

Biocontrol of Wilt-Inducing *Fusarium oxysporum* by Aqueous Leaf Extract from Egyptian *Ammi majus* and *Ammi visnaga*

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FUSARIUM *oxysporum* causes wilt disease of tomato crop. Fungal management using fungicides results in several problems in the environment. Plant metabolites represent an alternative method since they have minute impact on the environment. The present investigation is an attempt to develop eco-friendly management of *F. oxysporum* using aqueous leaf extract of *Ammi majus* and *Ammi visnaga* (Family: *Apiaceae*). Treating the fungus with aqueous leaf extract from the two species of *Ammi* plants resulted in the inhibition of the fungal growth. *Ammi* leaf extracts inhibited the activities of metabolic enzymes including glucose-6-phosphate dehydrogenase (G-6-PD, EC: 1.1.1.49), glutamine synthetase (GS, EC: 6.3.1.2) and nitrate reductase (NR, EC: 1.7.99.4) but increased L-phenylalanine ammonia lyase (PAL, EC: 4.3.1.5) activities of *F.oxysporum*. Both of the total amino acids and the total soluble protein contents of *F. oxysporum* were reduced under the same treatment. The leaf extracts inhibited the activities of the enzymes which are involved in pathogenicity including pectinase (EC 3.2.1.15), protease (EC: 3.4.21.14), cellulase (EC: 3.2.1.4) and xylanase (EC: 3.2.1.8). IC₅₀ values for the four enzymes were 93.32, 69.03, 71.27 and 74.93mg/ml, respectively. However, IC₅₀ values were 134.22, 106.31, 90.65 and 100.26mgml⁻¹ for the four enzymes in the same order under treatment with *A. visnaga* leaf extract. The total phenols and total flavonoids in leaf extracts from the two *Ammi* species were determined and they were higher in *A. majus* than those of *A.visnaga*. This study suggests efficiency of *Ammi* leaf extracts in suppression of *Fusarium oxysporum* pathogenicity.

Keywords: *Ammi majus*, *Ammi visnaga*, *Fusarium oxysporum*, Enzymes activities, Flavonoids, Phenolics.

Introduction

The major challenge in the production of plant crops, vegetables and fruits is the susceptibility of the various plants toward different plant pathogens. Fungi, viruses, bacteria as well as nematodes represent the main causal pathogens of several plant diseases. The parasitic fungi are the most severe plant pathogens and account about 90% of economic loss in agriculture (Gupta & Sharma, 2008; Maninegalai et al., 2011).

F. oxysporum is a soil-borne microflora which

causes a wilt disease in various plant crops and in particular tomato. This soil-borne fungus may be presented as pathogenic and nonpathogenic strains in soil rhizosphere and are competing with each other. The wilt inducing *Fusarium* strains are considered as hindrance in the production of various economically important plants (Srinivas et al., 2019).

The severe disease may be due to a combination of many factors including production of pathogenesis related enzymes, reaching of fungal mycelium in vascular bundle, antibiosis and

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deficiency of nutrients in rhizosphere (Goel & Sharma, 2016; Srinivas et al., 2019).

Plant pathogenic fungi are capable to produce various cell wall degrading enzymes (CWDE) that facilitate their passage through different plant cell barriers. Different CWDE play a vital role in the pathogenesis of different fungi by depolymerizing the major components of the cell wall of plant (Kubicek et al., 2014). In response, production of different protein inhibitors by host plant is a defense way against several phytopathogens (Juge, 2006).

Several biocontrol approaches including medicinal plant extract, fungicides and utilization of resistant breeding are relevant. The performance of synthetic fungicides to soil causes violent contamination to the environment. Therefore, the biocontrol of fungal diseases using various plant extracts is of excellent interest (Kilani-Jaziri et al., 2011; Rongai et al., 2012; Dwivedi & Sangeeta, 2015).

Plants develop several compounds that are able to inhibit microbial enzymes and cause impairment of the fungal cell wall (Polya, 2003; Mostafa et al., 2009; Goel & Sharma, 2016). *Ammi* plants are one of important medicinal plants belonging to family *Apiaceae* and they are widely distributed in Egypt. Several secondary metabolites of significant and numerous biological activities were found in *Ammi* plants such as coumarins, phenols, flavonoids and others (Al-Snafi, 2013; Aourabi et al., 2019). These compounds are used as crucial health care. The application of *Ammi* extracts in the biocontrol of many plant pathogens is of great importance (Hashim et al., 2014; Hussein et al., 2016; Adham & Abdula, 2017).

