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Molecular identification and control of somepathogenic *Fusarium* species isolated from maize in Egypt

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Abstract: *Fusarium* species are among the most common fungal pathogens on maize causing decrease of the yield and accumulation of one or more of*Fusarium* toxins. The current morphology based taxonomical system for *Fusarium* is inadequate, and detection and identification procedures are both time consuming and error-prone. Thus the aim of this study wasto isolate and identify pathogenic *Fusarium* species as well as studying the use of some plant extracts to control the *Fusarium* species.The molecular characterization was performed with partial 18S rRNA sequencing using primer (5'-AGAGTTTGATCCTGGCTCAG) targeted to internal transcribed spacer (ITS) region of rDNA complex. Sequence analysis identified the isolates as *Fusarium solani*and *Fusarium oxysporum* and the species identification based on sequence data correlated well with the morphologic classification. The growth of*F.oxysporum*and *F. solani*specieswere completely inhibitedusing100 ppm ofrocketandstar aniseextracts. The highly active extract of rocketandstar anise was followed byfennelandrosemary in descending order.*Fusarium oxysporum* was more sensitive for the plant extracts used. The MIC and IC50 of plant extracts were also determined.Inconclusion,the sequence variation within the ITS region allowed reliable and faster discrimination of the isolates at both the genus and species level. On the other hand, the plant extracts used in this study showed high antifungal activity against the *Fusarium* species.
Key words:*Fusarium s*pecies, maize, plant extract, antifungal activity, 18S rDNA.

Introduction

Maize (*Zea mays* L.) is a very versatile grain that serves as starting raw material for food and feed products. Itis prone to pre and pro-harvest fungal infection and is often unavoidable and is considered a worldwide problem. The susceptibility of maize grains to various fungi has been very well documented, whereas these fungi belong to the genera *Fusarium*, *Aspergillus* and *Penicillium.* The genus *Fusarium* comprises a diverse array of fungi which are distributed world-wide as important plant pathogens, as well as opportunistic colonizers of plant and agricultural commodities or as saprophytes on debris and cellulosic plant material[1].It has a wide range of host plants, such as tomatoes, potatoes, legumes, clove and grasses such as wheat, barley, oats, maize and sugarcane[2, 3]. In the last ten years, the increased level of colonization and infection by *Fusarium*, particularly of ripening ears of cereals, has attracted much attention: firstly, because of the significant effects on yield and the quality of harvested grains, andsecondly because of the ability of *Fusarium* species to produce a wide range of mycotoxins which can enter the human and animal food chains[4, 5, 6].

Fusarium solani are host-specific pathogens of a number of agriculturally important plants, including pea, cucurbits, and sweet potato[7]. Moreover, they are increasingly associated with opportunistic infections of humans and other animals, causing systemic infections with a high mortality rate[8], as well as localized infections in the skin and other body parts[9]. It is also associated with serious invasive mycoses in immune compromised and immune suppressed patients[10].*Fusarium oxysporum* is a soil-borne facultative parasite that

causes disease in more than 100 plant species, including important agricultural crops[11]. The fungus is a morpho-species that is divided into specialized groups according to the hosts they attack, and subdivided into races according to the susceptibility of specific host cultivars[12].

The routine analysis of maize and other cereals for toxigenic *Fusarium* species is hindered by difficulties associated with standard methods for isolating and identifying *Fusarium* species[13]. Traditionally *Fusarium* species have been differentiated by morphological characteristics such as presence or absence of micro-conidia, shape and size of macro-conidia, colony morphology, growth rates and pigmentation. Morphological identification of fungi is the first and the most difficult step in the identification process and is time consuming and requires considerable expertise and skill[2]. This is especially true for *Fusarium* species which have been differentiated by morphological characteristics, although differentiation of closely related cultures requires extensive molecular techniques[14]. Molecular techniques represent an important genetic resource for biotechnology study. Several recent studies have shown that genetic methods can be successfully used in the studies of pathogenic fungi. DNA-based techniques, particularly PCR can detect minute quantities of a pathogen[15, 16]. These methods have provided new opportunities to study and understand, for example, the biology of plant pathogenic fungi, pathogen population structure and dynamics, host- pathogen interactions, etc. [17, 18, 19].Thus, today *Fusarium* species are usually identified by combining the morphological, biological, and molecular data.

