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EVALUATION OF PLANT AQUEOUS EXTRACTS FOR THEIR ANTIFUNGAL ACTIVITY AGAINST CERTAIN PHYTOPATHOGENIC FUNGI

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ABSTRACT

Aqueous extracts from different organs of 36 plant species were evaluated for their antifungal activity against *Fusarium oxysporum* f. sp. *niveum*, *Rhizoctonia solani*, and *Botrytis cinerea*. The extracts of eight plants, clove, garlic, jojoba, liquarice, onion, pepper, rosselle and thyme highly suppressed the mycelial growth of the three tested fungi as well as the spore germination of *B. cinerea*. The reduction in mycelial growth ranged from 88 to 100%. Results revealed that mycelial growth was completely inhibited when tested fungi were exposed to garlic extract for 15 min or clove extract for 2 hr. Emergence of watermelon seedlings in soil infested with *F. oxysporum* f. sp. *niveum* was 100% when the soil was treated with clove or garlic extract. The same results were obtained when tomato seedlings grew in soil infested with *R. solani*. Scanning electron microscopy of *R. solani* mycelia treated with garlic, liquarice, rosselle and thyme extracts showed collapse of the hyphae and shriveling of their cell walls.

Keywords: Biocontrol, Tomato damping off, Watermelon wilt, Clove, Garlic.

INTRODUCTION

The American National Academy of Sciences (NAS) reported that the carcinogenic risk of fungicides' residues in food is more than that of insecticides and herbicides together (Research council, 1987). Therefore, plant pathologists seek efforts to find and use alternatives to synthetic fungicides. Present activities focus on finding compounds that are safe to human and environment (Wilson *et al.* 1997)

Plant extracts (Bianchi *et al.* 1997 and Wilson *et al.* 1997) and essential oils (Daouk *et al.* 1995; Zambonelli *et al.* 1996 and Wilson *et al.* 1997) show antifungal activity against a wide range of plant pathogenic fungi. Wilson *et al.*, (1997) described a rapid assay to determine the antifungal activity in plant extracts and essential oils. Among the 49 essential oils tested, palmarosa (*Cymbopogon martini*), red thyme (*Thymus zygis*), cinnamon leaf (*Cinnamomum zeylanicum*), and clove buds (*Eugenia caryophyllata*) demonstrated the highest antifungal activity against *Botrytis cinerea* (Wilson *et al.* 1977). Bianchi *et al.*, (1997) assessed the action of garlic on the ultrastructure of fungal cells. They proved that the series of modifications induced by the garlic extract on the tested fungi; *Pythium ultimum* var. *ultimum* and *Rhizoctonia solani* were similar to those observed during treatments with sterol-biosynthesis-inhibiting fungicides.

The objectives of this study were to assess *in vitro* the antifungal activity of certain plant aqueous extracts and to observe any morphological

alterations caused by the most effective extracts, as the *in vitro* tests indicates, on the fungal mycelia. Moreover, part of this study was directed to investigate the potential utility of these extracts in controlling the plant diseases caused by the tested pathogenic fungi.

MATERIALS AND METHODS

Test Fungi

Fusarium oxysporum f. sp. *niveum* (E.F. Smith) Snyder & Hansen, *Rhizoctonia solani* Khun, and *Botrytis cinerea* Pers. Ex. Fr., were the pathogenic fungi used in this study. These fungi were isolated from watermelon seeds cv. Giza 1, tomato seeds and cucumber fruits, respectively. The isolated fungi were purified either by single spore isolation or hyphal-tip technique. Identification of the purified fungi was accomplished on the basis of cultural and microscopical characteristics. The key given by Booth, (1971) was followed for the identification of the *Fusarium* isolates. *Rhizoctonia* were identified according to the key given by Sneh *et al.* (1994). The basis described by Harrison, (1983) was followed to identify *Botrytis* isolates. A preliminary pathogenicity test was carried out, and the most pathogenic isolate of each isolated fungal species was chosen for further studies.