The present work aimed to study the influence of leaf extract from *Ammi majus* and *A. visnaga* on growth of *F. oxysporum* and on the activities of some metabolic enzymes such as glucose-6-phosphate dehydrogenase (G6PD), L-phenylalanine ammonia lyase (PAL), glutamine synthetase (GS) and nitrate reductase (NR) of *F. oxysporum*. Also, it was aimed to test the influence of *Ammi* leaf extracts on the activities of pathogenicity related enzymes including pectinase, protease, cellulase and xylanase. In addition, it was designed to explore the influence of the leaf extracts on amino acids and protein

contents of *F. oxysporum*.

Materials and Methods

Fungal isolate

Fusarium oxysporum used in the present study was provided by Prof. Metwally A. Metwally, Professor of Microbiology, Botany Department, Faculty of Science, Tanta University. Fungal culture was grown on Czapek's-Dox agar containing % (sucrose, 3.0; NaNO₃, 0.2; K₂HPO₄, 0.1; FeSO₄.7H₂O, 0.1; KCl, 0.05 and MgSO₄ 7H₂O, 0.5 and pH 7.0) at 28°C for 5 days and was sub-cultured periodically for extended periods of storage.

Preparation of aqueous leaf extracts of *Ammi* plants

Collection of *Ammi* plants

A. majus and *A. visnaga* were collected from different locations in Egypt and identified by Prof. Dr. El-Sayed F. El-Halwany, Professor of Plant Ecology, Botany Department, Faculty of Science, Mansoura University.

Preparation of aqueous leaf extracts of *Ammi* plants

Preparation of aqueous leaf extract is based on the method of El-Shora et al. (2016). Leaves from each of *A. majus* and *A. visnaga* were collected and washed carefully under tap water then by distilled water. The collected leaves were air-dried and homogenously grinded using mechanical grinder to prepare fine powder. The powder (10% w/v) was soaked in sterilized distilled water and put on a shaker with a speed of 120rpm for 24hrs at room temperature (20±2°C). The resulting mixtures were filtered using Whatman number 1 filter paper then centrifuged for 20min at 10,000g to remove particulate materials. The resulted extracts were concentrated and then dried in lyophilizer. The dried extracts were collected in air tight containers and stored at room temperature for further use.

Extraction yield (%) = [dry wt. of dried extract / dry wt. of sample] x 100

Effect of *Ammi* leaf extracts on mycelium growth

The mycelium growth of *F. oxysporum* was evaluated in Petridishes (60mm) filled with PDA medium amended with various concentrations (0-50mg/ml) of aqueous leaf extract of *A. majus* or *A. visnaga*. Next, the Petri dishes were inoculated with 5mm diameter disc of fungal mycelium

which was taken from 7-day old pure culture. All dishes were incubated for 6 days at 25°C. After that, the mycelial growth was measured 6 days after inoculation. For each treatment, three replicates were maintained. Finally, the antifungal activity was expressed in terms of the inhibition percentage according to the following equation:

$$\text{Fungal inhibition (\%)} = (R_c - R_t) / R_c \times 100$$

where: R_c is the mean value of *F. oxysporum* radial growth in control and R_t is the mean value of fungal radial growth in plant extract treated plates.

Growth of F. oxysporum on modified Richard's liquid medium

Aqueous leaf extracts from dried *A. majus* or *A. visnaga* leaves was diluted to attain various concentrations (10, 20, 30, 40 and 50mg/ml) by mixing with sterilized modified Richard's liquid medium contained (g/L): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5; KH_2PO_4 , 5; KNO_3 , 10; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.02 supplemented with the substrate of the assayed enzyme (Zhang et al., 2014; Xue et al., 2018). Spore suspension of *F. oxysporum* (10^5 spores/ml) was inoculated into the broth medium and then incubated for 5 days at 28°C on an orbital shaker (180rpm).

Preparation of the mycelial extract

After the incubation period (5 days) the fungal mycelium was collected, washed and then homogenized for 30min in a homogenizer using 50mM phosphate buffer (pH 6.0). The resulting homogenate was centrifuged at 12,000g for 15min at 4°C and the supernatant represented the mycelial extract which was stored at -20°C. This supernatant was used for determination of metabolic enzymes activities, the total amino acids and total protein content. Broth medium without plant extract was used as control under the same conditions.

Assays of metabolic enzymes activities in mycelial extracts

Glucose-6-phosphate dehydrogenase (G6PD) activity

G6PD activity was assayed according to Betke et al. (1967). This method depends on measuring the production of NADPH spectrophotometrically at 340nm. The reaction medium of 3ml included 0.5ml of 0.6mM G6P, 0.5ml of 0.2mM of NADP and 1.5ml of 10mM MgCl_2 in 150mM Tris-HCl

buffer (pH 8.0). Enzyme preparation (0.3ml) was added to start the reaction and kept at 30°C 15min. One unit (U) of G6PD activity is defined as the amount of G6PD which produces one μmole of NADP per min under standard assay conditions.

