treatment of some plants with neem aqueous extract provided a control of many fungal diseases through a metabolic changes in plants including induction of phenol biosynthesis enzymes, antioxidant defensive enzyme and phenol accumulation (**Paul and Sharma, 2002; Guleria and Kumar, 2006; Aboellil, 2007**).

Willow is fast growing woody plant widely distributed in Egypt and has a high potential for inducing resistance (**Lambers *et al* 1998**). Aqueous and ethanolic extracts of willow leaves contain a high portion of phenolic compounds, glycosides, tannins and salicin (**Bravo, 1998**). Salicin the major compound in willow leaves aqueous extracts is a salicylic acid related compound. Willow is famous as a source of salicylic acid which is well known as systemic resistance inducer in many plants against several plant diseases (**Claudia, 2003**).

The objective of the present investigation is to evaluate the protective effects of neem and willow leaves aqueous extracts against *Fusarium oxysporum* in tomato seedlings. In addition, to study the effect of these natural extracts on the antioxidant defensive enzymes in tomato seedlings subjected to fusarium wilt.

MATERIALS AND METHODS

Plant material

Tomato seeds (*Lycopersicon esculantum Mill*, Cactlrook cultivar) were sterilized in hydrochloric acid 0.1 N for 5 min, washed 3 times with sterile distilled water then planted in trays with 50 individual cells (4×4×6 cm^3 per cell) containing sterilized sandy loamy soil (2:1 w/w) and then placed on benches in a glasshouse until transplanting. Four weeks old healthy tomato seedlings were selected and transplanted into pots containing sterilized sandy loamy soil (2:1 w/w 3 seedlings for each pot). Tomato seedlings were divided into 3 groups (30 pots /group):

1st group was not treated and serve as negative control.
2nd group was treated with 10% neem aqueous extracts.
3rd group was treated with 10% willow aqueous extracts.

After 4 days of different treatments, plant samples were collected and then each group was divided into two subgroups. One of them was inoculated with *Fusarium oxysorum* f.sp. *lycopersica* and the other as non infected. Plant samples were collected after 3 and 7 days of infection. Also, the percentage disease incidence was determined after 2, 4 and 6 weeks of infection.

Fusarium inoculation

Fusarium oxysporum f.sp. *lycopersica* strain was obtained from plant pathology department faculty of agriculture ain Shams University. Fungal suspension was prepared according to **Leslie and Summerell (2006)**. Corn maize grains (300 g) were inoculated with *Fusarium oxysporum* f.sp. *lycopersica* and incubated at 25 °C for 14 days. The inoculated corn maize medium was crushed and suspended in a volume of 1 liter of distilled water. Spores were obtained and used for infection of tomato seedlings by adding 30 ml of fungal spores suspension in the soil close to tomato seedlings roots in pots.

Determination of disease incidence

The disease incidence was recorded on a scale of 0 to 4 in accordance to the degree of wilt as reported by (**Song *et al* 2004**) scale zero refers to not suffer any wilt symptoms. On the other hand scale four refers to complete wilted plants. The scale 1, 2 and 3 refers to different degrees of wilt which indicates the scale of disease severity, number of 4 is the whole plant leaves become yellow as an indication of a complete infection.

Disease incidence is a parameter that includes both disease percentage and disease severity according to (**Song *et al* 2004**).

$$\text{Disease incidence}(\%) = \left[\frac{\sum \text{scale} \times \text{number of plants infected}}{\text{highest scale} \times \text{total number of plants}}\right] \times 100$$

Preparation of neem leaves aqueous extract

Neem leaves extract was prepared according to **Paul and Sharma (2002).** Neem plants were obtained from the Desert Research Center, Al-Mataria, Cairo, Egypt 100 g (fresh weight) of mature leaves were homogenized in a pre-chilled pestle and mortar using chilled, sterilized distilled water. The extract was filtered through four layers of a cheese cloth. The final volume was adjusted to 1000 ml with distilled water. The filtrate was centrifuged at 8000 rpm, 4°C for 15 min. The supernatant thus obtained was designated as concentrated leaf extract.

Preparation of willow leaves aqueous extract

The willow (*Salix babylonica)* leaves extract was prepared according to **El-Shemy *et al* (2007).** Willow leaves were collected from the willow farm of the Faculty of Agriculture, Cairo University, Giza, Egypt. On the day of harvest the young leaves (newly emerged) were immersed in hot water (at 10% w/v). About 10 g of fresh leaves were boiled (at 100 °C) in 100 ml distilled water for 20 min, then filtered through four layers of a cheese cloth and centrifuged at 15,000 x *g* for 15 min.