Preparation of plant aqueous extracts

Plant materials used in this study are listed in table (1). These materials were either collected dry, or dried in an oven at 40 °C for 4-5 days. Then, milled to fine powder. The aqueous extracts were obtained with the following procedure: 20 g of the tested powder were placed in 100 or 150 ml distilled water. The suspensions were shacked for 30 min, and then left for 24 h at 4°C. Centrifugation was applied at 2,000 rpm for 5 min. They were filtered through Whatman no. 1 filter paper, vacuum-filtered using Seitz filter under sterilized conditions. The extracts were kept at -4°C until used.

In vitro tests

(a) Effect of plant aqueous extracts on the mycelial growth

Potato Dextrose Agar (PDA) was prepared. Each 9.0 ml of the media were placed in a test tube and autoclaved. Three ml of the sterile plant extracts were added to the PDA at 40-45 C, and mixed well. Control plates were performed without extracts. The tested fungi were inoculated by using a disk of mycelial mat (0.5 cm in diameter), in the center of each dish. The mats were taken from the growing margin of 7-day-old cultures of the tested fungus. Each treatment was triplicated. Plates inoculated with *F. oxysporum* f. sp. *niveum*, or *R. solani*, were kept at 25-30° C, whereas those inoculated with *B. cinerea* were kept at 20° C. Inspections were made after 1, 2 and 3 days for *R. solani*, and after 2, 4 and 6 days for both *F. oxysporum* f. sp. *niveum*, and *B. cinerea*. The linear growth of the tested fungus was measured. Fungitoxicity was expressed in terms of percentage of mycelial linear growth inhibition and calculated according to the formula of Pandey *et al.* (1982).

$$\text{Fungitoxicity \%} = \frac{dc - dt}{dc} \times 100$$

Where dc = average diameter of fungal colony in the control
dt = average diameter of fungal growth in plant extract treated plates

(b) Effect of the plant aqueous extracts on the spore germination

Plant extracts used in this study were chosen according to their inhibition potency to the mycelial growth of *B. cinerea*. The spore suspension was prepared by washing-off the fungal growth of the Ten-day-old *B. cinerea* cultures from the surface of PDA media, using sterile malt extract. The suspension was adjusted at $1X10^5$ spores/ml. Three ml of the sterile plant extract was mixed with 9 ml spore suspension and placed in a test tube. Sterile distilled water was used for control treatment. Each treatment was represented by four replicates. Direct observations on spore germination were made using a light microscope, 40x objective, after 12 and 48 h. Two microscopic fields were chosen at random for each concentration.

(c) The exposure period for causing fungicidal effect

Petri dishes containing 7-day-old cultures of the tested fungus were used in this study. Two sterile filter papers were placed on the mycelial growth of each culture. Three ml of the sterile plant extracts were poured on the filter papers to soak them. Fungal inocula; 0.5 cm in diameter mycelial felts, were taken from each treated plate after different periods of time; 15min-48h, and placed on PDA. Cultures were investigated every 12h and 24h and the time required to cause total inhibition to the fungal growth was recorded.

Scanning electron microscopy

The mycelial growth of *R. solani* on PDA was used in this study. Glass covers were partly dipped in the agar media. The treated fungal mycelium grew on the covers. After 24h, each glass cover was dipped in FAA solution for 3 min for fixation. The mycelium was then dehydrated in graded aqueous series of ethanol (50, 60, 75, and 100%). The fungal hyphae were coated with gold palladium, then examined. The examination was conducted using the Electron Microscopy, at Faculty of Science, Alexandria University. The serial number of the film in the archive was 164 and the photographs demonstrated in the result section had the following numbers: 016430, 016431, 016433, 016435 and 016436.