The presences of toxigenic fungi in grains present a potential hazard to human and animal health. Currently, there is a strong debate about the safety aspects of chemical preservatives, and consumers tend to be suspicious of chemical additives and thus demand for more natural and more acceptable preservatives to been intensified[20]. Natural metabolites do not have any indiscriminate hazardous effects like synthetic fungicides. They are normally found in leaves and stem and concentrate in regions such as leaves, bark or fruit[21]. Bhardwaj[22] tested the aqueous extracts of twenty plants for their antifungal activity against *F. solani*, whereas Bajpai and Kang[23] studied the antifungal activity of *Magnolia liliflora* against several pathogenic fungi including *F. solani*.

Rocket (*Eruca sativa* L.)have gained greater importance as a salad vegetable and spice, especially among Middle Eastern populations and Europeans, and possess diversified medicinal and therapeutic properties including inhibition of tumorigenesis, anti-ulcer, and hepato-protective activities[24]. Fennel (*Foeniculum vulgare*L.) a plant belonging to the family Apiaceae, has a long history of herbal uses. Traditionally, fennel seeds are used as anti-inflammatory, analgesic, carminative, diuretic and antispasmodic agents. Recently there has been considerable interest in the antioxidant potential and antimicrobial activities of fennel seed extracts[25]. It is also used as a constituent of cosmetic and pharmaceutical products[26]. Rosemary (*Rosmarinus officinalis* L.)is a common household plant grown in many parts of the world. It is used for flavouring food, as well as in cosmetics and in folk medicine. Extract of rosemary relaxes smooth muscles of trachea and intestine, and has choleretic, hepato-protective and anti-tumerogenic activity. The most important constituents of rosemary are caffeic acid and its derivatives such as rosmarinic acid[27].Star anise (*Illiciumverum*) is an aromatic evergreen tree bearing purple-red flowers,growing almost exclusively in southern China and Vietnam. Its fruit is an important traditional Chinese medicine as well as a commonly used spice that closely resembles anise in flavour, obtained from the star-shaped pericarp[28].

In the present investigation morphological and molecular techniques were used to identify some *Fusarium* species isolated from pre-harvest maize, as well as evaluating the antifungal activity of some plant extracts to control the of the identified strains.

Materials and Methods

Isolation of *Fusarium* species

Fifteen samples of pre-harvest maize (each of three maize ears) were collected from Faculty of Agriculture, Cairo University. Each maize sample was individually surface sterilized in 1% sodium hypochloride solution for 1min, and then rinsed three times in sterile distilled water. Surface sterilization was necessary to curtail the development of only potential contaminants which would affect the recovery of molds. On Potato Dextrose Agar (PDA) (Difico, Detroit, MI)10 grains were plated and incubated at 25±2°C for 7 days. After incubation, the cultures were transferred onto PDA for species identification.

Morphological identification

The fungal species were identified according to the morphology using the descriptions of Nelson et al.[2]Thom and Raper[29], Gilman[30], and Barnett and Hunter[31].The isolates were grown on PDA at 25±2°C for 7 days in order to describe colony morphology and pigmentation as secondary criteria for identification. The isolates were morphologically identified as *F.solani*and*F. oxysporum*and were confirmed by polymerase chain reaction (PCR)

Molecular identification

DNA extraction

Genomic DNA was extracted from pure mycelial cultures of the *Fusarium* isolates; grown on PDA using Extract-N-Amp Plant PCR Kit (Sigma-Aldrich Co., USA) following the manufacturer's instructions. The crude lysate (freshly prepared) was subjected to18SrDNA PCR partial amplification by use protocol of Gene Jet genomic DNA purification kit[32].

