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Antifungal Activity of Ajowan Oil against *Fusarium oxysporum*

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Abstract

During the past decades, efforts in search of alternative fungicides have been focused on natural compounds from plant sources due to their less toxicity and more environmental compatible. In this study, essential oil extracted from mature seeds of ajowan (*Trachyspermum ammi* Lin.) was evaluated against three strains of *Fusarium oxysporum*, *F. oxysporum* f.sp. *lycopersici*, *F. oxysporum* f.sp. *cubense* and *F. oxysporum* f.sp. *capsici* the wilt pathogen of tomato, banana and chili respectively. The *in vitro* assay based on poison food method indicates that ajowan oil, in solution phase, possesses strong antifungal activity against the test fungi. The Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC) were 240 and 480 $\mu g \cdot mL^{-1}$, respectively. The antifungal activity of volatile headspace of the essential oil was evaluated under a modified atmosphere in inverted Petri-plates. Headspace vapor of ajowan oil has a strong activity with Minimum Inhibitory Quantity (MIQ) and the Minimum Fungicidal Quantity (MFQ) of 12.5 and 25.0 µl, respectively. Additionally, the effects of ajowan oil on the biomass production and sporulation of the test fungi indicate that it has significant retardation effect on the biomass production and sporulation of the fungi. Results suggested that ajowan oil has potential use as a biofungicide against the wilt pathogens.

Keywords: ajowan , *Fusarium oxysporum*, antifungal activity

1. Introduction

Fusarium oxysporum is a cosmopolitan fungus that includes pathogenic and non-pathogenic members. The pathogenic members are best known for causing *Fusarium* wilt diseases of many economically important crops [1]. Pathogenic strains of *F. oxysporum* infect hosts by penetrating through the rhizodermis into the root and ensuing colonization of cortex and endodermis up to the vascular system [2]. The vascular wilt diseases of plants often result when xylem vessels are blocked, with the blockage in at least some cases being due to gels composed of neutral sugars commonly found in the host plant's cell wall [3]. Until now, synthetic fungicides are used as primary means to control *Fusarium* wilts. However, today a number of synthetic fungicides have been found to cause adverse effects to humans and the environment. Methyl bromide is an example. It is a critical fumigant for both pre-plant and post-harvest management of *Fusarium* wilt

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diseases, which has been defined by the Montreal Protocol of 1991 as a chemical that contributes to the depletion of the ozone layer [4]. Moreover, traditional fungicides are becoming ineffective due to the development of new physiological races of the pathogens [5]. These lead to the urgent need for development of novel fungicides that are more effective and safer than the conventional fungicides.

Antifungal compounds from plant origins are of the most promise due to their being less toxic and more environmentally compatible by nature [6]. During the past decades, many plants have been extracted and screened for antifungal activities. Essential oils are among the plant derived natural products that have been reported to possess desirable bioactivities including fungicidal activities [7]. Ajowan is one of the aromatic seed spices, which is generally used for medicinal purposes as a digestive stimulant or to treat liver disorders. Thymol, the major phenolic compound present in Ajowan, has been reported to be a germicide, antispasmodic, and antifungal agent [8]. The aim of this study was to determine the *in vitro* efficacy of ajowan essential oil against three distinct phytopathogenic strains *Fusarium oxysporum*.

2. Materials and Methods

2.1 Fungal strain

The phytopathogenic strains of *Fusarium oxysporum*, *F. oxysporum* f.sp. *lycopersici*, *F. oxysporum* f.sp. *cubense* and *F. oxysporum* f.sp. *capsici* were obtained from the Plant Pathogen Collection of the Applied Biology Program, Phranakhon Si Ayutthaya Rajabhat University (Thailand). Fresh cultures of the strains were prepared by transferring conidia suspension from deep freeze culture on to potato dextrose agar (PDA) plates. The working cultures were sub-cultured monthly and stored on PDA slant at 4ºC.

Spore suspensions of *F. oxysporum* were prepared by transfer an agar plug of active growing mycelia into Bilay and Joffe's medium [9] with minor modifications (4.0g CMC-Na instead of 15g CMC (carboxymethyl cellulose), incubated on a rotary shaker at 100 rev·min^{-1} for 7 days . Broth containing conidia was serially diluted to 10^5 conidia·mL^{-1} of suspension.

2.2 Plant material and extraction of essential oil

The extraction of Ajowan (*Trachyspermum ammi* Lin.) oil from mature seeds was performed based on hydro-distillation using a modified Clevenger type-apparatus [10]. The seeds were ground to powder and 500g of seed powder were weighed and filled in a round bottom flask. An aliquot volume of distilled water was added to cover the material. The distillations were performed at a temperature around 100ºC for 4 hours. Oil distillates assembled in the reservoir were collected, dried with saturated anhydrous sodium sulfate and stored in a refrigerator at 4ºC.

