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Research Paper

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« *In vitro* evaluation of essential oil and water extracts from some medicinal and aromatic plants on colony growth of some soil and seed borne plant pathogens »

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Abstract: The effects of essential oil extracts from Lemongrass (cymbopogon citratus), Spearmint (Mentha spicata), and Basil (Ocimum gratissimum) and water extracts from Neem (Azadirachta indica), Argel(Solanostemma argel) and Bitter leaf (Vernonia amygdlina), after mixing with artificial medium at three different levels (10%, 20%, and 30%) in vitro against the well-known chickpea pathogens; Fusarium oxysporum. f. sp. ciceris. Fusarium solani. f.sp. ciceris and Alternaria alternata. The colonies growth was significantly reduced, of course with slight variation of the effectiveness of extracts according to their concentration used. According to the results of these studies, the extracts from the above mentioned medicinal and aromatic plants have the significant ability to inhibit the colonies growth of the different studied fungi. Argel and Neem aqueous extracts were found to be the more effective than the others, while Bitter leaf extract had moderate effect on the colonies growth of the above mentioned fungi.

Keywords: *Essential oils and Aqueous Extracts, Medium, Colonies, Fungi.*

Introduction:

At the beginning of the 21st century, humanity is facing challenges regarding food safety in relation to climatic changes and the energy crisis. Contrary to earlier predictions, the prices of agricultural commodities are increasing dramatically due to a shortage of production. The world's population is continuously growing and agricultural production must increase to ensure global access to safe food in sufficient amounts. At the same time, the use of fertilizers and chemical pesticides must be reduced to minimize the deleterious environmental impact of agriculture. In this context, there is a renewed interest in alternative approaches to pest, disease and weed control (Muller et al., 2008 and Toth et al., 2008).

Chickpea (Cicer arietinum L.) is the world's third most important pulse crop, after dry beans (Phaseolus vulgaris L.) and dry peas (Pisum sativum L.) (Vishwadhar and Gurha, 1998). Although, chickpea is predominantly consumed as a pulse, dry chickpea is also used in preparing a variety of snack foods, sweets and condiments and green fresh chickpeas are commonly consumed as a vegetable. It is a food crop of economic importance to human in the Sudan especially in the northern states. However, Chickpea (C. arietinum) is an annual leguminous winter crop that grows in several types of soils other than alkaline soil with bad drainage and sandy soils with good drainage are suitable for its cultivation. Its yield ranges between 0.83 and 2.8 t/ha, depending on weather conditions. Optimization of crop yields is a necessity to maintain its rank in the existing cropping system. Constraints that contribute to low productivity of chickpea include poor cultural practices done by farmers, lack of high yielding cultivars; stress inflicted by harsh environmental conditions, particularly high temperatures; diseases; insect pests; weeds and irrigation (Salih.et al., 1996). Fungal diseases are known to cause great damages all over the world. Different species of Alternaria, Aspergillus, Ceratobasidium, Cercospora, Cochliobolus, Curvularia, Drechslera, Fusarium, Gaeumannomyces, Microdochium, Penicillium, Pyricularia, Pythium, Rhizoctonia, Rhizopus, Sclerophthora, Trichoderma and Tricococcinella are most common associates of seeds all over the world, causing pre and post-infections and considerable quality losses viz. seed abortion, seed rot, seed necrosis, reduction or elimination

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of germination capacity, seedling damage and their nutritive value have been reported (Kavitha et al., 2005).

Most of chickpea cultivated varieties are susceptible to wilt and /or root rot disease complex. It is difficult to manage this disease either through crop rotation or application of chemicals because of soil nature persistence and its capacity to survive for long time even in the absence of host. Biological control provides an alternative to the use of synthetic fungicides with the advantages of greater public acceptance and reduced environmental impact (Reino et al., 2008). The use of natural products in disease prevention and control has received attention in recent years (Sati and Joshi, 2011). The medicinal plants have been recognized for their antimicrobial activity for many3 years. Touba and Zakaria, (2011) reported that all the seven spices studied viz. cardamom, chilli, coriander, onion, garlic, ginger, and galangale were showed significant anti-fungal activity at three concentrations (10%, 20% and 30%) of the crude extracts used against three Roselle pathogens i.e. Phoma exigua, Fusarium nygamai and Rhizoctonia solani using the 'poisoned food technique'.. In vitro the effects of medicinal and aromatic plant extracts Neem (A. indica) leaves, Argel (S. argel) shoot, Bitterleaf (V. amygdalina) leaves, Lemongrass (C. citratus) upper parts of the plant, Spearmint (M. spicata) shoots and Basil (O. gratissimum)) on colony growth of the pathogenic fungi were studied i.e , F. oxysporum. f. sp. ciceris. F. solani. f.sp. ciceris and A. alternata.

