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Full Length Research Paper

An overview of the efficiency of some plant extracts on *Fusarium* spp. isolated from carnation roots

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The present study deals comparatively with three control methods of *Fusarium* diseases of carnation, which include watery (cold and hot water extract) and organic extracts of *Eucalyptus*, Leek and Thyme. Cold water plant extract of Thyme and hot water plant extract of *Eucalyptus* proved to be most effective. One, they brought about the lowest percentages of carnation infection. Also, dipping rootless cuttings of carnation in suitable organic solvent extracts before planting in soil infested with the tested pathogens resulted in lowest percentages of infection. The effects of using different methods of plant extracts on some biochemical changes (contents of phenolics, oxidative enzymes, chlorophyll and carotenoides) of carnation cuttings planted in soil infested with pathogenic fungi tested were studied. Electrophoreses study of the tested *Fusarium* spp. showed complete similarity in five protein bands among *F. oxysporum, F. solani* and *F. moniliforme,* which may be a characteristic of the genus *Fusarium*. Carnation cutting treated with Thyme cold water extract resulted in similarity of seven bands separated from both treated cutting and non- treated ones. Differences in protein percentage refer to possible effect of the extract treatment on the plant. Moreover, some types of proteins were detected in infected plants, although they were not found either in healthy ones or in the fungal protein profiles. Such pathogenesis related proteins differed in their molecular weights among the three causal pathogens tested.

Key words: Plant extract, biochemical studies, protein pattern, *Fusarium* spp, carnation.

INTRODUCTION

Carnation plant *(Dianthus caryophllus* L.) is one of the ornamental plants that has great value and plays an important role in encouraging exportation from Egypt and increasing the national income of foreign currency. Plant diseases contribute significantly to the total crop losses both at global and national level. Carnation growers all over the world complain of losses due to certain fungal pathogens which cause wilt and root rot diseases that attack the root system, crown or basal stem parts. Snyder and Hansen (1945), Carver et al. (1996) and Wright et al. (1997) recorded that *Fusarium oxsporium f.* sp. *dianthi* [Schlecht] occupies an important rank among diseases of carnation plants. Under Egyptian environmental climate conditions, soil texture and irrigation system, the diseases of wilt and root rot lead to full collapse or death of plants in case of severe infection or at least lower the flower yield. Development of synthetic chemical fungicides for controlling plant diseases has become difficult due to strict regulations of health and environmental safety without lenient efficacy, selectivity and toxicity requirements as well as their general impact on environment (McLaren, 1986). Consequently, the need to find alternatives to these chemicals has been promoted in the last two decades. For example, certain higher plants are found to be a source of important natural extracts which may probably have alkaloid as a toxin on different plant pathogens (Tripathi et

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al., 1983; AL-Abed et al., 1993; Reddy et al., 2011; Salim, 2011). Such extracts of natural products are considered secondary products of plant metabolism or metabolite refuses. They have important ecological functions for the plants which synthesize them. One of these functions is to protect the plant against plant pathogens (McLaren, 1986; Reddy et al., 2011). The present research work aimed partly to study the effect of some natural extracts in controlling the investigated diseases taken into consideration as well as their possible role in biochemical changes in carnation plants (that is, phenolics compounds, oxidative enzymes, chlorophyll and carotenoides and protein pattern).

MATERIALS AND METHODS

Isolation and identification of *Fusarium* spp. associated with carnation wilt plants

Infected roots and basal steam parts (collected from Egypt) were cut into small fragments, washed thoroughly with tap water, and then sterilized with sodium hypochlorite solution of about 1% chlorine for one minute. They were rinsed several times in sterile distilled water and dried between two sterilized filter papers. Fragments were then placed on potato dextrose agar media in sterilized Petri dishes and incubated at 25°C for 7 days. The developed fungal colonies were recorded as percentage of frequencies for each fungus and purified using either hyphal-tip or single-spore technique .The isolated fungi were picked from the edges of growing colonies or germination spores and transferred onto water agar plates as described by Nelson et al. (1983) and Booth (1971). Then the purified colonies were transferred on PDA slants. All the obtained isolates were microscopically identified according to the morphological features using the description of Waterhouse (1956). Identification of the selected isolates was confirmed by the Fungal Taxonomy Department, Plant Pathology Research Institute, Agricultural Research Centre, Giza, Egypt.

Effect of soaking carnation cuttings in tested plant extracts

The three tested plants (*Eucalyptus*, Leek and Thyme) were tested for controlling the disease in four separated experiments. In the first three experiments, their effects were tested by soaking rooted cuttings of carnation in the obtained extracts for 30 min; and then planted in sterilized pots filled with infested soil containing each of the three pathogenic fungi as previously mentioned. While in the fourth experiment, their dry materials were mixed with soil at the rate of 5 g/kg soil. Ten cuttings of carnation were planted in each pot and four replicates were used for each treatment. Data were recorded 30 days after planting as percentage of infection.

Biochemical studies

The possible biochemical changes associated with each method used for controlling the disease in the present study were investigated. Healthy and infected plants of *F. oxysporum* f. sp. *dianthi*, *F. solani* and *F. moniliforme* treated with plant extracts, fungicide as well as the biocide were always sampled at the age of 30 days at the end of each pot experiment. The investigated parameter included changes in phenolic compounds, oxidative enzymes, chlorophyll and carotenoides contents and the electophoresis analysis of protein.

Phenolic compounds content

The phenolic compounds content was calorimetrically determined using the Follin reagent according to Snell and Snell (1953). About 10 g of dried ground grains or seeds of the tested maize hybrid and cotton cultivar inoculated separately with different isolates of *F. moniliforme* or *F. oxysporum*, respectively and then incubated for 15 days at 25 ± 2°C. Each treatment was plunged immediately into 95 % boiling ethanol for 10 min to kill the living tissues. The samples were then resumed for 10 to 12 h in a Soxhlet unit using 75% ethanol. The obtained ethanol extracts were filtered and evaporated to near dryness on a rotary evaporator at 60°C. The dried residue was re-dissolved in 6 ml of isopropyl alcohol (50%) and used to determine phenolic compounds content.