Effect of plant extracts on watermelon wilt and tomato damping off

Aqueous plant extracts showed high level of antifungal activity toward *F. oxysporum* f. sp. *niveum* and *R. solani*, namely; cinnamon, cloves, garlic, jojoba (seed cake), liquarice, onion, pepper, rosselle and thyme were selected to study their efficiency for controlling Fusarium wilt of watermelon and damping-off of tomato. Black plastic bags filled with 1 Kg of autoclaved soil were used in this study. *F. oxysporum* f. sp. *niveum* or *R. solani* were grown on sterilized barley grains kept in 250 conical flasks at room

temperature (20-25 °C) for two weeks, then the soil in the bags were infested by the tested fungi at the rate of 10 g /bag and watered daily for a period of 7 days. Then dried powder of the tested eight plants were separately added to bags at the rate of 20 g /bag. Bags containing autoclaved uninfested soil or infested soil without any plant extracts were used as controls. Tomato seedlings (5 seedling /bag) were transplanted in bags. Watermelon seeds (5 seeds/bag) were sown. Each treatment was represented with four replicates.

RESULTS

Effect of plant aqueous extracts

(a) Mycelial growth

The mycelial growth of the tested fungi was strongly inhibited by some of the tested plant extracts (Table 1). Cloves inhibited the radial growth of the three tested fungi, *F. oxysporum* f. sp. *niveum*, *R. solani* and *B. cinerea*, by 100%. Garlic extracts completely inhibited the radial growth of *F. oxysporum* f. sp. *niveum* and *B. cinerea* while caused 88% inhibition towards *R. solani*. Meanwhile, Roselle extract inhibited the radial growth of *R. solani* and *F. oxysporum* f. sp. *niveum* by 100% and the growth of *B. cinerea* by 50%. The other tested plant aqueous extracts showed varied degrees of mycelial growth inhibition which ranged from 5% to 77%.

(b) Spore germination

Inhibition of *B. cinerea* spore germination by the nine tested plants extracts was investigated; the results are presented in Table 2. Results revealed that all tested plant extracts completely inhibited the germination of *B. cinerea* spores up to 48 hours. On the other hand spores treated with Butcher's broom and colocynth were able to germinate after exposure to the plant extracts for 48 hr.

c) The exposure period for causing fungicidal effect

The minimum exposure period, required for the plant extract to cause inhibition of the fungal growth was recorded (Table 3). Results revealed that the time required for garlic extract to completely inhibit the growth of the three tested fungi was 15 min. Cloves gave the same inhibitory effect after 12 hr of exposure. The effect of the other tested extracts was less than that where they needed 48 h to cause 33 – 74 % mycelial inhibition of the tested fungi.

Scanning Electron Microscopy observations

R. solani treated with garlic, liquarice, roselle, and thyme extracts, showed morphological alterations of the hyphae (Fig. 1). Such alterations appeared as conspicuous collapse of the hyphae and shriveling of their walls.

Table (1): Effect of plant aqueous extracts on *in vitro* growth of three pathogenic fungi.