PCR partial amplification and sequencing of 18S rDNA

Identification of the fungal isolates was performed based on molecular genetic analysis using the internal transcribed spacer region (ITS). Partial sequences of the isolates 18S rDNA were obtained using a strategy based on Boekhoutet al.[33]. A divergent *5′* domain of the gene was amplified using primers forward (5'-AGAGTTTGATCCTGGCTCAG) and reverse (5'-GGTTACCTTGTTACGACTT).DNA amplification involved the following 25 cycles: initial 1 min denaturation at 94°C, 2 min annealing at 50°C, 1.5 min extension at 74°C and a final 5 min extension at 74°C. PCR amplification was performed using a PTC-100 thermal cycler (MJResearch Inc., Watertown, MA, USA).Amplified products were isolated with a silica matrix (Geneclean II Kit; Bio 101).

Identification of isolates

Sequencing results were individually inputted online into the nucleotide BLASTprogram(BLASTN 2.2.29) through the NCBI database (http://blast.ncbi.nlm.nih.gov/) to identify the isolate[34, 35].

Phylogenetic analysis

Sequencing results of the isolates were also inputted into a sequence alignment program called ClustalW to determine the phylogenetic relatedness of the different species. They were aligned using the UPGMA algorithm, which considers the rate of evolution to be constant between species, to develop a phylogenetic tree based on sequence homology. The resulting alignment was opened into a program called Tree View which allowed the phylogenetic tree to be viewed[36].

Plant materials

Four plants viz.,rocket (seeds), fennel (fruits),rosemary (leaves), and star anise (fruits) were used in this study, and they were purchased from local markets in Egypt. The plants were identified by the Department of Botany, Faculty of Science, Cairo University.

Preparation ofplant extracts

All plantsamples(seeds, fruits, and leaves) were thoroughly washed, air dried under forced circulation of heated air at 40°C and ground to powder. The plant materials were entailingextracted for 8 h with ethanol using aSoxhlet apparatus. Then the ethanol was evaporated using Rotary evaporator under vacuum at 30°C. The plant extracts were kept in a freezer at -20°Cuntil use.

Antifungal activity

The inhibitory effect of the plant extracts was calculated against the linear growth of *F.solani*and *F. oxysporum*. For each extract, seven concentrations 0, 50, 100, 200, 300, 400 and 500 ppm were prepared into sterilized molten and cooled PDA medium. Later 15mL of the molten medium was poured into sterilized Petri plates and then inoculated with a mycelium disc of 5 mm size and placed at the centre. Three replications were used for each concentration. The plates were incubated at 25±2°C and the radial growth was measured when fungus attained maximum growth in control plates.

Statistical analysis

Statistical analysis was performed using SPSS statistical program for windows (Version 16) (SPSS Inc., Chicago, IL, USA). All data were statistically analysed using analysis of variance.

Results

Fungal isolates

In this study, atotal of 50 fungal isolates (data not shown)were obtained from the pre-harvest maize samples. Among the*Fusarium* isolates twoisolateswere selected to identify themorphological andmolecular characteristics.

Morphological characterization

Thefirst isolate grown on PDA produced aerial mycelium (dense and floccose), white colony appearance, greenish or brownish shades on reverse, and fast growing (2.5 to 5.0 cm after4 days).The isolate also had longer macroconidia (3-4 septates),moderately curved 27x5.0 µm.Morphological observation revealed the taxonomic identity of the fungus, and the above characteristics designated the fungus to be *F. solani*. The second *Fusarium* isolate grown on PDA produced aerial mycelium, blue or purple colony colour, dark blue or dark purple shades on reverse, and fast growing.The isolate also had abundant macrocondia, only slightly sickle shaped, with foot shaped basal cell (3-5septates). Microconidiawere abundant, generally single celled, oval, and produced only in false heads. Morphological observation revealed the taxonomic identity of the fungus, and the above characteristics designated the fungus to be *F. oxysporum*.