Estimation of peroxidase activity

Enzyme extraction from the leaves was prepared as recommended by Maxwell and Bateman (1967). The leaf tissues were grounded with 0.1 M sodium phosphate buffer at pH 7.1 (2 ml buffer/g of fresh leaf tissues) in a mortar. These triturated tissues were strained through four layers of cheesecloth and the filtrates were centrifuged at 3000 rpm for 20 min at 6°C. The supernatant fluid was used for enzyme assays. Peroxidase activity was estimated according to the method of Allam and Hollis (1972).

Polyphenoloxidase assay

The activity of phenoloxidase was measured with the colorimetric method of Maxwell and Bateman (1967). The reaction mixture contained 0.2 ml enzyme extract, 0.5 ml sodium phosphate buffer at pH 7 and 0.5 ml of catechol brought to a final volume of 3 ml with distilled water. The activity of phenoloxidase was expressed as the change in absorbance/1 ml of extract per min at 495 nm.

Estimation of chlorophyll and carotene contents

Photosynthetic pigments were extracted from treated and untreated fresh leaves after 10 to 20 days of inoculation. Ten disks (1cm) were taken from each treated leaf and pigments were extracted for 48 h in the dark in a tube containing 10 ml of 85% acetone according to the methods described by Procter (1981). The total chlorophyll pigments were determined by measuring the optical density (O.D) at 663, 452 and 645 nm and calculated using the formula recorded by Arnon (1949).

Total chlorophyll = 8.02 × O.D at 663 + 20.20 × O.D at 645.

Carotene = 4.75 × O.D at 452 – total chlorophyll.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated to detect the variation among the pathogenic fungi (*F. oxysporum* f. sp. dianthi*, F. solani* and *F. moniliforme*) which when isolated from carnation plants were electrophoreticaly detected (Laemmili, 1970). The same technique was used for detecting protein patterns in healthy and infested plants and in treated and untreated with the best tested extracts.

Extraction of proteins from different fungi isolates

Proteins were prepared according to the methods described by Guseva and Gromova (1982). The grown mycelium (*F. oxysporum*

f. sp. dianthi, *F. solani F. moniliforme*) for 8 days at 20 to 30°C on liquid Czapeck's medium was harvested by filtration through cheesecloth, washed with distilled water several times, and freeze-dried. This frozen mycelium was suspended in phosphate buffer pH 8.3 (1 to 3 ml/g mycelium), mixed thoroughly with glass beads and ground in liquid nitrogen to a fine powder. The ground mycelium was centrifuged at 19,000 rpm for 30 min at 0°C. The protein content in supernatant was estimated according to Bradford (1976) by using bovine serum albumin as a standard protein.

Extraction of proteins from carnation plants

Freeze-dried root tissues (1g) of each tested basil sample were grinded using a mortar in liquid nitrogen, until the sample was completely homogenized. These samples were transferred into Eppendorf tubes (1 ml), each containing 200 µl of extraction buffer (50 m M tris-HCl buffer; pH 6.8; glycerol 10% w/v; ascorbic acid 0.1%; cysteine hydrochloride 0.1 w/v); and then centrifuged at 18,000 rpm for 30 min to remove debris. Protein content in supernatant was estimated according to the method of Bradford (1976), using bovine serum albumin as a standard protein.

Electrophoresis of dissociated protein (SDS-PAGE)

Each supernatant was mixed with an equal volume of a solution consisting of (by volume) 64% buffer (0.15 M Tris-HCl, pH 6.8), 20% glycerol; 6% SDS; 10% 2-mercaptoethanol and 0.1% bromophenol blue, before boiling in a water bath for 3 min. Twenty-microlitre samples (40 µg of protein) were subjected to electrophoresis in a 7.5% polyacrylamide gel prepared in 0.1% SDS with a 3.5% stacking gel. Electrophoresis was conducted at 10°C for 4 h at 15 and 30 mA, respectively, until the dye band reached the bottom of the separating gel (Laemmli, 1970). Electrophoresis was performed in a vertical slab mould (16 × 18 × 0.15 cm). Gels were stained with silver nitrate for the detection of protein bands (Sammons et al., 1981).

Gel analysis

Protein patterns obtained by SDS-PAGE were clustered by gel documentation system (Uvitec, Cambridge, UK) by the unweighted pair group method of arithmetic means (UPGMA) according to Sneath and Sokal (1973). Similarity coefficient matrix among protein banding patterns was calculated based on the number of shared bands (Nei and Li, 1979).

Statistical analysis

The obtained data were subjected to analysis of variance following Steel and Torrie (1960), whereas the differences between treatments were tested by calculating Least Significant Differences (L. S. D) at 5% level.

RESULTS

Isolation and identification of *Fusarium* spp. from carnation wilt

Fusarium isolates were identified into three species: *F. oxysporum* (Sehlect. Emend Snyd. and Hans), *F. solani* (Mart.) Apple and Wr. Emend. Snyd. and Hans, and *F. moniliforme* (Sheldon).