Common name	Latin name	Plant organ	Inhibition of mycelial growth (%)		
			F. oxysporum. f. sp. niveum	*R. solani*	*B. cinerea.*
Aniseed	*Pimpinella*	Fruits	05	10	12
Basil	*Ocimum*	Leaves	13	16	05
Butcher's	*Ruscus*	Rhizome	26	77	55
Cabbage*	*Brassica*	Leaves	67	50	25
Caraway	*Carum carvi*	Fruits	05	09	08
Carnation	*Diantus*	Shoot	23	55	54
Cinnamon	*Cimmondsia*	Bark	18	64	35
Cloves	*Eugenia*	Buds	100	100	100
Colocynth	*Citrullus*	Fruits	31	36	49
Coriander	*Corindrum*	Fruits	08	12	18
Eruca*	*Eruca sativa*	Leaves	28	41	36
Fennel	*Foenculum*	Fruits	00	00	00
Fog fruit	*Lippia nodiflora*	Whole	08	10	11
Garlic	*Allium sativum*	Bulbila	100	88	100
Jericho-	*Anastatica*	Shoot	30	31	35
Jojoba	*Simmondsia*	Leaves	30	20	00
	chinensis	Seed	60	38	69
		Seed	11	15	05
Liquarice	*Grlycyrrhiza*	Roots&L	67	71	64
Lupine	*Lupinus termis*	Seeds	21	08	50
Majoram	*Marjorana*	Shoot	18	20	09
Mastic tree	*Pistacia*	Dry gum	13	12	15
Nigella	*Nagella sativa*	Seeds	26	28	16
Olibanum	*Boswellia*	Dry	19	18	30
Onion	*Allium cepa*	Bulbs	43	45	22
Orange	*Citrus sinensis*	Pericarp	44	45	40
Parsely	*Petroselinum*	Leaves	23	32	22
Pepper	*Capsicum*	Fruits	43	59	41
Peppermint	*Mentha*	Leaves	22	36	08
Pomegranat	*Punica*	Pericarp	22	18	24
Porrum-leek	*Allium porrum*	Leaves	31	33	20
Radish*	*Raphanus*	Roots&L	43	41	21
Rosselle	*Hibiscus*	Flowers	100	100	50
Common	*Rosmarinus*	Leaves&	18	28	28
Sage	*Salvia*	Shoot	18	28	28
Senna*	*Cassia senna*	Leaves	14	09	11
Thyme*	*Thymus*	Leaves&	50	61	29
Tooth brush	*Savadors*	Branche	20	12	00

*20 g milled dried plants were placed in 150 ml water.

Table (2): The effect of nine tested plants extracts on spore germination of *Botrytis cinerea:* (+) spore germination, (-) no spore germination.

Plant extract	Spore germination	
	12 hr	48 hr
Burcher's broom	-	+
Carnation	-	-
Cloves	-	-
Colocynth	-	+
Garlic	-	-
Jojoba (seed cake)	-	-
Liquarice	-	-
Lupine	-	-
Rosselle	-	-

Table (3): The inhibitory effect of the tested plant extracts on mycelial growth of the tested fungi.

Common name	Exposure time*	% Inhibition		
		F. oxysporum f. sp. *niveum*	*R. solani*	*B. cinerea*
Cinnamon	48 hr	33	42	45
Cloves	12 hr	100	100	100
Garlic	15 min	100	100	100
Jojoba (seed cake)	48 hr	70	52	64
Liquarice	48 hr	74	69	69
Onion	48 hr	46	55	48
Pepper	48 hr	57	55	53
Rosselle	48 hr	83	74	66
Thyme	48 hr	66	68	60

*** Time of exposure to plant extracts.**

Effect of plant extracts on watermelon wilt and tomato damping off

Data presented in Table 4 revealed that the tested fungi were pathogenic to their corresponding host plants. Data also revealed that plant aqueous extracts added to the infested soil increased the percentage of the emerged watermelon and tomato seedlings comparing with the control. The highest emergence was that in soil treated with cloves and garlic (100%). Jojoba and thyme gave 72 and 93% watermelon emergence, respectively; while liquarice, pepper and rosselle increased the emerged tomato seedlings by 100, 95 and 72%, respectively.

DISCUSSION

Natural fungicides that exist in plants and microorganisms include examples that would serve as safe and effective alternatives to synthetic fungicides.

The present study showed that mycelial growth of *Rhizoctonia solani* was more sensitive to the tested extracts than these of *F. oxysporum f. sp. niveum* and *B. cinerea* the most effective plant extracts showing antifungal activity among the tested 36 plant extracts were species of cloves, garlic, thyme and Rosselle. The aqueous extracts of four plants highly inhibited the mycelial growth of the tested pathogenic. Spore germination of *B. cinerea* was inhibited by extracts of Bucher's broom, carnation, cloves, colocynth,