Molecular characteristics

PCR amplification of ITS region produced about 800 bp of DNA fragments. The fragments were then sequenced to determine the species of fungus based on the similarity with other references of identified species. The sequences of the isolates studied were compared with those of NCBI databases using BLAST network. The partial sequences of 18S rRNA obtained from the first *Fusarium*isolates was aligned with the available 18S rRNAsequences in GenBank data base, and the fungus was found to have 99% similarity with *Fusarium solani* strain (Accession Number Gen Bank: KF572456.1) and*Fusarium solani*CEF-325 (Accession Number Gen Bank: KF999012.1)(Table 1). The second *Fusarium* isolate was found to have 99% similarity with*Fusarium oxysporum* isolate (Accession Number Gen Bank: AY928414.1) (Table 2).

Table 1: Sequence producing significant alignments for the first *Fusarium*isolate

Accession	Description	Max score	Total score	Query coverage	E value	Identity
KF572456.1	*Fusarium solani* strain	953	953	97%	0.0	99%
KF999012.1	*Fusarium solani*strain CEF-325	952	952	96%	0.0	99%
HG798753.1	*Fusarium solani*strain TUFs8	948	948	96%	0.0	98%
KC808261.1	*Fusarium keratoplasticum* f101	946	946	96%	0.0	95%
JF740923.1	*Fusarium lacertarum* isolate 091029	946	946	96%	0.0	94%
GQ505464.1	*Fusarium* NRRL45994 isolate FUS	946	946	96%	0.0	94%
KC808252.1	*Fusarium petroliphilum* isolate Fs2	944	944	96%	0.0	94%

Table 2: Sequence producing significant alignments for the second *Fusarium*isolate

Accession	Description	Max score	Total score	Query coverage	E value	Identity
AY928414.1	*Fusarium oxysporum*isolate	959	959	97%	0.0	99%
JN400705.1	*Fusarium oxysporum* f101	959	959	96%	0.0	98%
KC808261.1	*Fusarium keratoplasticum* f101	959	959	96%	0.0	97%
JF322999.1	*Fusarium solani* isolate Fs1	957	957	96%	0.0	95%
GQ505464.1	*Fusarium* NRRL45994 isolate FUS	957	957	96%	0.0	95%
JF740923.1	*Fusarium lacertarum* isolate 091029	957	957	95%	0.0	94%
KC808252.1	*Fusarium petroliphilum* isolate Fs2	957	957	95%	0.0	94%

The 18S rRNA partial sequence of the *F.solani* is presented in Fig (1), whereas the 18S rDNA partial sequence of the *F. oxysporum* is presented in Fig (2). As a result, phylogenetic trees were mapped using the neighbour joining method, and are shown in Fig (3 and 4). From data in Table (1)and Fig (3), it can be noticed that the first *Fusarium*isolateswas closely related to the *Fusarium solani* strain, while data in Table (2) and Fig (4), showed that the second *Fusarium*isolate is closely related to the *Fusarium oxysporum*isolate. Data in Table (3) showed a comparison between the two identified isolates.

GGGCCTCTTAAGCAGGTAGGCTCATCACCCTGTGACATACCTATAACGTTGCCTCG
GCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCCTAACT
CTGTTTCTATAATGTTTCTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACA
ACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGT
ATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTG
GCGTTGGGGATCGGCGGAAGCCCCCTGCGGGCACAACGCCGTCCCCCAAATACAG
TGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACCTCGCAACTGGAGAG
CGGCGCGGCCACGCCGTAAAACACCCAACTTCTGAATGTTGACCTCGAATCAGGTA
GGAATACCCGCTGAACTTAAGCATATCAATAAGCCGGGAGGAA

Figure (1): Partial sequence of 18S ribosomal RNA gene ofthe first *Fusarium*isolate

GGCAATCTACATGATTCGAGGTCACATTCAGAAGTTGGGTGTTTTACGGCGTGGCC
GCGCCGCTCTCCAGTTGCGAGGTGTTAGCTACTACGCAATGGAAGCTGCGGCGGGA
CCGCCACTGTATTTGGGGGACGGCGTTGTGCCCGCAGGGGGCTTCCGCCGATCCCC
AACGCCAGGCCCGGGGGCCTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCGC
CAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTG
CAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAG
ATCCGTTGTTGAAAGTTTTAATTTATTTGCTTGTTTACTCAGAAGAAACATTATAGA
AACAGAGTTAGGGGGTCCTCTGGCGGGGGCGGCCCGTGTTACGGGGCCGTCTGTTC
CCGCCGAGGCAACGTTATAGGTATGTTCACAGGGTTGATGAGTTGTATAACTCGGT
AATGATCCCTCCGCAGGTCCCCCTACGGAGGG