Effect of soaking carnation cuttings in cold, hot and solvent extracts, Topsin M-70 and plant guard treatments on percentage of infection

Carnation cuttings were soaked in cold water extracts of the plants *Eucalyptus*, Leek and Thyme as well as Topsin M-70 (fungicide) and Plant Guard (bioproduct) to study their effect on percentage of infection in soil infested with the tested pathogenic fungus, *Fusarium* spp. under greenhouse conditions. Table 1 revealed that all cuttings treated with cold water plant extract, Topsin M-70 or Plant-Guard significantly decreased the infection percentages more than check treatment. Cold water extract of Thyme proved to be the most effective extract in decreasing infection percentages. Moreover, there was no significant difference in infection percentages between the effectiveness of the fungicide Topsin M-70 and the cold extract of Thyme or Plant Guard. *Eucalyptus* gave moderate effectiveness, while Leek cold water extract was the least effective in this respect. Also, all hot water extracts significantly decreased % infection compared with untreated control. Hot water plant extract of Thyme was superior in decreasing % infection (0.0%) in all the pathogenic tested fungi. Moreover, no significant differences were recorded in % infection among hot water plant extract of Thyme, Topsin-M70 and Plant Guard treatments except for Topsin M-70 treatment in soil infested with *F. oxysporum*. On the contrary, hot water plant extract of Leek recorded the least effectiveness. In addition, carnation cuttings were soaked in solvents plant extracts (*Eucalyptus*, Leek and Thyme). The acetone Thyme plant extract was the best in decreasing % infection (5.0, 5.0, 0.0 and 0.0%) with no significant differences from Topsin M-70 or Plant Guard. In this respect, ethanol Thyme plant extract came in the second rank followed by *Eucalyptus* extracted by the same solvent; while Leek extracts gave the least effectiveness.

Biochemical changes

The possible biochemical changes were investigated in leaves samples of carnation plants treated with extracts of Thyme, *Eucalyptus* and Leek. Medicinal plant extracts were tested in three different forms (extracts in cold water, hot water and in the best two organic solvents for each plant based on previous results). Each experiment included 2 other methods of disease control (the fungicide Topsin M-70 and the biocide Plant Guard) as well as check treatments (carnation planted in soil infested with any of the pathogens tested). There was determination of the biochemical changes of two oxidative enzymes and electrophoresis protein patterns. Similar results were obtained in all experiments regarding the check treatments

Table 1. Effect of soaking carnation cuttings in different extracts and Topsin M70 and Plant-Guard on %infection under greenhouse conditions.

Treatment	Solvent	*F. oxysporum* f. sp. *dianthi*	*F. solani*	*F. moniliforme*
		% infection, 30 days after planting (cold extracts)		
Eucalyptus		22.5	15.0	12.5
Leek		47.5	42.2	40.0
Thyme		10.0	5.0	5.0
Topsin M70		7.5	0.0	0.0
Plant-Guard		5.0	2.0	2.5
Check		80.0	50.0	60.0
LSD 0.05		Treatment (T) = 4.94, Fungi (F) = 2.78 and T×F = 6.80		
		% infection, 30 days after planting (hot extracts)		
Eucalyptus		27.5	27.5	22.5
Leek		42.5	37.5	40.0
Thyme		0.0	0.0	0.0
Topsin M70		7.5	0.0	0.0
Plant-Guard		5.0	2.5	2.5
Check		80.0	50.0	60.0
LSD 0.05		Treatment (T) = 6.42, Fungi (F) = 3.22 and T×F = 7.90		
		% infection, 30 days after planting (organic solvents xtracts)		
Eucalyptus	Ethanol	22.5	12.5	15.0
	Chloroform	52.5	35.0	32.5
Leek	Ethanol	50.0	42.5	37.5
	Hexan	62.5	50.0	42.5
Thyme	Ethanol	10.0	10.0	5.0
	Acetone	5.0	0.0	0.0
Topsin M70		7.5	0.0	0.0
Plant-Guard		5.0	2.5	2.5
Check		80.0	50.0	60.0
LSD 0.05		Treatment (T) = 4.94, Fungi (F) = 2.33 and T×F = 7.03		

(Topsin M-70, Plant Guard and the check treatments in infested soil).

Phenolic compounds

Data in Tables 2 clarify that cold and hot water extracts of Thyme proved to be the superior in increasing free, conjugated and total phenolics followed by *Eucalyptus* water extract in most cases. The lowest values of phenolics compounds among extracts were obtained from Leek compared with other treatments in soil infested with all tested pathogenic fungi. Data in the same table indicated that values of phenolics compounds in carnation cuttings soaked in Leek (extracted by hexan or ethanol), *Eucalyptus* (extracted by chloroform or ethanol) and Thyme ((extracted by acetone or ethanol) were higher than in untreated infected ones. However, ethanol Thyme extract was the superior treatment in increasing values of total phenolics compounds in carnation cuttings planted in soil infested with any of the three tested fungi. In contrast, the lowest values of total phenolics compounds were obtained in carnation cuttings soaked in Leek extracted by hexane. Moreover, there was significant difference between all treatments and check treatment, except treatment with cold and hot Leek extracts in case of the three tested pathogens.

Oxidative enzymes

Data in Table 3 show evaluated values of the activity of oxidative enzymes (peroxidase and polyphenoloxidase) in carnation cuttings treated with extracts of Leek, Thyme and

Table 2. Effect of soaking carnation cuttings in different extracts, Topsin M70 and Plant-Guard on phenolic compound (mg/g fresh weight).