Determination of Lipid peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA) reaction as described by (**Heath and Packer, 1968**). One gram of seedling tissue (FW) was homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 x *g* for 5 min and 4 ml of 20% TCA containing 0.5% (w/v) TBA was added to 1 ml of the supernatant, the mixture was heated at 95ºC for 30 min and then quickly cooled on ice. The contents were centrifuged at 10000 x *g* for 15 min and the absorbance of suspension was measured at 532 nm in spectrophotometer (UV-visible-160A, Shimadzu). The concentration of MDA was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$. MDA content expressed as µmol g^{-1} FW (Fresh weight).

Extraction of antioxidant defensive enzymes

Plant seedlings were homogenized in 100 mM chilled sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone (PVP) (w/v) at 4ºC. The extraction ratio was 4 ml buffer for each one gram of plant material. Homogenate was centrifuged at 15.000 x *g* for 15 min at 4ºC. Supernatant was used to measure the activities of enzymes. The soluble protein content in supernatant of enzyme crude extract was measured according to **Lowry *et al* (1951)**.

Assay of peroxidase activity

The activity of POX (EC1.11.1.7) was assayed by the method of **Hammerschmidt *et al* (1982**). The reaction mixture (3 ml) consisted of 0.25 % (v/v) guaiacol in 10 mM sodium phosphate buffer (pH= 6 containing 10 mM hydrogen peroxide H_2O_2). Volume of 100 µl of the crude enzyme extract was added to initiate the reaction which was measured spectrophotometrically (UV-visible-160A, Shimadzu). The activity was calculated by measuring the ratio at 470 nm/min = 0.01, which is defined as 1 unit of activity. The specific activity expressed as (IU. mg^{-1} protein).

Assay of catalase activity

The activity of catalase (EC 1.11.1.6) was determined by **Aebi (1974)**. Enzyme extract (100 µl) was added to 2.9 ml of a reaction mixture containing 20 mM H_2O_2 and 50 mM sodium phosphate buffer (pH 7.0). The activity of CAT was measured by monitoring the reduction in the absorbance at 240 nm as a result of H_2O_2 consumption. The amount of consumed H_2O_2 was calculated by using a molar extinction coefficient of 0.04 cm^2 μmol^{-1}. Catalase activity was expressed as units min^{-1} mg^{-1} protein. One unit of enzyme activity was defined as the decomposition of 1 µmol of H_2O_2 per min.

Assay of Superoxide dismutase

The activity of SOD (EC 1.15.1.1) was assayed by the method of **Beauchamp and Fridovich (1971)** by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT). The reaction mixture (3 ml) contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 100 µl of the crude enzyme extract. The test tubes were shaken and placed 30 cm below light source consisting of 15W fluorescent lamp. The absorbance was taken at 560 nm. The activity of SOD was expressed at unit mg^{-1} protein one unit of SOD activity is the amount of protein required to inhibit 50% initial reduction of NBT under light. Enzyme activity was expressed as (IU. mg^{-1}Protein).

Peroxidase isozyme analysis

Electrophoretic separation of isozymes was determined on discontinuous native polyacrylamide gel electrophoresis (5% and 10% acrylamide stacking and separating gels, respectively) performed according to **Jonathan and Weeden (1990)**. Tomato plant extract containing 10 µg protein was loaded directly on wells in the stacking gel. Mixture of 50 µl enzyme extract with 10 µl bromophenol blue in an eppendorf tube then the 60 µl mixture was loaded into gel wells. The POX activity was revealed by treatment of the gel with a

freshly prepared solution of 0.25 mg Benzidine dihydrochloride moistened with 0.5 ml of glacial acetic acid then up to 100 ml distilled water and added ten drops of 1% fresh H_2O_2 solution on the gel which was shaked for one minute in the dark to visualize bands, at room temperature, until red–brown bands appeared **(Graham *et al* 1965)**.