L-phenylalanine ammonia-lyase activity

The assay of L-phenylalanine ammonia-lyase (PAL) activity was carried out by the method adopted by Chen et al. (2006). In 3ml, the reaction mixture composed of 0.2ml of 0.5mM L-phenylalanine, 1.5ml of 50mM Tris-HCl buffer (pH 8.9), 0.8ml of 3.6mM NaCl and 0.5ml of the mycelial extract (crude enzyme). The reaction mixture was incubated at 37°C for 1hrs and was terminated using 0.5ml of 35% (w/v) trifluoroacetic acid (TFA). The mixture was centrifuged for 5min at 5,000g to pellet the denaturated protein. The cinnamic acid was estimated spectrophotometrically by measuring the absorbance at 290nm. One unit (U) was expressed as the amount of PAL required to catalyze the conversion of one μmol of L-phenylalanine per min.

Glutamine synthetase (GS) activity

GS was assayed by the method adopted by El-Shora & Khalaf (2008) and El-Shora & Ali (2011). The assay mixture contained in 3ml: 50mM imidazole-HCl (pH 7.0), 55mM L-glutamate, 3mM ATP, 15mM MgCl_2 , 30mM NH_4Cl and 0.5ml enzyme extract. The starting of the reaction was initiated by enzyme extract and the incubation at 30°C. The reaction was terminated by 5ml freshly prepared $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.8% in 0.015N H_2SO_4) after 15min. The produced inorganic phosphate (Pi) was estimated by 0.5ml of 6.6% ammonium molbedate. The absorbance was recorded spectrophotometrically at 600nm.

Nitrate reductase (NR)

NR was assayed according to Evans & Nason (1953). One ml Tris buffer-enzyme extract was mixed with 0.5ml of 100mM KNO_3 and incubated for 5min at 30°C. The enzyme reaction was initiated by 5ml of 0.5 μM NADPH and kept for 15min at 30°C. The enzyme reaction was terminated by 0.3ml of 100mM $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$ followed by 1ml of absolute ethanol.

A sample (0.2ml) of the assay mixture was added to 1.0ml of 1.0% sulphanilamide dichloride in distilled water. The resulting pink color from diazotization was left for developing for 20min.

The volume was raised up to 5.0ml using distilled water. The absorbance was recorded at 540nm spectrophotometrically. Sodium nitrite solution was using for preparing standard curve.

Influence of aqueous leaf extracts on total free amino acid of F. oxysporum

The total free amino acid of *F. oxysporum* was estimated by ninhydrin method according to Moore & Stein (1948). One ml of the sample was added to one ml of ninhydrin solution and then left for 15min in boiling water bath. Add 5ml of diluent (water + n-propanol in equal volume) and incubate for 15min at Lab temperature. The absorbance was recorded spectrophotometrically at 570nm. The estimation was done in triplicates and the results were expressed as mgg^{-1} sample.

Influence of aqueous leaf extracts on total soluble protein content of F. oxysporum

The total soluble protein of *F. oxysporum* was determined according to Bradford (1976). About 50 μl of mycelial extract was made up to 100 μl with 150mM NaCl in a test tube. One ml of Bradford's reagent was added and mixed well. The absorbance was read at 595nm spectrophotometrically. Bovine serum albumin (BSA) was used as standard (Bradford, 1976).

Influence of leaf extracts on the activities of pathogenesis related enzymes

F. oxysporum (10^5 spores/ml) was grown on modified Richard's liquid medium amended with different concentrations (0-50mg/ml) of leaf extract from *A. majus* or *A. visnaga* and specific enzyme substrate as the sole carbon source for each investigated enzyme. Flasks were incubated on an orbital shaker (180rpm) at 28°C for 5 days. After the incubation time, the mycelial pellets were collected through filtration using Whatman filter paper No. 1 (Whatman, Piscataway, NJ, USA) and discarded. The filtrate was centrifuged at 12,000g for 15min at 4°C and the supernatant was collected and stored at -20°C as the crude extract of extracellular enzymes for determination of pathogenicity related enzymes activities (Zhang et al., 2014; Xue et al., 2018).

Assay of pectinase activity

Polygalacturonase activity was assayed according to Aguillar & Huitron (1990). A sample of 0.1ml of enzyme preparation was incubated with 0.1ml of polygalacturonic acid (PGA, 1.0%, w/v) for 10min at 40°C. After adding 0.4ml of dinitrosalicylic acid reagent (DNSA), the assay

medium was boiled for 10min. The mixture was finally made up to 5ml using 4.4ml distilled water. The absorbance was recorded at 530nm spectrophotometrically. One unit (U) was defined as the amount of enzyme which catalyzes the formation of one μmol of galacturonic acid per min.