Treatment	Solvent	*F. oxysporum* f. sp. *dianthi*			*F. solani*			*F. moniliforme*		
		Free	Conj.	Total	Free	Conj.	Total	Free	Conj.	Total
Content of phenolic compounds after soaking carnation cutting in hot water extracts										
Leek		0.94	0.85	1.79	1.34	0.63	1.97	1.06	0.52	1.58
Thyme		2.46	1.75	4.21	1.91	2.07	3.98	3.07	1.88	4.95
Eucalyptus		1.19	0.97	2.16	2.05	1.14	3.19	2.81	2.01	4.82
Topsin M70		1.48	0.98	2.46	1.32	0.87	2.19	1.61	1.53	3.14
Plant-Guard		1.70	1.64	3.34	1.40	1.12	2.61	1.50	1.43	2.93
Check		0.62	0.12	0.74	0.75	0.14	0.89	0.35	0.11	0.46
LSD 0.05		0.53	0.11	0.93	0.41	0.62	0.67	0.89	0.38	1.11
Content of phenolic compounds after soaking carnation cutting in cold water extracts										
Leek		1.10	0.79	1.89	1.01	0.72	1.73	1.03	0.60	1.63
Thyme		2.91	2.33	5.24	2.28	1.62	3.90	1.69	1.53	3.21
Eucalyptus		2.10	1.42	3.52	1.50	1.62	3.12	1.22	1.31	2.53
Topsin M70		1.48	0.98	2.46	1.32	0.87	2.19	1.61	1.53	3.14
Plant-Guard		1.70	1.64	3.34	1.40	1.12	2.61	1.50	1.43	2.93
Check		0.62	0.12	0.74	0.75	0.14	0.89	0.35	0.11	0.46
LSD 0.05		0.58	0.29	1.65	0.55	0.62	0.45	0.92	0.33	1.20
Content of phenolic compounds after soaking carnation cutting in organic solvents extracts										
Leek	Hexan	0.64	0.70	1.34	1.11	0.62	1.73	1.76	0.50	2.26
	Ethanol	0.85	0.66	1.51	1.57	0.97	2.54	1.89	0.73	2.62
Thyme	Acetone	1.72	1.81	3.53	1.64	1.12	2.76	1.79	0.62	2.41
	Ethanol	1.98	2.01	3.99	1.79	1.21	3.00	1.71	1.77	2.94
Eucalyptus	Chloroform	0.80	0.75	1.55	1.17	0.76	1.93	0.92	1.11	2.03
	Ethanol	2.20	1.72	3.92	1.02	0.98	2.00	1.09	1.22	2.31
Topsin M70		1.48	0.98	2.46	1.23	0.87	2.19	1.61	1.53	3.14
Plant-Guard		1.70	1.64	3.34	1.40	1.12	2.61	1.50	1.43	2.93
Check		0.62	0.12	0.74	0.75	0.14	0.89	0.35	0.11	0.46
LSD 0.05		0.28	0.47	1.05	0.44	0.33	0.93	0.76	0.34	1.14

Free = free phenolic compound, conj.= conjugated phenolic compound, total = total phenolic compound.

Eucalyptus as well as Topsin M-70 or Plant-Guard and planted in soil infested with the pathogenic tested fungi. These oxidative enzymes were also evaluated in non treated cuttings and planted in infested soil (check).

The highest levels of the two oxidative enzymes were recorded in carnation cuttings soaked in water extract of Thyme (cold and hot) in soil infested with any tested fungi while Leek water

Table 3. Effect of soaking carnation cuttings in different extracts, Topsin M70 and Plant-Guard on activity of peroxidase (PO)and polyphenoloxidase enzymes (PPO).

Treatment	Solvent	*F. oxysporum* f. sp. *Dianthi*		*F. solani*		*F. moniliforme*	
		po	ppo	po	ppo	po	ppo
Activity of po and ppo enzymes after soaking carnation cutting in hot water extracts							
Leek		1.59	0.37	1.72	0.46	1.23	0.33
Thyme		2.94	0.62	2.39	0.49	2.64	0.59
Eucalyptus		1.67	0.39	1.98	0.46	2.30	0.56
Topsin M70		1.72	0.42	1.61	0.39	1.98	0.56
Plant-Guard		1.84	0.47	1.77	0.54	1.81	0.41
Check		0.61	0.23	1.22	0.29	0.72	0.28
LSD 0.05		0.56	0.11	0.60	0.28	0.41	0.21
Activity of po and ppo enzymes after soaking carnation cutting in cold water extracts							
Leek		1.69	0.31	1.73	0.73	1.45	0.29
Thyme		3.18	0.63	2.53	0.95	2.16	0.48
Eucalyptus		2.01	0.52	1.95	0.47	1.77	0.43
Topsin M70		1.72	0.42	1.61	0.39	1.98	0.56
Plant-Guard		1.84	0.47	1.77	0.54	1.81	0.41
Check		0.61	0.23	1.22	0.29	0.72	0.28
LSD 0.05		0.46	0.07	0.37	0.31	0.22	0.16
Activity of po and ppo enzymes after soaking carnation cutting in organic solvents extracts							
Leek	Hexan	1.40	0.24	1.59	0.31	1.75	0.35
	Ethanol	1.49	0.25	1.98	0.42	1.85	0.38
Thyme	Acetone	2.32	0.58	1.87	0.41	1.75	0.39
	Ethanol	3.43	0.76	2.65	0.62	2.01	0.47
Eucalyptus	Chloroform	1.53	0.29	1.79	0.59	1.69	0.56
	Ethanol	1.96	0.49	1.83	0.44	2.15	0.79
Topsin M70		1.72	0.42	1.61	0.39	1.98	0.56
Plant-Guard		1.84	0.47	1.77	0.54	1.81	0.41
Check		0.61	0.23	1.22	0.29	0.72	0.28
LSD 0.05		0.56	0.15	0.19	0.11	0.30	0.14

Po = peroxidase enzyme, ppo = polyphenoloxidase enzyme

extract was recorded the least effective in this respect. In addition, treated cuttings with thyme extracted by ethanol gave the best effect on oxidative enzymes. Thyme extracted by acetone occupied the second rank. In contrast, Leek extracted by hexane or ethanol recorded the least levels of peroxidase and polyphenoloxidase activity, even more than the check.

In general, there was significant difference

between all treatments and check treatment, except treatments with cold, hot and hexan leek extracts in plant infected with *F. moniliforme.*

Chlorophyll and carotenoides

Regarding determination of the chlorophyll and carotenoides contents, the data in Table 4 revealed that cuttings soaked in water extracts (cold or hot) of Thyme and *Eucalyptus* increased contents of chlorophyll and carotenoides as compared to untreated cuttings planted in either soil infested with the tested pathogenic fungi. In contrast, the lowest levels of chlorophyll and carotenoides contents were obtained from cuttings soaked in water extracts (cold or hot) of Leek in soil infested with the tested pathogen. On the other side, data in Table 4 clearly indicate that values of chlorophyll and carotenoides contents increased mostly in cuttings treated with solvents than untreated cuttings. However, ethanol extracts of Thyme recorded the highest level of chlorophyll a&b. In contrast, ethanol extracts of Leek gave the lowest values of chlorophyll a and b. regarding carotenoides contents, highest levels were obtained in cuttings soaked in acetone extract of Thyme in soil infested with *F. oxysporum* or *F. moniliforme* followed by chloroform extract of *Eucalyptus*.