Statistical Analysis

The results presented are the means ± standard deviation of three replicates. The recorded data were treated statistically using the one way analysis of variance as described by **Snedecor and Cochran (1969).** The means were compared by Least Significant Difference test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Disease incidence

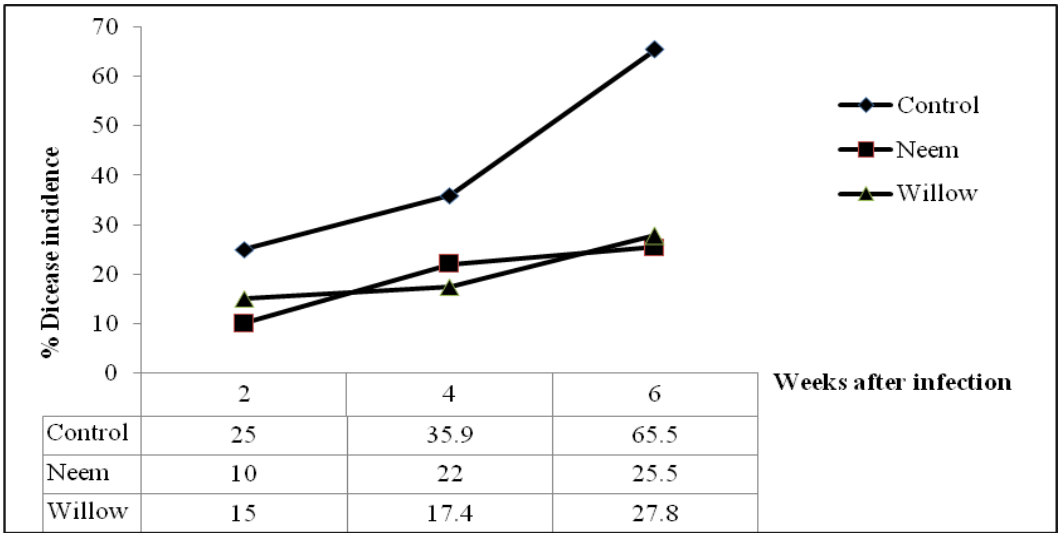
The induced resistance in tomato seedlings by neem and willow aqueous extracts was measured by the reduction of wilt disease severity in tomato seedlings infected with *Fusarium oxysporum*. Data in **Figure (1)** showed that the highest of percentage of disease incidence in tomato seedlings was recorded in the untreated seedlings (control) after 2, 4 and 6 weeks of infection. The obtained results clearly demonstrated that the percentage of disease incidence was gradually increased with increasing time of infection in non infected control as well as in neem or willow treatment. **Figure (1)** indicated that pretreatment with neem and willow aqueous extracts reduced the percentage of disease incidence from 25% in control to 10 and 15% after 2 weeks of infection, from 35.9% to 22 and 17.4% after 4 weeks of infection, from 65.5% to 25.5 and 27.8% after 6 weeks of infection. The aqueous extract of neem seems to be more effective than aqueous extract of willow after 6 weeks of infection. The observed disease control by willow and neem aqueous extracts may be due to the presence of biological active constituents which have either direct antimicrobial activities (**Amadioha, 2000**) or induce host plants defense response resulting in a reduction of fusarium wilt development (**Schneider and Ultrich, 1994**). The significant reduction of fusarium wilt disease in tomato plants treated with neem extract could be due to the presence of gedunin i.e tetranortriterpenoid which posses antifungal properties (**Sadre *et al* 1983**) or due to presence of Azadirachtin (tetranortriterpenoid).

The inhibitory effect of willow aqueous extract against fusarium wilt disease could be explained by the presence of salicin i.e. glucoside of salicyl alchohol. Salicin may bind with receptors on the surface of pathogen cells and penetrates to the fusarium cells which could be killed through denaturation of some enzymes and proteins. Salicin may be the major compound that shows the antifungal effect but other metabolites may increase the potency of the willow extracts compared with pure salicin (**El-Shemy *et al* 2007**).

Growth promotion

Shoot and root lengths of tomato seedlings grown in *Fusarium oxysporum* pathogenized soil are markedly less than that of non infected seedlings. The reduction of tomato shoot and root growth increased significantly with increasing the time of infection compared to healthy untreated seedlings (**Table 1**). Application of aqueous extract of neem or willow leaves enhanced shoot and root lengths of infected seedlings. The effect of neem and willow aqueous extracts on shoot and root lengths were significantly similar after 3 and 7 days of infection with *Fusarium oxysporum*. Moreover before infection neem and willow extracts exhibited a significant increase in shoot and root lengths before infection. Neem and willow aqueous extracts cause a similar promotive effect on shoot lengths before and after infection. Whereas willow aqueous extract causes a significant higher promotion on the root growth than that of neem extract. So, it can be concluded that neem and willow aqueous extracts pretreatments prevented the growth inhibition caused by *Fusarium oxysporum*.