Figure (2): Partial sequence of 18S ribosomal RNA gene of the second*Fusarium* isolate

Figure (3): Phylogenetic tree based on partial 18S rDNA sequences of *Fusarium solani*

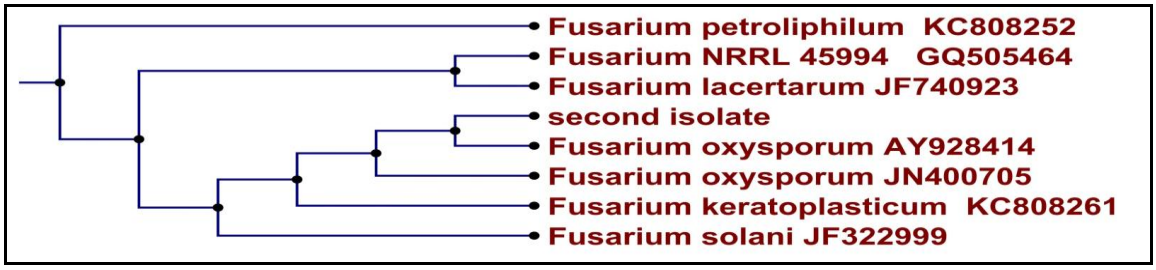

Figure (4): Phylogenetic tree based on partial 18S rDNA sequences of *Fusarium oxysporum*

Table 3: Identification percentage between fungal isolates and related species

Source of isolate	Fungal isolates	Identified isolates	Identity percentage (%)	Query coverage (%)
Pre-harvest maize	1st isolate	*Fusarium solani* KF572456.1	99	97
Pre-harvest maize	2nd isolate	*Fusarium oxysporum* AY928414.1	99	97

Antifungal activity

Data in Fig (5 and 6) revealed that rocket and star aniseextracts showed high antifungal activity and completely inhibited *F. solani* at a concentration of 100 ppm, whereas *F. oxysporum* was completely inhibited at a lower concentration (50 ppm). Results also revealed that the fennel and rosemary extracts completely inhibited the growth of the two isolates under study at 400 and 500 ppm respectively. Data in Table (4) showed the minimum inhibitory concentration (MIC) and inhibitory concentration at 50% (IC50) of the plant extracts. Both rocket and star anise extracts were highly effective against *F.solani* and MIC was recorded as low as 88.00 ppm, while MIC of fennel and rosemary extracts was recorded at high concentrations,341.76 and 442.61ppm respectively.