Electrophoresis studies

Electrophoresis studies included two runs to identify the protein fractions of the three *Fusarium* species used in the study namely *F. oxysporum, F. solani* and *F. moniliforme.* The photographs of the two runs are shown in Figure 1 for separation of protein of the fungal species and in Figure 2 for separation of protein of healthy carnation plants (either treated or non treated with Thyme extract) and carnation plants infected with the three *Fusarium* spp. The results of the computer analysis of the obtained profiles of protein fractions are shown in Table 5.

Variation among protein patterns of Fusarium spp. Tested

Separated protein bands were 10 for *F. oxysporum,* 7 for *F. solani* and 6 for *F. moniliforme.* The three tested species of the genus *Fusarium* showed almost complete similarity in the 5 bands separated at the molecular weight of 61 to 62 kd, 45 to 48 kd, 35 to 38 kd, 29 to 30 kd and 15 to 16 kd in descending order .These 5 bands resembled 63.87, 88.25 and 88.98% of the total protein separated from *F. oxysporum, F. solani* and *F. moniliforme,* respectively. These specific five bands may be a characteristic of the genius *Fusarium* and require more studies to confirm this finding. The pathogen *F. moniliforme* was characterized by missing two specific bands separated at molecular weight of 74 to 75 kd and 23 to 26 kd, although these particular two bands were found in *F. solani* and *F. oxysporum* where they resembled 11.7 and 11.91% of the total separated protein, respectively. The pathogen *F. solani* was characterized by two specific bands separated at molecular weight of 57 to 58 kd and 41 to 42 kd in descending order where they resembled 7.49 and 10.19% of the total protein *F. oxysporum*. These two particular bands separated were completely missed in both *F. solani* and *F. moniliforme.*

Changes in protein patterns between healthy carnation plants treated and non-treated with* Thyme *extract

The non treated healthy carnation plants were characterized by 11 protein bands separated at molecular weight that ranged from 11 to 18 kd to 77 to 78 kd in ascending order. However, healthy carnation plant treated with extract resulted in separations of 21 protein bands. Complete similarity was observed in 7 bands either separated from non-treated healthy plant without extract treatment and healthy carnation plant treated with the extract. These 7 bands were separated at molecular weight of 77 to 78 kd, 71 to 72 kd, 61 to 62 kd, 39 to 40 kd, 33 to 34 kd, 29 to 30 kd and 17 to 18 kd. Few differences were observed in the protein % amount as related to the total protein in only 4 of these 7 bands. However, the band separated at molecular weight of 39 to 40 kd resembled 5.64% of the total protein in healthy plant without treatment and this protein increased to 17.33% in treated plant. In the same trend, the band separated at molecular weight of 33 to 34 kd increased from 10.06% in non treated healthy plant to 16.88% in treated healthy plant. The protein was also changed in the band separated at 17 to 18 kd where it decreased from 21.37% in healthy plants without treatment to be 15.9% of the total separated protein in healthy plants treated with the extract. It seems that the extract had slight influence on specific protein bands that resulted in some changes in the molecular weight and the protein amount of separated protein as related to total separated bands. The following observation confirms that hypothesis. The non complete similarity was found in 4 bands in healthy plants without treatment and 5 bands in healthy plants treated with the extract. Two bands of them separated at molecular weight of 57 and 55 kd in healthy plants without extract treatment were accumulated in only one band separated at 54 kd in healthy plants treated with the extract, where the protein of this particular band was7.73% and the total protein of the original two bands was 7.75%. Also, the band was separated at molecular weight of 25 to 26 kd with protein

Table 4. Effect of soaking carnation cuttings in different extracts, Topsin M70 and Plant-Guard on chlorophyll (a and b) and carotenoides (mg./ml).

Treatment	Solvent	*F. oxysporum* f. sp. *dianthi*			*F. solani*			*F. moniliforme*		
		a	b	c	a	b	c	a	b	c
Contents of chlorophyll (a and b) and carotenoides (mg./ml) after soaking carnation cutting in hot water extracts										
Leek		1.12	0.64	0.49	0.57	0.27	0.54	1.46	0.85	0.59
Thyme		1.76	0.71	0.96	2.35	0.91	0.89	1.90	0.84	0.74
Eucalyptus		1.41	0.74	0.65	1.51	0.81	0.58	1.76	0.98	0.78
Topsin M70		1.31	0.81	0.86	1.15	0.64	0.75	1.02	0.67	1.67
Plant-Guard		1.47	0.82	1.11	1.16	0.63	1.30	1.24	0.78	2.02
Check		0.24	0.32	0.32	0.30	0.25	0.27	0.35	0.17	0.52
LSD 0.05		0.44	0.24	0.27	0.19	0.28	0.15	0.36	0.12	0.23
Contents of chlorophyll (a and b) and carotenoides (mg/ml) after soaking carnation cutting in cold water extracts										
Leek		0.49	0.17	1.20	0.70	0.39	0.56	0.59	0.27	0.76
Thyme		2.57	1.89	2.63	3.04	1.42	1.85	2.79	1.24	1.92
Eucalyptus		3.91	1.10	1.86	1.96	0.87	1.42	1.72	0.97	1.69
Topsin M70		1.31	0.81	0.86	1.15	0.64	0.75	1.02	0.67	0.57
Plant-Guard		1.47	0.82	1.11	1.16	0.63	1.30	1.24	0.78	1.03
Check		0.24	0.32	0.32	0.30	0.25	0.27	0.35	0.17	0.24
LSD 0.05		0.64	0.61	0.55	0.80	0.28	0.21	0.53	0.47	0.36
Contents of chlorophyll (a and b) and carotenoides (mg/ml) after soaking carnation cutting in organic solvents extracts										
Leek	Hexan	1.59	0.67	0.64	1.77	0.91	1.41	1.95	0.82	1.50
	Ethanol	1.21	0.54	0.73	1.32	0.54	0.94	1.44	0.71	1.21
Thyme	Acetone	1.56	0.78	2.41	1.97	0.83	1.71	1.72	0.62	1.98
	Ethanol	2.71	1.31	1.66	1.89	0.72	1.52	2.43	0.99	1.43
Eucalyptus	Chloroform	1.89	0.83	2.11	1.45	0.74	1.80	2.33	1.61	1.92
	Ethanol	2.11	0.98	1.71	1.73	0.93	1.21	1.57	0.62	1.41
Topsin M70		1.31	0.81	0.86	1.15	0.64	0.75	1.02	0.67	0.57
Plant-Guard		1.47	0.82	1.11	1.16	0.63	1.30	1.24	0.78	1.03
Check		0.24	0.32	0.32	0.30	0.25	0.27	0.35	0.17	0.24
LSD 0.05		0.77	0.32	0.40	0.44	0.63	0.51	0.83	0.33	0.45