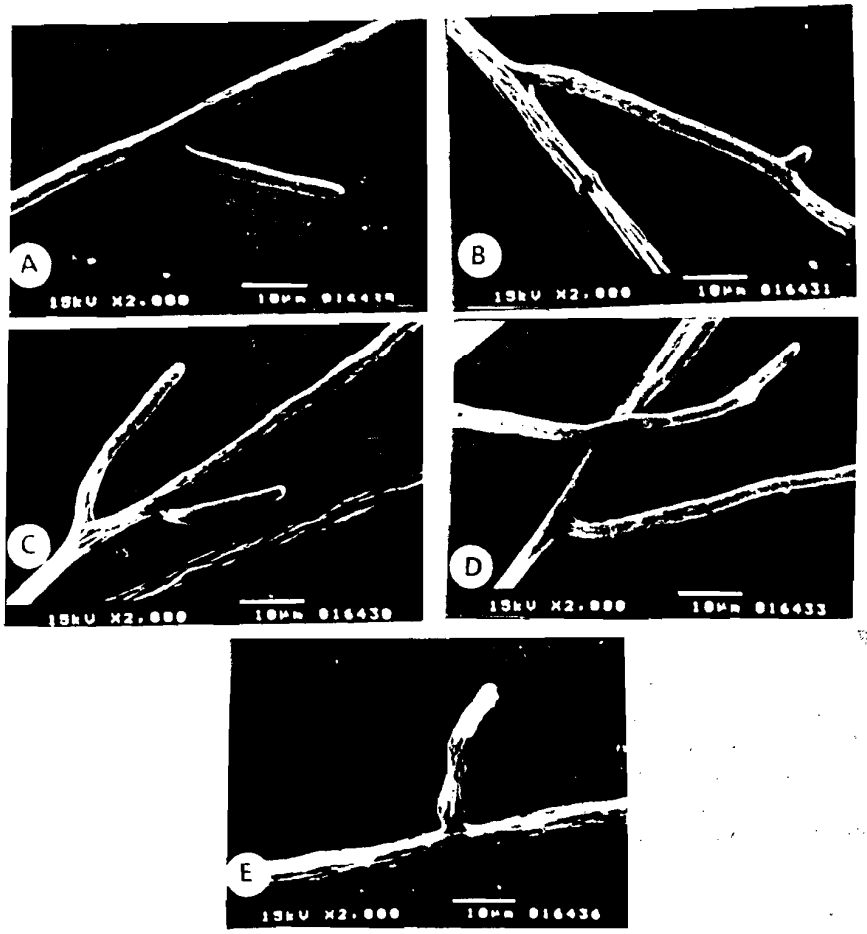

Fig (1): Morphological alteration of *Rhizoctonia solani* due to certain plant extracts. Normal untreated (control) hyphae (A), and collapsed hyphae treated with garlic (B), Liquarice, (C), Rosselle (D) and thyme (E) extracts.

garlic, jojoba, liquarice, lupin and Rosselle. As biocontrol agents, extracts of clove buds and garlic bulbils greatly increased the percentage of emerged seedling of watermelon and tomato growing in soil infested with *F. oxysporum* f. sp. *niveum* and *R. solani*, respectively. Extracts of liquarice, pepper and Rosselle increased the percentage of emerged tomato seedlings. These results are partially in agreement with researchers who recommended garlic extract and garlic powder to reduce the mycelial growth and/or spore germination of certain pathogenic fungi and to control diseases caused by them (Carcia and Lawas, 1990; El-Shami *et al.*., 1985; Singh *et al.* 1995, Bianchi *et al.* 1997 and Mohamed, 2001).

Table (4): The effect of the tested plant extracts on percentage of emerged seedlings of watermelon and tomato.