The results confirm the findings of (**El-Khallal, 2007**) who reported that the growth rate of shoot and root was markedly inhibited in tomato seedlings in response to Fusarium wilt disease. The reduction of shoot and root length in infected seedlings could be a morphological expression of hormonal imbalance. This hormonal imbalance in tomato seedling after Fusarium infection included that accumulation of ABA and reduction of IAA, GA_3 and cytokinines (**El-Khallal, 2007**). In addition, the short root and shoot lengths in tomato seedlings infected with *Fusarium oxysporum* could be due to production of some toxins by fungi and the accumulation of phenolic compounds produced from the cell wall degredation mainly lignin via depolymerization results from fungal elicitors **(Steijl *et al* 1999)**.



	2	4	6
Control	25	35.9	65.5
Neem	10	22	25.5
Willow	15	17.4	27.8

Fig. 1. Effect of aqueous leaves extract from neem and willow on % of disease incidence of fusarium wilt in tomato seedlings

Table 1. Effect of aqueous leaves extract from neem and willow on shoot and root length in infected and non infected tomato seedlings

	Treatments	Before infection	after 3 days of infection		after 7 days of infection	
			Non infected	Infected	Non infected	Infected
Shoot length Cm	Control	$5^{b} \pm 1$	$15.5^{a} \pm 1.3$	$11.38^{b} \pm 1.8$	$16.9^{bc} \pm 1.88$	$14^{c} \pm 1.27$
	Neem	$8.17^{a} \pm 1.53$	$16.2^{a} \pm 2.8$	$16.13^{a} \pm 1.3$	$20^{ab} \pm 3.37$	$17.36^{b} \pm 2.99$
	Willow	$7.83^{a} \pm 1.61$	$16.3^{a} \pm 1.1$	$14.5^{a} \pm 2.5$	$22.5^{a} \pm 3.2$	$19.46^{ab} \pm 1.1$
Root length Cm	Control	$6.03^{c} \pm 0.55$	$10.63^{ab} \pm 0.75$	$7.3^{b} \pm 2.7$	$15.9^{ab} \pm 1.1$	$10.8^{c} \pm 1.5$
	Neem	$7.7^{b} \pm 0.25$	$12.1^{a} \pm 2.78$	$13.25^{a} \pm 2.1$	$15.08^{ab} \pm 2.98$	$14.5^{ab} \pm 2.1$
	Willow	$9^{a} \pm 1$	$11^{a} \pm 2.9$	$10.88^{a} \pm 2.1$	$16.6^{a} \pm 3.29$	$13.1^{bc} \pm 0.96$

Data represent the means ± standard deviation of three replicates. Means with the same letter are not significantly different at ($p \leq 0.05$).

The promotive effect of neem aqueous extract on tomato seedlings growth could be due to triterpene (azadirachtin) which acts by delaying the transformation of ammonium nitrogen into nitrate nitrogen as reported by **(Akhtar, 1999 a & b)**. The slow nitrogen conversion led to continuous availability of nitrogen during plant growth and growth promotion. The high shoot and root lengths in willow treated plants could be explained by the presence of salicylic acid related compounds or plant growth promoter i.g. IAA and cytokinine (**El-Khallal, 2007**).

Lipid peroxidation

Reactive oxygen species (ROS) frequently cause cellular damage through peroxidation of membrane fatty acids as a result of infection with *Fusarium oxysporum* (**El-Khallal, 2007**). The level of MDA is a biochemical marker of lipid peroxidation of plant cellular membrane. MDA is formed by peroxidation reaction between ROS and lipid molecules **(Shimizu *et al* 2006)**. **Table (2)** showed that neem and willow aqueous extract caused significant increases in MDA contents in tomato seedlings after 4 days of treatments (4.02 and 4.23 µmol MDA.g^{-1} FW) compared to 2.85 µmol MDA. g^{-1} FW in control. Significantly increase in MDA content in control group after 3 and 7 days of infection 6.47 and 6.93 µmol MDA.g^{-1} FW compared with the non infected 3.1 and 3.7 µmol MDA.g^{-1} FW respectively. Both neem and willow aqueous extracts significantly reduced the level of MDA in infected tomato seedlings with *Fusarium oxysporum* After 3 and 7 days of infection.