A = chlorophyll a, b = chlorophyll b, c = carotenoides.

amount of 21.67 in healthy plants without treatment with extract. This observed band separated to be supported into two bands at 23 to 24 kd and 21 to 22 kd in the healthy plants treated with the extract with total protein of about 21%. The healthy plants treated with the extract were

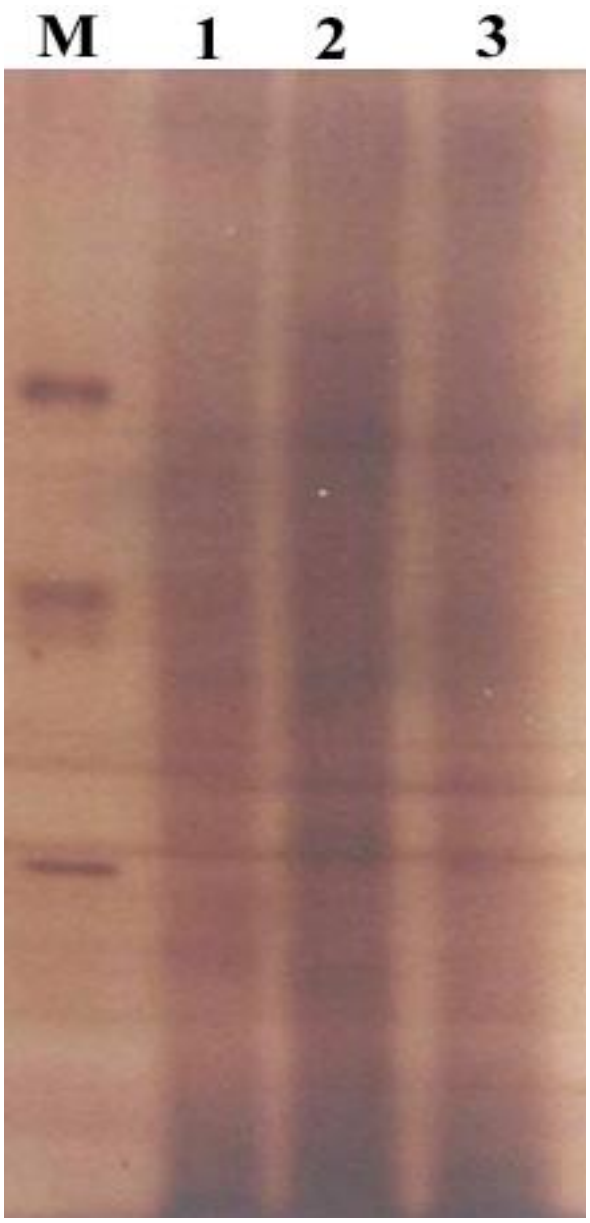

Figure 1. Photography of the protein profiles of the three investigated of *Fusarium oxysporum* (1), *F. solani* (2) and *F. moniliforme* (3) as compared to three molecular markers (M) of molecular weight of 66, 48 and 29 kd in ascending order.

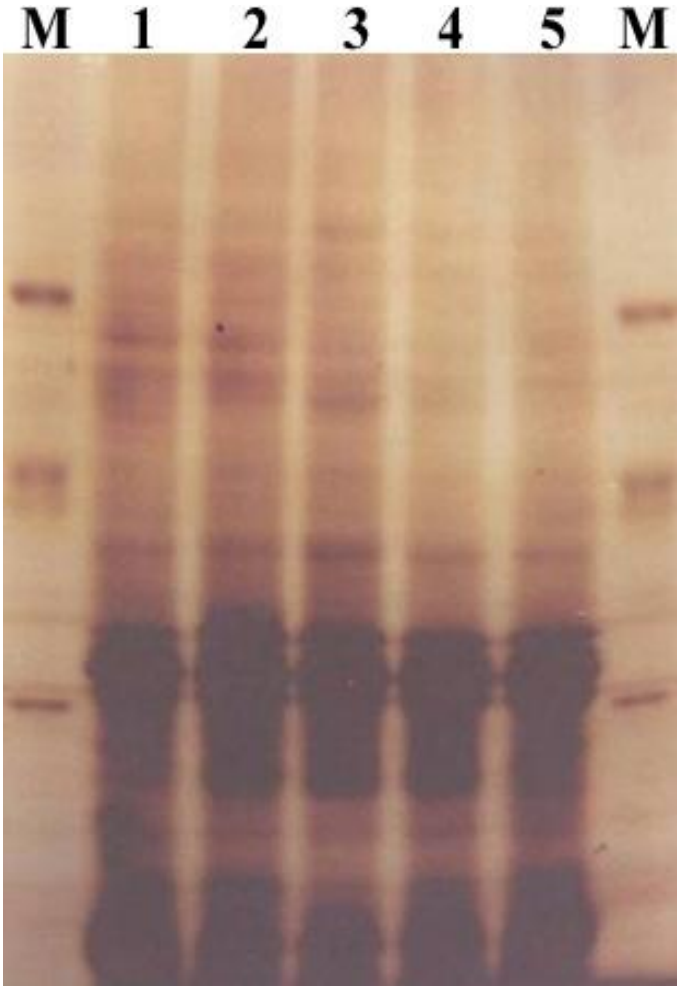

Figure 2. Photography of the protein profiles of the three investigated of healthy carnation plants either treated with thyme extract (1) or not (2) and infected plants with *Fusarium oxysporum* (3), *F. solani* (4) and *F. moniliforme* (5) as compared to three molecular markers (M) of molecular weight of 66kd,48kd and 29kd in ascending order.