Assay of protease activity

Protease assay was carried out by the adopted method of El-Shora & Metwally (2008) and El-Shora et al. (2016). A sample (2.5ml) of casein solution (0.65%) prepared in 100mM Tris-HCl buffer (pH 8.5) was mixed with 0.5ml enzyme extract. The reaction was kept for 10min at 37°C. After the incubation time, the reaction was terminated by 2ml of 120mM trichloroacetic acid (TCA). The mixture was held in water bath for 20min at 37°C and then centrifuged for 15min at 12,000g. One ml of the resulting supernatant was added to 2.5ml of 500mM Na_2CO_3 and 0.5ml of Folin-Ciocalteu reagent. The mixture was left at 37°C for 25min. The absorbance was recorded at 660nm. One unit (U) was defined as the amount of protease which liberates $1\mu\text{gmin}^{-1}$ tyrosine at 37°C and pH 8.5. Tyrosin was used for preparation of standard curve.

Assay of cellulase activity

The assay of cellulase activity was carried out by estimating the reducing sugar released from carboxymethyl cellulose (CMC) using dinitrosalicylic acid reagent (Goel et al., 2019). Enzyme preparation (0.5ml) was mixed with 0.5ml 1% CMC in 50mM phosphate buffer (pH 7.0). The mixture was incubated for at 30°C 30min. The reaction was boiled for 15min at 100°C in a water bath followed by adding 3ml of dinitrosalicylic acid. The absorbance was recorded at 540nm spectrophotometrically to estimate the enzyme activity using glucose standard curve.

Assay of xylanase activity

The assay of xylanase activity was performed by the adopted method of Bailey et al. (1992). The assay medium contained 0.9ml of xylose (1% w/v) in 50mM citrate buffer (pH 5.0) and 0.5ml of enzyme preparation followed by incubation at 30°C for 20min. The mixture was boiled at 100°C for 10min and then 1ml of dinitrosalicylic acid reagent was added to terminate the reaction. After cooling, the absorbance was recorded spectrophotometrically at 540nm using standard curve of xylose and the results were expressed in

terms of one μmol of reducing sugar produced per min.

Determination of total phenolic content in leaf extracts of *Ammi* plants

The total phenolic content of *A. majus* and *A. visnaga* extract was estimated by the adopted method of Kaur & Kapoor (2002). The reaction medium contained 0.5ml plant leaf extract or gallic acid mixed with 2.5ml distilled water as well as 0.25ml of 2N Folin-Ciocalteu reagent for 5min. Aqueous Na_2CO_3 (0.75ml, 20% w/v) was then added with 0.95ml distilled water and incubated for 1hr in dark at 37°C . The absorbance was recorded at 765nm. Gallic acid (GA) was used as a standard and the results were expressed as mg GA g^{-1} dry weight.

Determination of total flavonoid content in leaf extracts of *Ammi* plants

The total flavonoid of leaf extracts from *A. majus* and *A. visnaga* was determined by the adopted method of Gulati et al. (2012). A sample of leaf extract (0.5ml) was raised up to 1.0ml using distilled water, followed by 0.3ml NaNO_2 (5%) and 0.3ml aluminum chloride (10%). The mixture was mixed and incubated for 10min at 25°C and then 2ml of 1M NaOH were added. The final volume of the reaction was made up to 10ml with distilled water. The absorbance was recorded at 510nm after 5min. Quercetin was used as standard and the results are calculated as $\text{mg quercetin equivalent (QE) g}^{-1}$ dry weight.

Statistical analysis

All the experiments were done in triplicate and their mean values were presented with the standard deviation (S.D.).

Results and Discussion

Aqueous leaf extract was prepared from each of *A. majus* and *A. visnaga* as described in Materials and Methods. The extraction yield of this process was 17%. The antifungal activity of leaf extract from *A. majus* and *A. visnaga* against *F. oxysporum* was carried out using agar plate assay and presented in Fig.1. The results reveal the capability of aqueous leaf extract from both species of *Ammi* plants to reduce the fungal radial growth and the reduction was dependent on the extract concentration.

The reduction in mycelial growth of *F.*

oxysporum by aqueous leaf extract of *A. majus* and *A. visnaga* was about 93% and 74%, respectively at 50% concentration. Anti-mycotic activity of *Ammi* leaf extracts was associated with low content of total amino acids as well as the total soluble protein. Dwivedi & Sangeeta (2015) reported the capability of aqueous extract from four medicinal plants at various concentrations to decrease the radial growth of *F. oxysporum*. The antimicrobial activity of *A. majus* and *A. visnaga* extracts was reported by Belkacem et al. (2016) and Al-Hadhrami et al. (2016). The antifungal potentiality of various medicinal plants was attributed to their phenolic and flavonoids contents (Shankaracharya et al., 2000; Hadizadeh et al., 2009; Singh et al., 2013). *Ammi* species