Table 2. Effect of neem or willow aqueous leaves extract on malondialdhyde (MDA) contents in infected and non infected tomato seedlings

Treatments	MDA content μmol.g^{-1}FW				
	Before infection	after 3 days of infection		after 7 days of infection	
		Non infected	Infected	Non infected	Infected
Control	2.8539^{b} ± 0.39	3.1^{e} ± 0.83	6.47^{a} ± 0.42	3.74^{c} ± 0.08	6.93^{a} ± 0.27
Neem	4.0201^{a} ± 0.49	4.12^{cd} ± 0.35	4.48^{bc} ± 0.11	4.54^{b} ± 0.21	4.64^{b} ± 0.24
Willow	4.2333^{a} ± 0.39	3.21^{de} ± 0.46	5.33^{b} ± 0.71	3.95^{c} ± 0.19	4.76^{b} ± 0.34

Data represent the means ± standard deviation of three replicates. Means with the same letter are not significantly different at ($p \leq 0.05$).

Antioxidant defensive enzymes

The enzyme activities of peroxidase, catalase and superoxide dismutase were increased after 4 days of treatment with neem and willow aqueous extracts compared to control as shown in **Table (3)**. Willow extract treatment caused a higher POX activity before infection than neem treatment. In contrast CAT activity in willow treated tomato seedlings was lower than in tomato seedlings treated with neem extract before infection. While, neem and willow aqueous extracts treatments caused significantly similar elevation in SOD activity before infection.

The results indicated that infection with *Fusarium oxysporum* caused a significant increase in the activites of POX, SOD and CAT in tomato seedlings after 3 and 7 days of infection compared to non infected seedlings in control group. These observed high POX, CAT and SOD activities in infected tomato seedlings of control group did not seem to be enough to avoid the development of Fusarium disease symptoms or reduction of the level of MDA. The high MDA in infected seedlings reflects the higher production of ROS e.g. H_2O_2 and could be related to increase of the activity of SOD and not coordinates with sufficient increase in H_2O_2 scavenging enzymes CAT and POX. Data in **Table (3)** showed that after 3 and 7 days of infection POX, CAT and SOD activities were higher in infected seedlings treated with neem and willow aqueous extracts than that of control. Neem aqueous extract caused a higher POX, CAT and SOD activities than that of willow extract after 3 days of infection. On the other hand, willow extract caused a higher POX activity than neem extract after 7 days of infection. CAT activity of infected seedlings treated with neem was higher than activity of CAT in infected seedlings treated with willow extract after 7 days of infection. While SOD activities of infected seedlings pretreated with neem and willow aqueous extracts were similar after 7 days of infection. These data clearly suggested that neem and willow aqueous extract prevent *Fusarium oxysporum* disease development and reduced the level of lipid peroxidation through a mechanism involved activation of antioxidant defensive enzymes.

Moreover, it could be concluded that neem and willow aqueous extract caused a coordinated activities of H_2O_2 releasing enzymes i.g. SOD and H_2O_2 scavenging enzymes i.g. POX and CAT.

POX isoenzymes

The electrophoretic isoenzyme pattern of POX indicated that, 10 different detected POX isoforms were found in all tomato seedlings samples before infection (lane 1-3). The detected POX isoenzymes were presented from 1 to 10. **Fig. (2)** indicated that the quanitative increases in POX activity after 4 days of treatment with neem and willow aqueous extracts are corresponding to increasing the expression of three POX isoenzymes (5, 6 and 8).