characterized with one specific protein band. It seems that this particular band belongs to the extract itself. It was at molecular weight of 65 kd and separated only in the case of healthy plants treated with the extract.

C: Changes in protein patterns of healthy carnation plants and infected plants

Table 5 showed that healthy plants gave 11 bands while plants infected by *F. oxysporum* gave 11 bands, plants infected by. *solani* gave 13 bands and 10 bands for *F. moniliforme.* Regarding comparison between healthy carnation plants and those infected by. *F. oxysporum*, there was similarity of molecular weight in 7 bands separated at 77 to 78 kd, 71 to 72 kd, 61to 62 kd, 30 to 40 kd, 33 to 34 kd, 29 to 30 kd and 17 to 18 kd. Changes were realized at molecular weight of 53 to 58 kd where two separated bands of healthy plants were accumulated in only one band in infected plants. The infected carnation by *F. oxysporum* was therefore characterized with a low molecular weight of 15 to 16 kd which probably belongs to the fungus and a higher molecular weight band separated at 67 to 68 kd. This particular band was not observed in healthy plants or in the fungus alone (Table 5).

Comparing healthy plants and infected with *F. solani* indicated complete similarity of 7 bands separated at 77 to 78 kd, 71 to 72 kd, 61 to 62 kd, 57 to 58 kd, 39 to 40 kd, 29

Table 5. Number of separated bands, related molecular weight and % of amount of area in *F. oxysporum* f. sp. *dianthi*, *F. solani F. moniliforme* and infected ones with three tested pathogens and carnation cuttings treated with Thyme extract and non-treated.

M. W (Kd)	% Amount							
	F	S	M	IF	IS	IM	H	T
79 - 80						0.76		
77 - 78				1.77	0.87		1.24	1.49
75 - 76	1.72							
73 - 74		2.38						
71 - 72				3.02	2.43	1.66	2.43	2.42
69 - 70								
67 - 68				2.91	1.91			
65 - 66								2.46
63 - 64								
61 - 62	10.74	13.25	15.36	4.35	3.86	5.73	5.72	5.81
59 - 60								
57 - 58	7.49				6.57	2.66	3.03	
55 - 56							4.72	
53 - 54				8.21		5.65		7.33
51 - 52	5.22		11.01					
49 - 50								
47 - 48	13.82							
45 - 46		35.51	24.20		5.45			
43 - 44							11.75	
41 - 42	11.50							
39 - 40				17.94	11.88		5.64	17.33
37 - 38	5.60							
35 - 36		5.78	9.78					
33 - 34				19.31			16.06	16.88
31 - 32					19.66	32.86		
29 - 30	14.84	16.41	21.41	5.82	6.92	6.85	6.38	6.12
27 - 28								
25 - 26	10.19					15.17	21.67	
23 - 24		9.32		19.88	17.32			16.34
21 - 22					3.88	8.07		4.03
19 - 20					7.96			
17 - 18				14.03	11.30	20.95	21.37	15.90
15 - 16	19.14	17.35	18.23	2.77				3.89

F = *Fusarium oxysporum,* S= *Fusarium solani,* M= *Fusarium moniliforme,* IF= plant infected with *Fusarium oxysporum,* IS= plant infected with *Fusarium solani,* IM= plant infected with *Fusarium moniliforme,* H= Healthy plant and non treated with plant extract, T= treated with Thyme extract.

to 30 kd and 17 to 18 kd. Similarity was realized somewhat in three other bands separated in healthy plants at molecular weight of 43 to 44 kd, 33 to 34 and 25 to 26 kd while separated in infected plants at 45 to 46 kd, 31 to 32 kd and 23 to 24 kd, respectively. Changes were realized in one band of healthy plants that was separated at 55 to 56 kd and seem to be accumulated with the band separated at 57 to 58 kd (in healthy plant) to form one band in the infected samples separated at the same molecular weight. The carnation plant infected by *F. solani* was therefore characterized with three bands separated at molecular weight of 67 to 68, 21to 22 and 19 to 20 kd. These particular bands were separated either in healthy plants or in the fungus alone.

Regarding healthy plant compared with carnation plant infected with *F. moniliforme,* there was one characteristic band that distinguished infection where the protein band separated at molecular weight of 21 to 22 kd which was never separated from the fungi alone or healthy plant (non infected). Complete similarity was recognized in 6 bands separated at molecular weight of 71 to 72, 61to 62, 57 to 58, 29 to 30, 25 to 26 and 17 to 18 kd, whereas three bands showed close similarity to somewhat become separated at molecular weight of 77 to 78 kd, 53 to 56 and 31 to 34 kd,

respectively. However it is clear that infection with *F. moniliforme* resulted in the disappearance of two bands separated at 43 to 44 kd and 39 to 40 kd and they resembled 17.39% of the total amount of the host protein.