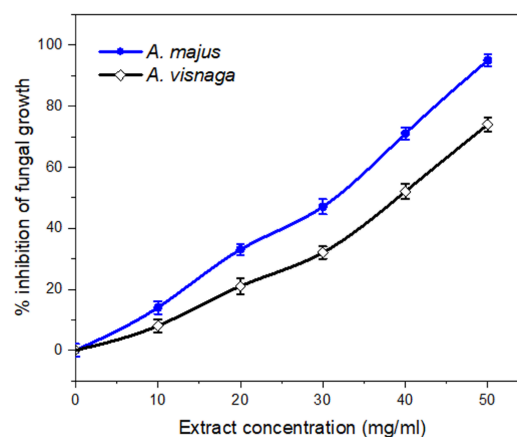


Fig. 1. Susceptibility of *F.oxysporum* radial growth to aqueous leaf extract of *A. majus* and *A. visnaga* at different concentrations.

contain various bioactive compounds (mainly coumarins, phenols and flavonoids) which are of important biological activities (Al-Snafi, 2013).

Several enzymes are involved in the physiological processes and pathogenesis of fungi. The inhibitory effect of leaf extracts from *A. majus* (Fig. 2A) and *A. visnaga* (Fig. 2B) on fungal enzymes activities of *F. oxysporum* was investigated. The production of G6PD, GS and NR by *F. oxysporum* was inhibited by the aqueous leaf extracts from both plant species and the inhibition was concentration-dependent. On the other hand, PAL activity of *F. oxysporum* increased in concentration-dependent manner by leaf extract of both *Ammi* species.

Fu et al. (2018) and Chamizo-Ampudia et al. (2017) reported that NR in different microorganisms is the key enzyme in nitrogen

assimilation. Cytosolic-NR catalyzes the conversion of nitrate into nitrite, followed by the reduction of NO_2^- into NH_4^+ for amino acid biosynthesis (Kolbert et al., 2008; Stéphanie & Dimah, 2009). Processes of nitrate reduction and glutamate formation require various reductants which supplied by G6PD. In addition, Hall & Tomsett (2000) reported that NADPH is the electron donor for NR in fungi. Therefore, the results confirm the correlation between reduction of G6PD production and NR activity. The

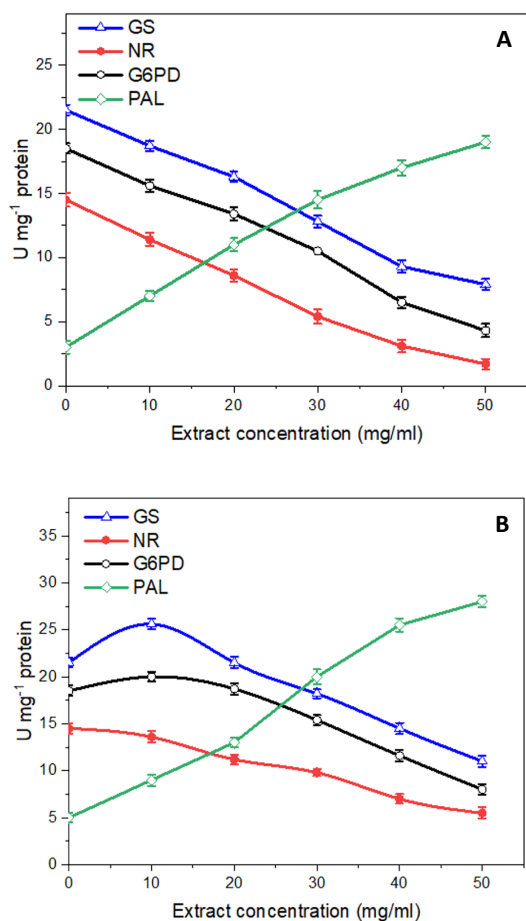


Fig. 2. Influence of aqueous leaf extracts from *A. majus* (A) and *A. visnaga* (B) on metabolic enzymes of *F. oxysporum*.

deactivation of NR caused reduction of nitric oxide level and amino acid production by some pathogens and consequently reduction of fungal growth (Yamamoto-Katou et al., 2006).

GS is a major regulatory enzyme in ATP-dependent assimilation of ammonium with glutamate (Zhang et al., 2016). The obtained glutamine was then incorporated with specific amino acids, producing precursors for other

amino acids, proteins and nitrogen-containing compounds (Forde & Lea, 2007). It has been reported that inhibition of GS results in reduction of *Aspergillus nidulans* growth (Macheda et al., 1999).