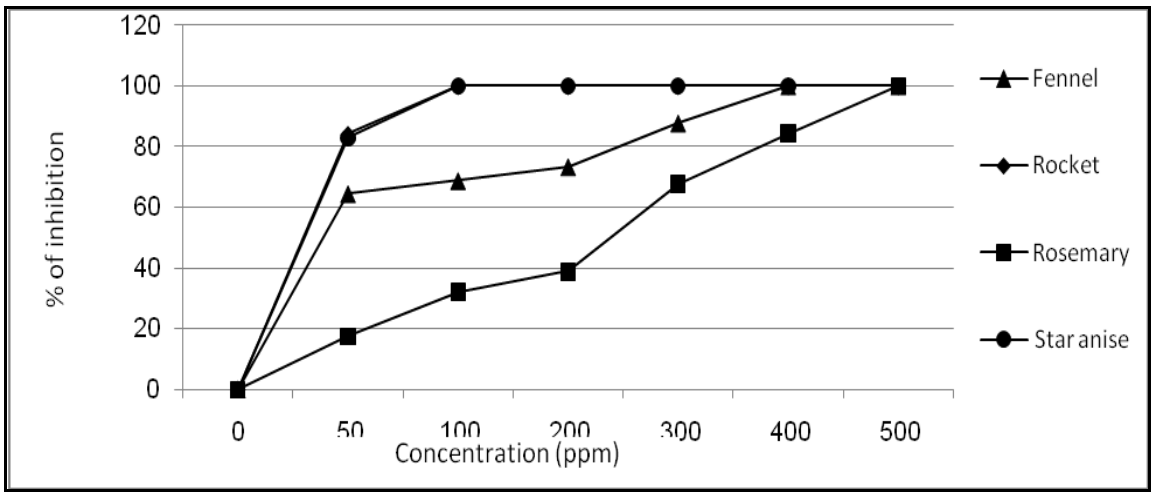

Figure (5): Antifungal activity of some plant extract against *F. solani*

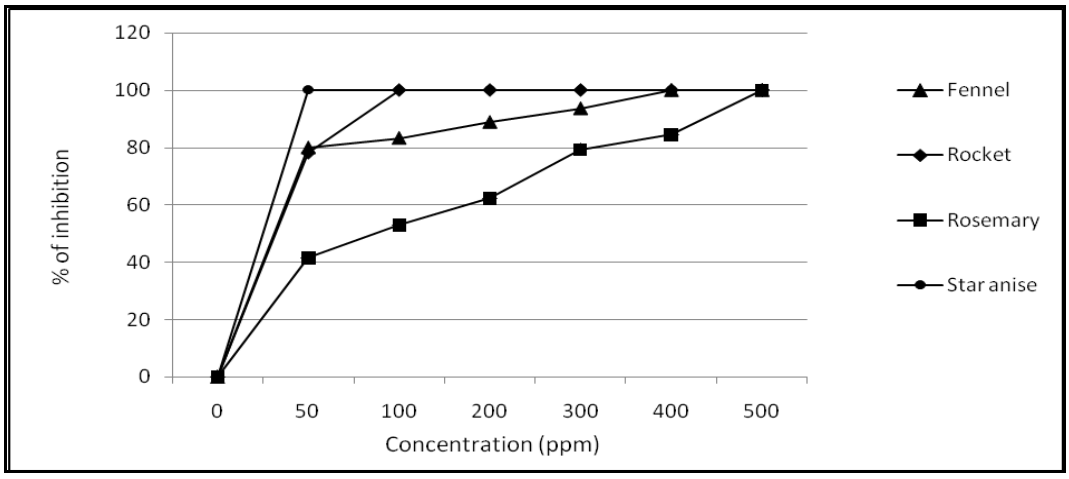

Figure (6): Antifungal activity of some plant extract against *F. oxysporum*

Table 4: Inhibitory concentration at 50% and minimum inhibitory concentration ofsome plant extracts against *F. solani*and *F. oxysporum*growth

Plant extract	*Fusarium solani*		*Fusarium oxysporum*	
	IC50*	**MIC***	**IC50***	**MIC***
Fennel	38.79	341.76	31.25	320.00
Rocket	29.73	88.00	31.90	100.00
Rosemary	221.40	442.61	94.44	484.89
Star anise	30.13	88.35	22.95	500.00

IC50: Inhibitory concentration at 50%
MIC: Minimum inhibitory concentration
* : ppm

Discussion

The importance of this investigationis to shed light on morphological and molecular techniques used to identify some *Fusarium* species isolated from pre-harvest maize, as well as evaluating the antifungal activity of some plant extracts to control the growth of the identified strains.In a recent studyNajim[6]showedthat *F. solani* produced deoxynivalenol, fumonisins,zearalenone,and T2 toxin. Moreover, Manisha[37] reported thatstrains of *F. oxysporum*were able to produce several toxins including fusaric acid. *Fusariumsolani*, asdefined based on morphology, is actually a diverse complex of over 45 phylogenetic and/or biological species[7,38]. These morphologically similar species are generally identified broadly under the name *F. solani*[2]. The morphological concept of *F. solani* proposed by Nelson et al.[2]and Snyder and Hansen[39]is characterized by the production of slightly curved, usually three septate macroconidia with a blunt apical cell and foot-shaped basal cell, from usually cream-colored but sometimes green, blue or red sporodochia, abundant resistant chlamydospores, and production of one- or two-celled aerial conidia that vary in shape, from long monophialides that often have a distinct collarette. Concerning *F.oxysporum*,our results revealed that the above mentioned morphological characters were considered as secondary criteria for the identification of *F. oxysporum*[3]. Meanwhile, Booth[40] considered the conidiogenous cell bearing microconidia as a primary taxonomic criterion and growth rate as a secondary criterion for the identification of *F. oxysporum*. The shape of macroconidia produced in sporodochia is one of the primary defining characteristics of *Fusarium* species and are preferred in identification purpose. The molecular characterization can also be a useful tool to phylogenetically relate the fungi on the basis of their characteristic morphological features[41]. Our results indicated that molecular identification of the organisms exhibited high specificity and sensitivity and can be used for classifying microorganisms at taxonomical level.