DISCUSSION

Carnation plants *(Dianthus caryophllus* L.) are subject to serious diseases. They are attacked by several fungi but *Fusarium* spp. were the most dominant isolated fungi from carnation wilt. Therefore, *F. moniliforme. F. solani* and *F. oxysporum* were chosen for further studies in this work. Successful control of the disease investigated was reported by El-Habbaa et al. (2002). By using fungicides or biocides, it was proved in the present study that Topsin M-70 and Plant Guard were inclusively used, compared to the extracts of medicinal plants, for the need to find safe alternatives. The development of non-toxic alternative, despite chemical fungicides, would be useful in reducing the undesirable effects on environment and public health. Fortunately, many medicinal plant extracts including certain Egyptian medicinal and aromatic plants were reported to be effective in controlling *Fusarium* diseases (Abdel-Gafor, 2000; Harrison et al., 2001; Selim et al., 2002; Joseph et al., 2008). The water extracts (cold and hot) and organic solvent ones (acetone, ethanol, chloroform, benzen and hexane) of Leek, *Eucalyptus* and Thyme were found to have inhibitory effects on the pathogens tested.

However, they markedly differed in their fungitoxicity according to the kind of extracted plants, type of extraction (cold, hot water or organic solvents) and kind of the fungi tested. Perusal of the earlier literature indicated naturally presence of some substances in certain plant extracts (including the present investigated ones), with antifungal prosperities which were recognized when tested against a wide range of fungi infecting many crops and commercially important plants (Mishra et al., 1990; Hilal et al., 2002; Sharma and Kumar, 2009; Satish et al., 2009).

In general, variable results of these extracts tested in greenhouse might be due to differences in the antifungal toxic substances according to their solubility in water, the quantitative presence of inhibitory, their selective toxicities which differed with fungal species and quantitative differences in the antifungal principles present in each medicinal plant. The present study concentrated on some biochemical changes which occurred as a result of soaking carnation cuttings in the plant tested extracts. Phenolic compounds considerably increased in plants treated with extracts of *Eucalyptus*, Leek and Thyme than those untreated and planted in soil infested with tested pathogenic fungi, as well as more than other treatments including those of Topsin M-70 and Plant Guard. The present results are in harmony with those reported by Daayf et al. (1997) and Nada (2002). They reported that plant extract of *Reynouria sachalinemis* stimulated the production of fungitoxic phenolic compounds. Moreover, the amounts of these compounds in treated plants were nearly five times higher than the level found in the control plants. It seems that the increased Phenolic compounds gave surely an increase in the capability of plants to induce defense against disease infection process and consequently delayed the disease development. Increase in phenolic compounds is involved in the activation of inducible defense (Kalaichelv and Nagarajan, 1992; Dai and Mumper, 2010; Sarwar et al., 2011; Al-Sohaibani et al., 2011). Moreover, the phenolic toxic compounds in plant cells acting through structure of the bond form with cell wall compounds of plant tissues (Mahadevan and Sridhar, 1986; Dai and Mumper, 2010), the enhancement of the host resistant and probably stimulating the host defense mechanisms (Subba Rao et al., 1988; Sarwar et al., 2011). Such activation may also present the extent of fungal growth with plant tissues (Soni et al., 1992) by penetrating the microorganisms and causing considerable damage to the cell metabolisms (Kalaichelvan and Elangovan, 1995; Abyaneh et al., 2005). It is worth noting also that oxidative enzymes play a partial role in activating inducible defense of plant (Vera-Estrella et al., 1994; Sarwar et al., 2011). The results were also somewhat similar to those obtained by Sallam et al. (2001), Selim et al. (2002), Thiribhuvanamala and Indral (2002) and Al-Sohaibani et al. (2011). Concerning chlorophyll a and b, and carotenoides contents, considerable increase occurred in carnations as a result of soaking them in tested plant extracts than untreated control. These results are in agreement with those obtained by Sallam et al. (2001), Selim et al. (2002) and Karthikeyan et al. (2009). However, Faye et al. (1995) reported that the deleterious effect of the infection by *Fusarium* on the pigments of photosynthesis might be due to the secretion of toxic substances by the fungi that might stimulate the activity of chlorophyll and chlorophyll degradation or decrease the carotenoides that prevent chlorophyll photo destruction, inhibit Fe uptake and transport to plant leaves. Bonner and Varner (1965) hypothesized that the stimulated effect of the tested plant extracts on the photosynthetic pigments might be due to substances that act as activators for chlorophyll synthesis and/or inhibit the effect of causal fungi on chlorophyll degradation and Fe uptake as well as carotenoides.

According to the phytoalexin theory, plant disease develops when metabolism of the parasite fits that of the host cell. On the other hand, less existence of coordination between their metabolisms, simultaneously, results in appearance of nonspecific phytoalexins that prevent further development of the parasite. Spraying hosts with plant extracts subsequently might encourage the host to form phytoalexin compounds. The possibility of using protein patterns of the studied pathogenic fungi belonging to *Fusarium* spp. for fast detection of the involved causal of the disease was confirmed. Obvious variations of the separated protein patterns of the three

tested pathogenic fungi could be related to the different features of the three species. However, the three species tested of the genius *Fusarium* showed some complete similarity in the 5 bands separated. These specific bands may be a characteristic of the genius *Fusarium* and require more studies to confirm that.

The possible influence of the treatment with cold water extract of Thyme on the protein patterns of the healthy host plant was also investigated. It seems that the extract had slight influence on specific protein bands that resulted in some changes in the molecular weight and the protein amount of separated bands. There are recognized results in the activation of synthesis of pathogenesis-related (PR) proteins (Selitrennikoff, 2001). These results may be due to gene theory as reported by Hussien (2001) Sang and Joo-Yeon (1992) and Al-Sohaibani et al. (2011) which lead to changes in protein pattern. It was suggested that PR protein has specific function to the host pathogen interaction. The infected carnations by *F. oxysporum, F. solani* and *F. moniliforme* were characterized with a different band. These particular bands were not separated either in healthy plants or in the fungus alone. The present results could be considered as pathogenesis related protein, according to Bowles (1990), Van Loon et al. (1994) and Datta and Mulhukrishnan (1999).

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