Hyun et al. (2011) reported the presence of PAL in various ascomycetes, deuteromycetes and basidiomycetes. Different fungi can utilize the amino acid phenylalanine through various metabolic pathways involved in the deamination process to cinnamic acid. The function of PAL in fungi is to obtain the requirements of nitrogen and carbon from amino acids. It was suggested that PAL activity is vital in secondary metabolism and pathogenesis and the increase of PAL activity by the *Ammi* leaf extracts in agreement with report of Gholamnezhad (2019).

PAL activity was increased under treatment with *Ammi* leaf extracts to convert the primary metabolites to defensive metabolites for scavenging the reactive oxygen species (ROS) produced within the fungal mycelium due to the chemical stress resulted by *Ammi* leaf extracts which contain various chemical compounds responsible for such stress (Kaboodi et al., 2016). Among these chemicals found in *Ammi* extracts are coumarins, flavonoids, tannins, anthraquinones, saponin and terpenoids (Zaher et al., 2019).

The total amino acids (Fig. 3A) and total soluble protein (Fig. 3B) of *F. oxysporum* were remarkably declined with increasing concentration of the aqueous leaf extracts from *A. majus* and *A. visnaga*. Leaf extract of *A. majus* was more effective in reducing the total amino acids as well as the total soluble protein of *F. oxysporum* than that of *A. visnaga*. Phenolics from plant parts were reported to be the main reason for inhibition of protein synthesis by fungi and the occurrence of sever pathogenesis (Nateqi & Mirghazanfari, 2018).

Fungi produce the various enzymes to depolymerize the major polysaccharides in the plant cell wall including pectin, cellulose and hemicellulose (Kubicek et al., 2014). The extracellular fungal enzymes are efficient in the degradation of the structure barriers of plant cell and hence facilitate the penetration and invasion of host cells (Walton, 1994; Kumar et al., 2016).

Phytopathogenic fungal diseases are mainly

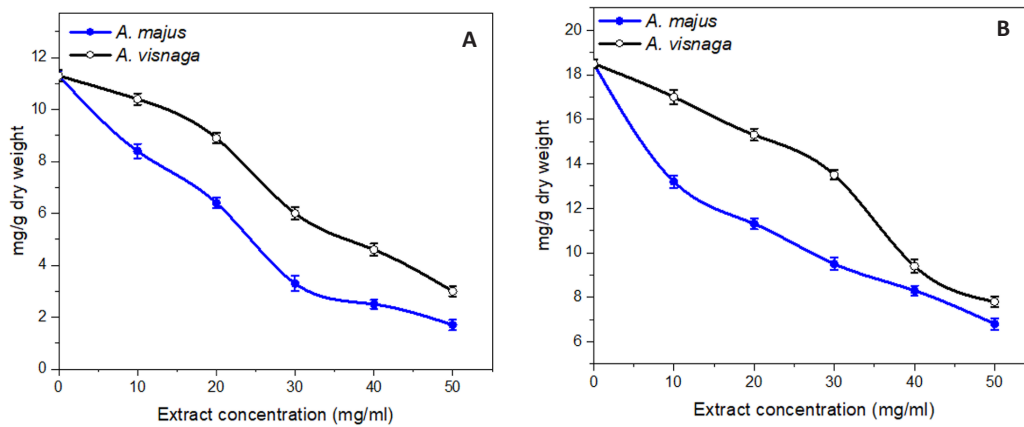


Fig. 3. Influence of aqueous leaf extracts from *A. majus* and *A. visnaga* on total amino acids (a) and total soluble protein (b) produced by *F. oxysporum*.

depending on the hydrolases and proteases which are involved in cell wall degradation, maceration and different stages in disease development. High correlation between the aggressiveness of disease and the synergistic action of various enzymes was reported (Chandrasekaran et al., 2016).

The response of the pathogenesis-related enzymes of *F. oxysporum* toward treatment with leaf extracts of *A. majus* and *A. visnaga* was investigated and the results are presented in Fig. 4. The results reveal that all studied extracellular enzymes from *F. oxysporum* were inhibited in a concentration-dependent manner. Under treatment