After 3 days of infection only 9 POX isoenzymes were detected in all treatments where an isoenzymes of POX (POX 7) was disappeared. *Fusarium oxysporum* infection led to over expression in two POX isoenzymes (8 and 9) in neem and willow treatments as well as untreated control. After 7 days of infection only 6 POX isoenzymes were detected. All detected POX isoenzymes after 7 days of infection showed over expression as a result of Fusarium infection as well as willow and neem aqueous extract treatments. The observed high POX activity in infected tomato seedlings

Table 3. Effect of neem and willow aqueous leaves extract on the activities antioxidant defensive enzymes in infected and non infected tomato seedlings

	Treatments	Before infection	after 3 days of infection		after 7 days of infection	
			Non nfected	Infected	Non infected	Infected
POX activity IU. $min^{-1}.mg^{-1}$ protein	Control	317.7^{c}±7.3	379.41^{d} ±42.35	558.7^{c}±29.6	891.5^{d}±76.7	1140.8^{c}±123.5
	Neem	548.7^{b}±93.9	607.9^{c}±61.44	1475.9^{a}±66.7	1211^{c}± 65.3	1453.4^{b}±82.2
	Willow	797.5^{a}±24.1	571.9^{c}±89.6	712.2^{b}±19.8	1150.8^{c}± 94.1	2262^{a} ±93
CAT activity IU. $min^{-1}.mg^{-1}$ protein	Control	5.7^{c} ±0.24	4.97^{d}±1.2	9.5^{c}±0.76	6.1^{d}±0.1	9.5^{c} ±1.4
	Neem	14.01^{a}±0.7	10.4^{c}±1.4	13.97^{b}±0.95	16^{a}±1.3	15.9^{a}±1.6
	Willow	10.8^{b}±1.57	13.9^{b}±0.5	19.2^{a}±1.5	11.9^{b}±0.99	10.2^{bc}±1.03
SOD activity IU. $min^{-1}.mg^{-1}$ protein	Control	34.2^{b}±3.56	30.97^{d}±4.09	46.00^{c}±1.63	35.17^{d}±1.93	43.28^{c}±2.02
	Neem	51.07^{a}±3.91	47.19^{c}±2.75	66.39^{a}±2.94	58.10^{a}±2.07	53.51^{ab}±7.15
	Willow	46.60^{a}±0.67	53.81^{b}±3.28	55.39^{b}±1.49	43.25^{c}±3.75	49.38^{bc}±0.57

Data represent the means ± standard deviation of three replicates. Means with the same letter are not significantly different at ($p \leq 0.05$)

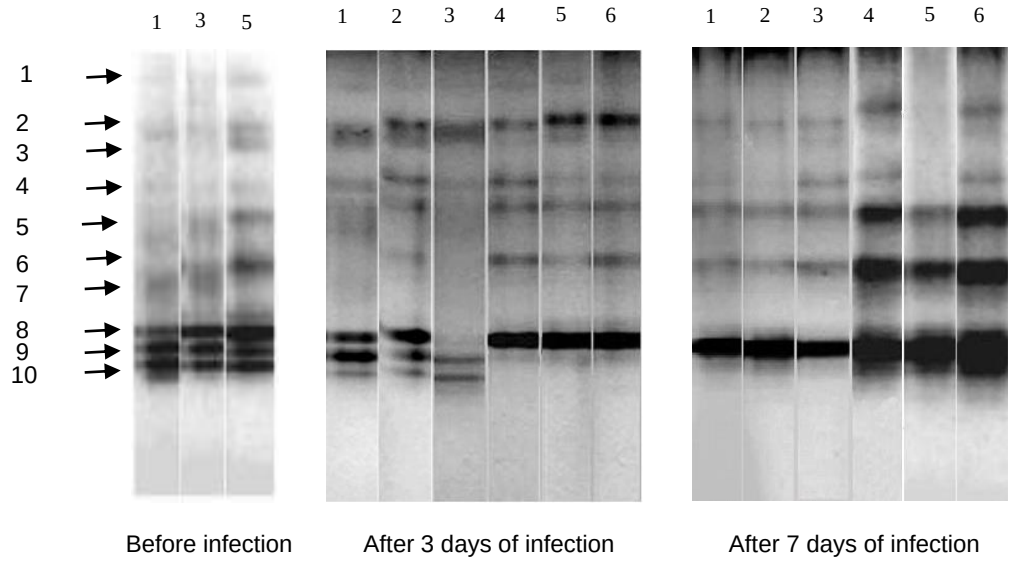


Fig. 2. Native – PAGE for POX isoenzymes in tomato seedlings treated with neem and willow before or after infection with *Fusarium oxysporum*

1; refer to non infected control
2; refer to infected control
3; refer to non infected neem treated seedling
4; refer to infected seedling treated with neem
5; refer to non infected willow treated seedlings
6; refer to infected seedling treated with willow

could be attributed to increasing the gene-expression of some POX isoenzymes especially POX 6 and 7. Also the electrophoretic separation of POX isoenzymes clearly demonstrated that both neem and willow aqueous extract induce a high POX activity through induction the expression of some POX isoenzymes especially POX 6 and POX 7. Many researchers have reported that the increase in the expression of anodic and cathodic POX after infection is involved in the synthesis of lignin like compounds (**Gotthardt and Grambow, 1992; Kerby and Somerville, 1992**).