2.3 Poisoned food assay

The contact antifungal activity of ajowan oil was evaluated using poison food method as described previously by Tripathi *et al.* [1]. Aliquot (20ml PDA) was poured into sterilized Petri plates and a measured amount of oil was added to obtain a series of two fold concentrations (30, 60, 120, 240, 480 and 960 µg·mL^{-1}). Tween-80 (0.05%) was also added for even distribution of the oil in the medium. For control sets, the medium was supplemented with the same amount of distilled water and 0.05% Tween-80 instead of oil. After solidification, a 6 mm well was made in the center of each PDA plate using a sterile cork borer and a 6 mm mycelia plug of the test fungus was then aseptically inserted into the well. Plates were incubated at 25 ± 1°C for 5 days. The antifungal activities were determined by means of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) which were defined as the lowest concentration that completely

inhibited growth of the fungal mycelia and the lowest concentration that totally killed the fungal inocula respectively. Experiments were done in triplicate.

To determine whether the fungal inocula was totally killed, inhibited mycelia plugs were removed from poison food plates, immersed in sterile distilled water to remove residual oil, and then reinoculated separately into Petri plates containing fresh medium. Plates were incubated at 25 ± 1°C. The revival of the fungal inocula was observed after 5 days. The fungal inocula were assumed to be killed totally if they fail to revive on the fresh medium.

2.4 Volatile activity assay

Antifungal effect of the volatile vapor of Ajowan oil was determined based on volatile activity assay in invert Petri plate (50 mm diameter) [1]. An aliquot (5 mL of PDA) were poured into each Petri plate. After solidification, an aliquot (30 µL spore suspension) of each fungal strain was dropped separately on to the center of each Petri plate and allowed to dry. The plates were then inverted and a sterile paper disc was placed on the lid of each inverted plate. Different doses of essential oil (6, 12.5, 25, 50 and 100 µl) were applied on to the paper disc. For the control set, an equal volume of Dimethyl sulfoxide (DMSO) was applied on the paper disc instead of essential oil. All the inverted plates were incubated at 25 ± 1ºC in an environmental control chamber for 7 days. The antifungal activity was expressed in terms of MID and MFD which was defined as the lowest concentration that completely inhibited the fungal growth and the lowest concentration that totally killed the fungal inocula respectively. The killing effect was determined as previously described in 2.3.

2.5 Effects of ajowan oil on biomass production

To determine the effect of essential oil on the fungal biomass production, four different concentrations of ajowan oil (0, ½MIC, MIC and MFC) in 50 mL Potato Dextrose Broth (PDB) were prepared in conical flasks and inoculated with each strain of *Fusarium oxysporum* to the desired concentration of 10^6 cfu·mL^{-1}. The flasks were incubated on a rotary shaker with 120 rev·min^{-1}. After 30 days dry weight of mycelium was determined. Flasks containing mycelia were autoclaved and subsequently filtered through No.1 filter papers (Whatman). The mycelia were washed several times with distilled water and allowed to dry at 40°C overnight. The filter paper containing dry mycelia were weighed. Percent growth inhibition based on dry weight was calculated as:

$$\% \text{ inhibition} = \frac{w_o - w}{w_o} \times 100$$

Where r_0 and r are dry weight of control and dry weight of sample, respectively.

2.6 Anti-sporulation activity of ajowan oil

Four different concentrations of ajowan oil (0, ½MIC, MIC and MFC) in 50 mL Bilay and Joffe's medium were prepared in conical flasks. A 6 mm mycelia plug of the test fungus was inoculated into each flask. The flasks were incubated on a rotary shaker with 120 rev·min^{-1} for 7 days. Conidia density was determined using a hemacytometer.

3. Results and Discussion

The antimicrobial potential of plant essential oil has long been recognized, and numerous researchers reported that aromatic medicinal plants are good sources of essential oils. In this study ajowan, an aromatic plant of the family Umbelliferae, was evaluated *in vitro* against *F oxysporum*. Ajowan oil was extracted from mature seeds of the plant by hydro-distillation and the oily fraction (ajowan oil) obtained was clear, yellowish and rich in aroma. The specific gravity of the oil was determined to be *ca*. 0.981 g·mL^{-1}.

A series of bioassays were conducted on the oil. Poison food based bioassay was carried out to determine the antifungal activity of ajowan oil solution (contact activity). It was observed that ajowan oil strongly inhibited the growth of fungal strains. The MIC and MFC of the essential oil were determined to be 240 and 480 µg·mL^{-1}, respectively (Table 1 and Figure 1B).

Table 1 *In vitro* antifungal activity of ajowan oil against the strains of *F. oxysporum*

Fungal strains	Poison food		Volatile activity	
	MIC[a]	MFC[b]	MIQ[c]	MFQ[d]
F. oxysporum f.sp. *lycopersici*,	240	480	12.5	25.0
F. oxysporum f.sp. *cubense*	240	480	6	25.0
F. oxysporum f.sp. *capsici*	240	480	12.5	25.0

[a] Minimum inhibitory concentration (µg·mL^{-1})
[b] Minimum fungicidal concentration (µg·mL^{-1})
[c] Minimum inhibitory quantity (µL)
[d] Minimum fungicidal quantity (µL)

Volatile constituents of the essential oil may possess antimicrobial activities supporting their potential use as biofumigant against certain microbes in pre- or post-harvest preservation of economically important crops [11]. Therefore, in this study the antifungal activity of the volatile headspace of ajowan oil was also determined.