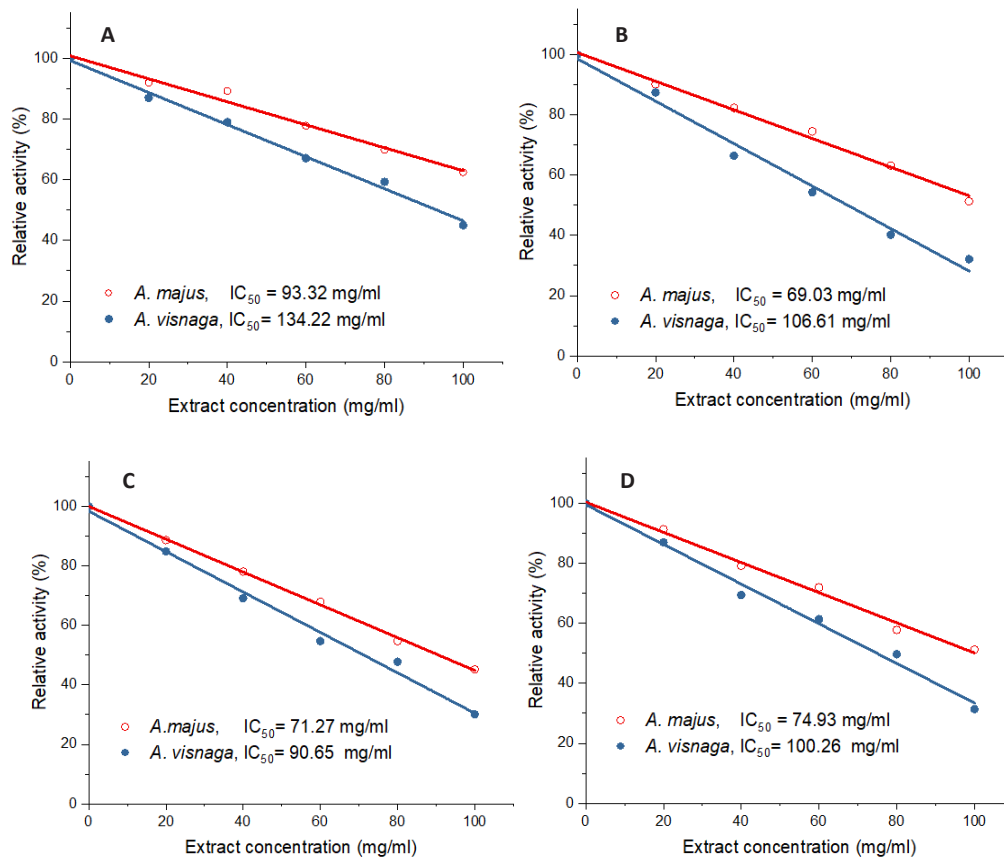


Fig. 4. Influence of leaf extracts of *A. majus* and *A. visnaga* on production of pectinase (A), protease (B), cellulase (C) and xylanase (D) by *F. oxysporum*.

with *A. majus* leaf extract IC_{50} values for pectinase (Fig. 4A), protease (Fig. 4B), cellulase (Fig. 4C) and xylanase (Fig. 4D) were 93.32, 69.03, 71.27 and 74.93mg/ml, respectively. However, IC_{50} values were 134.22, 106.31, 90.65 and 100.26mg/ml for the same enzymes in the same order under treatment with *A. visnaga* leaf extract.

Pectinases represent a determining factor in plant susceptibility toward the wilt-producing *Fusarium* sp. as the suppression of fungal pectinase induces resistance of plant against fungal infection (Herbert et al., 2003). Pectinase catalyzes the cleavage of glycosidic linkage causing a depolymerization of pectins (Kashyap et al., 2001; King et al., 2002). Powell et al. (2000) suggested that the inhibition of polygalacturonase is the reason for significant reduction in virulence of *Botrytis cinerea*.

Xylanases have the capability to depolymerize xylan which is secondary plant cell wall polysaccharides (Wong et al., 1988). However, other researchers reported that xylanase is not essential for fungal pathogenicity (Gomez-Gomez et al., 2002; Calero-Nieto et al., 2007). It has been reported that deletion of xylanase gene expression causes significant reduction in pathogenicity (Brito et al., 2006). Interestingly, fungal xylanase may elicit the defense mechanisms in the plants (Belien et al., 2006).

It has been reported that flavonoids, phenolics and other compounds in plant leaf extracts may be the reason for the inhibition of pectinase, proteases, xylanase and cellulase activities (Mehta & Mehta, 2005; Nayebi et al., 2013). Therefore, the total phenols and total flavonoids in the two *Ammi* species were determined (Fig. 5). The total phenol constituents detected in the leaf extracts of *A. majus* and *A. visnaga* were 31.4 ± 0.8 and 21.5 ± 0.6 mg GA (gallic acid) g^{-1} dry weight, respectively. However, the total flavonoid in leaf extracts of *A. majus* and *A. visnaga* were 19.6 ± 0.6 and 11.8 ± 0.5 mg QE (quercetin equivalent) g^{-1} dry weight. These results are in harmony with those of Hussein et al. (2016) who found the presence of secondary phenolic constituents in *A. majus*. Furthermore, the phenolics and flavonoids of *A. visnaga* were in the range of 18.66 to 172.66mg GAg^{-1} and 3.3 to 22mg QE g^{-1} , respectively (Belkacem et al., 2016).