The antifungal activity of the plant extracts under study to control the growth of *F. solani*and *F. oxysporum* was measured as the inhibition %.It was noticed that the extent of inhibition of fungal growth varied

depending on the concentrations of plant extract used. The results also revealed that the plant extracts can be divided into two groups according to their antifungal activity to high antifungal activity (rocket and star anise) and low antifungal activity (fennel and rosemary).The high antifungal activity of rocket extract was confirmed by Rani et al.[42]. Similar results were reported by Sabry[43]who foundthat rocket extract and essential oil had the most powerful effect on dry mycelium weight and/or aflatoxin production by *A. flavus* even at a concentration as low as 50 ppm.

In the present study, the effectiveness of fennelethanolic extract against both *Fusarium* species was recorded. Our results are in agreement with those reported by Thakur et al.[44] who reported the antifungal activity of the alcoholic and the aqueous extracts of the *F.vulgare* seeds against several species of fungi. Results are also confirmed by Prabhaet al.[45] who reported the ability of *F.vulgare* extracts to inhibit the growth of *Fusarium oxysporum*. On the other hand, several authors reported the efficacy of *F. vulgare* essential oil as antifungal agent[26,46, 47].

Similar results were reported for star anise extract, which was found to be highly effective and caused 100% reduction of fungal growth. Yazdani et al.[48] reported that star anise fruits extract inhibited the growth of *A. niger*, one of the most important saprophytic funguses known to be associated with mycotoxin production in agricultural products and foods, at 16 mg/mL. In the same trend, Singh et al.[49] showed that the star anise essential oil prevented the growth of *Fusarium moniliforme* at 6 µl dose.

The antifungal activity of rosemary against both *Fusarium* species is similar to results reported by Dellavalleet al.[50]who found that rosemary exhibited antifungal effects against *Alternaria* species even at low concentrations.

The antifungal activities of plant extracts are most likely due to the presence of chemical compounds with antifungal properties. Particularly worth noting is erucin, which accounted for approximately 78.69% of the rocket extractwhichplay an important role as an antifungal agent[43].On the other hand, the antifungal activity of fennel and star anise extracts may be due to presence of anethole[51, 52]. Moreover, De et al.[53] indicated that anethole was effective against bacteria, yeast as well as fungal strains.In a recent study Aly et al. [54]identified trans-anethole as the main component of star anise essential oil (83.32%). Concerning rosemary extract, Okamura et al.[55] reported that the antifungal effects of *Rosmarinus officinalis* oil can be attributed to the Monoterpens combination and in particular α-pinene whose antifungal effects of this combination has been proved. Similar results were reported by Moghtader and Afzali[56] who revealed that the antimicrobial impacts of rosemary essential oil can be related to the high percentage of α-pinene, camphor, verbenone and 1, 8-cineole. On the other hand, the difference in the sensitivity of the *Fusarium* isolates to the plant extracts might be due to the genetic differences between these isolates as shown in Figs (1, 2, 3 and 4).

Conclusion

Molecular characterization showed significant promise in allowing precise and rapid identification of fungal species. Furthermore, complex studies (microbiological, biochemical and molecular) are essential, when the identification of new fungal isolates is the purpose of the investigation.

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