Finally the present study clearly indicates that neem and willow aqueous extract treatments increase the resistant level of tomato plants to *Fusarium oxysporum* through induction of some POX isoforms and increase the activity of POX to a level coordinated with the activity of H_2O_2 releasing enzyme i.g. SOD. Our results are in agreement with (**Luhova *et al* 2006)** who reported that the high POX activity after infection of tomato seedlings by *Fusarium oxysporum* may participate in ROS removal and inhibition of their accumulation in plant tissues. Also, POX contributes in to the induced resistance through catalyzing oxidation and polymerization of some phenols to form lignin and antifungal oxidizes phenols **(Misaghi, 1982).**

REFERENCES

Aboellil, A.H. (2007). Trilogy, a product of neem (*Azadirachta indica*) induces resistance in cucumber against *Podosphaera xanthi*. **Research J. Microbiology 2(5): 402-414.**

Aebi, H. (1974). Catalase. In: **Methods of Enzymatic Analysis**. **pp. 673-677.** Bergmeyer, H.U. (Ed.). Verlag Chemie/Academic Press Inc., Weinheim/NewYork, USA.

Agbenin, N.O.; A.M. Emeehebe and P.S. Marley (2004). Evaluation of neem seed powder for *Fusarium* wilt and *Meloidogne* control on tomato. **Archives of Phytopthology and Plant Protection 37(4): 319-326.**

Akhtar, M. (1999a). Biological control of plant-parasitic nematodes in pigeonpea filed crops using neem-based products and manurial treatments. **Applied Soil Ecology 12: 191-195.**

Akhtar, M. (1999b). Plant growth and nematode dynamics in response to soil amendments with neem products, urea and compost. **Bioresource Technology 69: 181-183.**

Amadioha, A.C. (2000). Controlling rice blast in vitro and in vivo with extracts of *Azadirachta indica*. **Crop Protection 19: 287-290.**

Beauchamp, C. and I. Fridovich (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. **Anal. Biochem. 44: 276–287.**

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. **Nutr. Rev. 56: 317-333.**

Claudia, O. (2003). Aspirin protects against cancer of the upper aerodigestive tract. The Lancet Oncology. **Newsdesk. Volume 4, Issue 4, p. 200.**

El-Khallal, S.M. (2007). Induction and Modulation of Resistance in Tomato Plants Against *Fusarium* Wilt Disease by Bioagent Fungi (*Arbuscular Mycorrhiza*) And/or Hormonal Elicitors (Jasmonic Acid & Salicylic Acid): 2-Changes in the Antioxidant Enzymes, Phenolic Compounds and Pathogen Related-Proteins. **Australian J. Basic and Applied Sci. 1(4): 717-732.**

El-Shemy, H.A.; A.M. Aboul-Enein; K.M. Aboul-Enein and K. Fujita (2007). Willow Leaves' Extracts Contain Anti-Tumor Agents Effective against Three Cell Types. **PLOS ONE 2(1): e178.**

Gotthardt, U. and H.J. Grambow (1992). Near-isogenic wheat suspension culture established, elicitor induced peroxidase activity and potential use in the study of host / pathogen-interaction. **J. Plant Physiol. 139: 659–665.**

Graham, R.C.; U. Lundholm and M.J. Karnovsky (1965). Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole**. J. Histochem. Cytochem 13: 150–152.**

Guleria, S. and A. Kumar (2006). *Azadirachta indica* leaf extract induces resistance in sesame against *Alternaria* leaf spot disease. **J. Cell and Molecular Biology 5: 81-86.**

Hammerschmidt, R.; E. Nuckles and J. Kuc (1982). Association of enhanced peroxidase activity with induced systemic resistance of cucumber to Colletotrichum lagenarium. Physiol. **Plant Pathol. 20: 73-82.**

Heath, R.L. and L. Packer (1968). Photoperoxidation in isolated chloroplasts 1. Kinetics and stoichiometry of fatty acid peroxidatoin. **Arch. Biochem. Biophys 125: 189–198.**

Jonathan, F.W. and N.F. Weeden (1990). Visualization and interpretation of plant isozymes. In: Soltis D.E. and P.S. Soltis (eds.), **Isozymes in Plant Biology. pp. 5- 45.** Chapman and Hall, London.