Treatment	Emerged seedlings (%)*	
	Watermelon	Tomato
Autoclaved soil (control 1)	95	90
Infested soil (control 2)	22	20
+ Cinnamon	42	42
+ Cloves	100	100
+ Garlic	100	100
+ Jojoba (seed cake)	72	21
+ Liquarice	51	100
+ Pepper	30	95
+ Rosselle	35	72
+ Thyme	93	42

* Percentage of emerged seedlings in soil artificially infested with *F. oxysporum* f. sp *niveum* or *R. solani.*

The antifungal effect of garlic extract is attributed to the formation of the high sulfur containing compound ajoene [(E,Z)-4,5,9- trithiododeca-1,6,11- trien-9- oxide]. Ajoene, also has high antibacterial, antiviral and antiprotozoal activities (El Shami *et* al, 1985; Singh *et al.*, and O'gara *et al.*,, 2000). Hippe (1991) detected certain morphological modifications in pathogenic fungi as a result of treatment with garlic powder or its extracts. These cytomorphological modifications are undulations of plasma lemma, accumulation of lipidic osmiophil bodies and thickening of cell walls. Such modifications are similar to those observed in certain fungal species treated with sterol-biosynthesis- inhibiting fungicides (Hippe, 1991).

It was also suggested that the sulfur-reduced compounds present in garlic might act as groups binding with sulfhydrelic groups of essential amino acids, proteins and enzymes (Barone and Tansey, 1977)

Reports attributed the fungicidal effect of thyme, on mycelial growth and spore germination of *Aspergillus niger*, to the presence of the essential oil , thymol which belongs to the phenolic alcohols (Hitoko *et al.*,, 1980; Karapinar, 1990; Antonov *et al*, 1995, Arras *et al.*,, 1995; Riebau *et al.*,, 1995 and Zambonelli *et al.*,, 1996). The high antifungal activity of thymol oil is probably a result of chitin penetration of the hyphal wall which damages the lipoprotein cytoplasmic membranes, leading to escape of cytoplasm, as well as, the emptying and squashing of the hyphae (Zambonelli *et al.*,, 1996).

Some other authors (Sancholle *et al.*,,1988 and Yang and Anderson, 1999) attributed the fungicidal effect of certain plant extracts to their enzymatic activity or their capacity to interfere certain metabolic processes leading to toxification of the pathogen.

The essential oil eugenol, which is chemically related to terpenoids, is a product of clove. It has wide spectrum as antimicrobial agent. The mechanism of terpens and their derivative, terpenoids, is not fully understood but it is speculated to involve membrane disruption by the lipophilic compounds (Cowan, 1999).

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تأثير المستخلصات المائية لبعض النباتات على نشاط بعض الفطريات الممرضة للنبات

وفاء القفاش و أسامة المنوفى

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أجريت التجارب على المستخلصات المائية لعدد ٣٦ نوع نباتى لدراسة تأثيرها على نمو الفطريات فيوزاريوم أوكسيسبورم نيفيام، رايزوكتونيا سولانى وبوترايتس سينيريا. بينت النتائج أن المستخلصات المائية لبراعم القرنفل، بصيلات الثوم، أوراق وكسب الهوهوبا، جذور وسوق العرقسوس، أزهار الكركدية وأوراق الزعتر تثبط نمو الفطريات المختبرة بنسبة تراوحت بين ٨٨ و ١٠٠%، كما تمنع إنبات جراثيم الفطر بوترايتس سينيريا منعا تاما. كذلك دلت النتائج على أن تعريض ميسيليوم الفطريات المختبرة لمستخلص بصيلات الثوم لمدة ١٥ دقيقة أو مستخلص براعم القرنفل لمدة ١٢ ساعة أدت إلى قتل الفطريات المختبرة بما يمنع نموها تماما. وأفادت مستخلصات بصيلات الثوم وبراعم القرنفل فى تحسين إنبات بذور الشمام والطماطم فى تربة معدية بالفطر فيوزاريوم أوكسيسبورم نيفيام والفطر رايزوكتونيا سولانى على التوالى حيث كانت نسبة الإنبات ١٠٠% مقارنة بالبذور النابتة فى تربة غير معدية بالفطريات المختبرة. بينت الفحوص باستخدام الميكروسكوب الإلكتروني الماسح أن هيفات الفطر رايزوكتونيا سولانى المعاملة بمستخلصات الثوم والعرقسوس والكركدية والزعتر تؤدى إلى حدوث انقباضات واضحة فى الهيفات وانكماش و تجعيد فى جدرها.