The presence of phenols and flavonoids

in *Ammi* plants reveal and confirm that these compounds seem likely to play an important part in the inhibition of cell wall degrading enzymes of *F. oxysporum*. Surendran et al. (2018) reported a

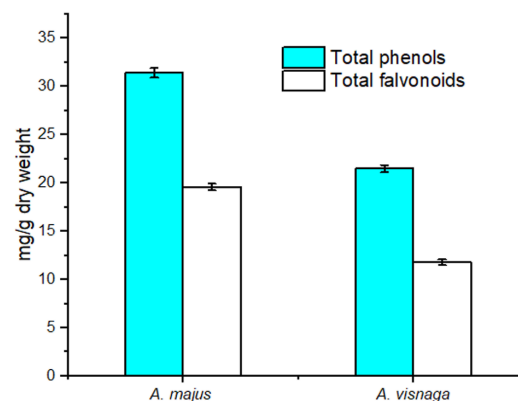


Fig. 5. The contents of total phenol and the total flavonoid in *A. majus* and *A. visnaga*.

remarkable inhibition of cellulase of *Ganoderma* sp. by different phenolic compounds. The degree of inhibition of cellulase and polygalacturonase by phenolics was concentration-dependent (Mehta & Mehta, 2005). Furthermore, it was reported that various phenolics inhibited the activity of cell wall degrading enzymes of *F. oxysporum* (Modafar & Boustani, 2001).

Since leaf extracts of *Ammi* plants contain remarkable content of phenol, their toxicity mechanism against *F. oxysporum* may include interference with cell wall synthesis, changing the permeability of cell, interfering with the electron transport, the nutrient absorption, the ATP production, metabolic processes of the cell, inactivation of different enzymes in the cells and protein denaturation (Marjorie, 1996; Al-Amiery et al., 2012). Furthermore, studies have indicated that the hydroxyl groups of phenol are responsible for the toxicity against microorganisms. This implies that increased hydroxylation leads to increased toxicity. It seems likely that phenolic toxicity to microbial enzymes is possibly through reaction with sulfhydryl group through non-specific interactions with membrane proteins (Arif et al., 2009).

Conclusion

The findings of the present study lead to the conclusion that the aqueous leaf extracts from both *A. majus* and *A. visnaga* have antifungal activity against the wilt-producing *F. oxysporum*.

The leaf extracts caused remarkable reduction in the growth of *F. oxysporum*, the activities of the different metabolic enzymes, pathogenicity related enzymes, total amino acids and total soluble protein. The present work presents beneficial information for developing of natural eco-friendly and inexpensive formulations using *Ammi* leaf extracts for protection of tomato against *F. oxysporum*. However, more studies are required to explain the crucial mechanisms of the fungal inhibition by *Ammi* leaf extracts and to determine the compounds responsible for the inhibition which seems likely complex and multifactorial.

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التحكم الحيوي في مرض الذبول المستحث بفطر فيوزاريوم أوكسيسبوروم بواسطة مستخلصات مائية لأوراق نوعين من نبات الخلة هما الخلة البرية و الخلة البلدية

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يسبب فطر فيوزاريوم أوكسيسبوروم مرض الذبول لمحصول الطماطم والتحكم في نمو ونشاط الفطر باستخدام مبيدات الفطريات يؤدي إلى العديد من المشاكل البيئية. وتمثل مواد الأيض النباتية طريقة بديلة للتحكم في نشاط الفطر حيث أن لها تأثير طفيف على البيئة. لذلك هدف هذا البحث إلى محاولة استخدام مستخلصات مائية لأوراق نوعين من نبات الخلة هما الخلة البرية والخلة البلدية والمنتمي للفصيلة الخيمية. أدت معاملة الفطر بالمستخلصات المائية لأوراق نباتي الخلة إلى تثبيط نمو الفطر. كما تثبتت المستخلصات المائية أنشطة بعض الإنزيمات الأيضية مثل ديهيدروجينيز الجلوكوز-6-فوسفات و الإنزيم المصنع للجلوتامين وإنزيم ريدكتيز النترات ولكن زاد نشاط كل من إنزيم فينيل ألانين أمونيا لبيز للفطر. كما أدت المعاملة إلى انخفاض المحتوى الكلي للأحماض الأمينية الحرة والمحتوي الكلي للبروتينات الذاتية للفطر. وثبتت المستخلصات المائية أنشطة بعض الإنزيمات التي تشارك في حدوث مرض الذبول مثل البكتينيز والبروتيزو السيلوليز و الزيلانيز. تم دراسة المحتوى الكلي للفينولات و الفلافونويدات في مستخلصات أوراق نباتي الخلة وكان محتوى كل منهما أعلى في نبات الخلة البرية عن نظيره الموجود في نبات الخلة البلدية. تقترح هذه الدراسة إمكانية استخدام المستخلصات المائية لنباتي الخلة في قمع نشاط ونمو فطر فيوزاريوم أوكسيسبوروم.