Kerby, K. and S.C. Somerville (1992). Purification of an infection-related extra-cellular peroxidase from barley. **Plant Physiol. 100: 397–402.**

Leslie, J.F. and B.A. Summerell (2006). The Fusarium Laboratory Manual. **1st Ed.; pp. 2-10.** Blackwell Publishing Co., Ames, Iowa, USA.

Lambers, H.; F.S. Chapin and T.L. Pons (1998). **Plant Physiological Ecology. p. 429.** Springer-Verlag, New York.

Luhova, L.; A. Lebeda; E. Kuteova; D. Hedererova and P. Pec (2006). Peroxidase, catalase, amine oxidase and acid phosphatase activities in *Pisum sativum* during infection with *Fusarium oxysporum* and *F. solani*. **Biologia Plantarum 4: 675-682.**

Lowry, D.H.; N.J. Rosebrough; A.L. Farr and R.J. Randall (1951). Protein measurement with the folin phenol reagent, **J. Biol. Chem. 193: 265–275.**

Misaghi, I.J. (1982). Physiology and Biochemistry of Plant Pathogen Interaction. p. 163. Plenum Press, New York, USA.

Paul, P.K. and P.D. Sharma (2002). *Azadirachta indica* leaf extract induces resistance in barley against leaf stripe disease. **Physiological and Molecular Plant Pathology 61(1): 3-13.**

Sadre, N.L.; V.Y. Deshpande; K.N. Mendulkar and D.H. Nandal (1983). Male Antifertility Activity of *Azadirachta indica in* Different Species. pp. 473-482. In: Natural Pesticides from Neem tree *(Azadirachta indica A. Juss)* and other tropical plants. Deutsche Gesellschaft for Technische Zusammenarbeit (GTZ), Eschborn, Germany.

Sateesh, K.; T. Marimuthu; B. Thayumana van; R. Nandakumar and R. Samiyappan (2004). Antimicrobial activity and induction of systemic resistance in rice by leaf extract of *Datura metel* against *Rhizoctonia solani* and *Xanthomonas oryzae pv. Oryzae*. **Physiological and Molecular Plant Pathology 65: 91–100.**

Schneider, S. and W.R. Ultrich (1994). Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. **Physiological and Molecular Plant Pathology 45: 29 I-304.**

Shimizu, N.; N. Hosogi; G.S. Hyon; S. Jiang; K. Inoue and P. Park (2006). Reactive oxygen species (ROS) generation and ROS induced lipid peroxidation are associated with plant membrane modifications in host cells in response to AK-toxin from *Alternaria alternate* Japanese pear pathotype. **J. Gen. Plant Pathol. 72: 6-15.**

Singh, U.P. and B. Prithiviraj (1997). Neemazal, a product of neem (*Azadirachta indica*), induces resistance in pea (*Pisum sativum*) against *Erysiphe pisi.* **Physiological and Molecular Plant Pathology 51: 181-194.**

Singh, U.P.; H.B. Singh and R.B. Singh (1980). The fungicidal effect of neem (*A. indica*) extracts on some soil born pathogens of gram (*Cicer arietinum*). **Mycologia 72: 1077-1093.**

Singh, H.N.P.; M.M. Prasad and K.K. Sinha (1993). Efficacy of leaf extracts of some medicinal plants against disease development in banana. **Letters in Applied Microbiology 17: 269-271.**

Snedecor, G.W. and W.G. Cochran. (1969). Statistical Methods. 6 Ed. Th Iowa Univ. Press, Ames, Iowa, USA.

Song, W.; L. Zhou; C. Yang; X. Cao; L. Zhang and X. Liu (**2004).** Tomato Fusarium Wilt its chemical control strategies in a hydroponic system. **Crop Protection 23: 243-247**

Steijl, H.; G.J. Niemann and J.J. Boon (1999). Changes in chemical composition related to fungal infection and induced resistance in carnation and radish investigated by pyrolysis mass spectrometry. **Physiological and Molecular Plant Pathology 55: 297-311.**