The results from Table 1 clearly indicates that the vapor headspace of ajowan oil possesses a strong inhibitory activity against the fungal strains tested since it completely suppressed the fungus at a relative low dosage. Under the experimental condition, the lowest quantity that completely killed the test fungus was determined to be 25 µL. Due to its highly volatile activity, ajowan oil should be considered as a novel biofumigant for pre- or post-harvest application.

The effects of ajowan oil on biomass production of the fungus were evaluated in liquid cultures. Results showed that the supplement of ajowan oil in culture medium caused considerable reduction of the fungal biomass (Table 2). It should be noted that supplement of ajowan oil at the MIC level (240 $\mu g \cdot mL^{-1}$) did not completely suppress the biomass production. It is thus reasonable that a little growth the fungus should be detected during longer incubation times.

From the anti-sporulation assay, ajowan oil at the level ½MIC and MIC sharply reduced the sporulation of the test fungus as compared to the control (Table 2 and Figure 1C). At the MFC level the disappearance of *Fusarium* conidia was observed. However this may be due to the fungicidal effect rather than the anti-sporulation effect of ajowan oil.

All the above results represented the prominent antifungal activities of ajowan oil against representative strains of *F. oxysporum.* The values of antifungal indices in Table 1 suggest that ajowan oil affected all the fungal strains to a similar degree (not strain dependent). This leads to the assumption that the active constituents of ajowan oil would cause disruptive effects on the non-strain specific physiological components of *F. oxysporum*. However, this phenomenon should be intensively studied in further research.

Previous researches have elucidated that Ajowan oil constitutes of thymol as the major component, *ca.* 40-60 % of its whole composition [12-13]. The antimicrobial activity of essential oils is affected by their composition**.** Thus it is highly possible that thymol is responsible for the antifungal activity of the oil. Ajowan oil has been reported to possess nematocidal [11] and insecticidal [12] activities. However, reports on the antifungal activity of ajowan oil are relatively rare, especially against phytopathogenic strains of *F. oxysporum*.

Table 2 Effects of ajowan oil on biomass production and sporulation of *F. oxysporum*

Fungal strains	Conc. of oil ($\mu g \cdot mL^{-1}$)	Biomass		Sporulation
		Dry weight[a] (mg)	% inhibition	($cfu \cdot mL^{-1}$)
F. oxysporum f.sp. *lycopersici*,	0	662.33	0.00	3.73×10^6
	120[b]	267.00	59.69	2.20×10^6
	240[c]	118.67	82.08	8.00×10^5
	480[d]	6.33	99.04	0
F. oxysporum f.sp. *cubense*	0	673.00	0.00	3.85×10^6
	120	263.33	60.87	1.83×10^6
	240	80.67	88.01	6.25×10^5
	480	4.00	99.41	0
F. oxysporum f.sp. *capsici*	0	706.00	0.00	4.15×10^6
	120	279.67	60.39	1.80×10^6
	240	110.33	84.37	8.00×10^5
	480	0.00	100.00	0

[a] means of triplicate samples
[b] the concentration of ½MIC
[c] the concentration of MIC
[d] the concentration of MFC

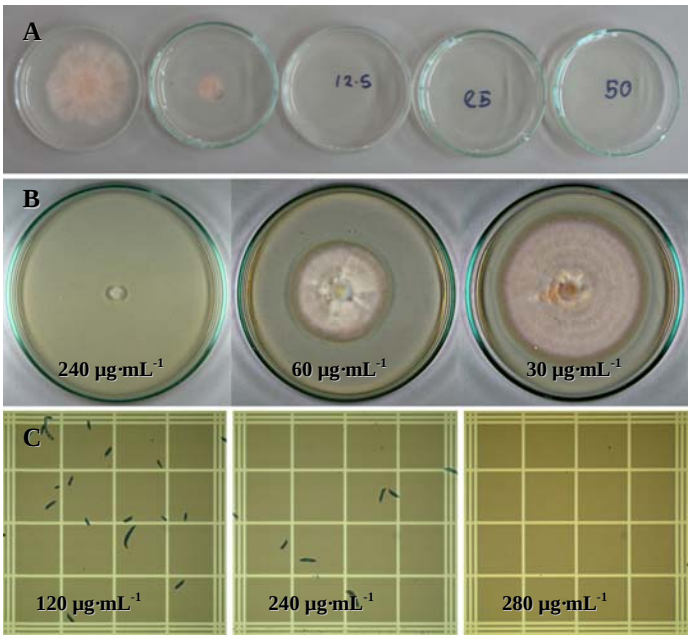

Figure 1 Antifungal activities of ajowan oil against *F. oxysporum* f.sp. *lycopersici*, (A) volatile activity assay, (B) poison food assay, (C) anti-sporulation effects.

4. Conclusions

In this study essential oil extracted from mature seeds of ajowan was evaluated *in vitro* against representative strains of three different *formae speciales* of *F. oxysporum*. All the results obtained indicate that ajowan oil is toxic to the fungi, either in solution or gaseous phases, and thus has potential use as an alternative to conventional fungicides for the control of *F. oxysporum*.

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