Materials and methods:

Isolation, identification and multiplication of the pathogens

Isolation of pathogens:

Chickpea (Cicer arietinum) diseased specimens of wilt and/or rot were collected from Hudeiba and Merawi areas at the Nile river and the Northern States respectively, in the Northern Sudan. The organisms associated with the diseased material were isolated by the standard isolation procedure (Riker and Riker, 1936). Isolations were made from rotted roots and wilted leaves and stalks of naturally infected Chickpea plants. The roots and stalks were thoroughly washed under running tap-water to remove the adhering soil particles, and left to dry for 10 – 20 minutes. Infected plant roots and stalks were cut into small pieces of 3 mm in length. These pieces were surface sterilized with 1% sodium-hypochlorite (Chlorine) for two minutes followed by two washings with sterilized distilled water. Then, placed on sterilized Petri plate containing filter paper to absorb the access of moisture. Finally, these pieces were placed in Petri plates containing wetted filter papers, and left under room temperature for 3 – 4 days. The hyphal tips of the fungal colonies growing from the plated material were transferred to Petri-plates containing potato-dextrose agar medium (PDA). The medium was already steam sterilized in the autoclave at 1.1 kg.cm-2 for about 15 minutes. To avoid bacterial contamination, streptomycin sulphate (1:1000) was added to the medium after sterilization and before pouring.

Re-isolation of the pathogens:

Suspension stocks should be regularly maintained by re-isolating the fungi from plants of highly susceptible accessions to avoid loss of pathogenicity. Suspensions (1x106 conidia /ml by using a haemocytometer were prepared in autoclaved distilled water for inoculation.

Pathogenicity Test:

Pathogenicity test was conducted using Koch's Postulates to confirm the Fusarium species as the causal agents of wilt and/or root rot of Chickpea. It was carried out both under in vitro and pot culture studies. The Fusarium isolates used represent the isolates, which were successfully isolated and identified from the roots and stalks of infected Chickpea plants. The inocula of the two Fusarium species were mixed together4 and tested for their pathogenicity on apparently healthy Chickpea plants. The roots of the uprooted healthy Chickpea plants were washed and surface sterilized before inoculation. The spore suspension used, was adjusted to 1x106 cfu /ml by using a Haemocytometer. The roots of the uprooted healthy plants (7-10 days old) were soaked in a mixture of 20 milliliters (10 milliliters from spore suspension of each of the two Fusarium spp.) on a petri-plate for 30 minutes. The roots of the uprooted control plants were soaked in 20 milliliters of sterile distilled water. All the uprooted, treated plants were immediately grown in sterilized sandy soil and irrigated every three days with sterile distilled water. The treatments were replicated three(3) times in a completely randomized design in a green house. The disease symptoms were observed at 30, 45 and 60 days after inoculation.

Extracts Preparation:

The extracts of essential oils obtained from certain medicinal and aromatic plants viz, lemongrass (C. citratus). Basil (O. gratissimum L.), Spearmint (M. spicata) and other water soaked extracts from Neem leaves (A. indica), Argil (S. argel), and Bitter leaf (V. amygdalina) shoots and leaves were collected from the experimental farm and extracted in the laboratory of the Medicinal and Aromatic Plants Research Institute (MAPRI)-Khartoum. These essential oils and water extracts were used to test their antifungal activity against the above mentioned fungi in vitro.

Essential oils extraction:

Distillation of essential oils from Basil, Spearmint and lemongrass was carried out using the method described by (Sukhdev et al., 2008); Two hundred and fifty grams of the chopped plants were placed in 2000 ml rounded bottom capacity flask. 1000 ml of distilled water was added and the Clevenger receiver (lighter than water) and condenser attached to the top of the flask. The system was heated at 100ºC for about four hours till the volume of oil above water layer at the receiver was constant. The oil was pipetted, dried over sodium sulphate anhydrous and stored in a dark container in a refrigerator till used.

Preparation of the aqueous extracts:

Two hundred and fifty grams of the plant sample was soaked in 1000 ml hot distilled water placed in 2000 ml rounded bottom capacity flask, and left till cooled down with continuous stirring at room temperature. The extract was then filtered and freezed in a deep freezer. Freezed extract was dried using a freez dryer (Freez dryer, Trivac, USA) till powdered extract was obtained (Harborne, 1984).

The antifungal activities of these essential oils and water extracts against the above mentioned pathogens were tested in vitro at three different levels (concentrations) of 10, 20 and 30% respectively. The oil and water extracts were respectively, diluted with methanolic solvent and distilled water at constant rate on the base of (w/v) 3g into 100 ml of each solvent, and then blended with Potato Dextrose Agar (PDA) in the following5 concentrations (after sterilization of the medium), according to the previous work of Singh and Singh, (1997):

Actual concentrations used according to (w/v)

1- 10 ml of extracts made up to 100 ml of medium (10%).

(10*3)/100 = 0.3 gm

2- 20 ml of extract made up to 100 ml (20%).

(20*3)/100 = 0.6 gm

3- 30 ml of extract made up to 100 ml (30%).

(30*3)/100 = 0.9 gm

4- 100 ml of PDA medium used as control for each pathogen

Twenty ml of treated medium was poured in each sterilized Petri-plate. Suitable checks were maintained without addition of extracts. Five millimeters mycelial disc of actively growing inoculum of the six different isolates was taken from the periphery of 7 days old culture was placed in the plate centre and incubated at 28 ± 2ºC for one week for full growth of the fungus. Three replications were maintained for each treatment. The diameter of the colony growth was measured in two directions and the average was recorded.

Lab. studies:

Species of Fusarium oxysporum. f.sp. ciceris . Fusarium solani .f.sp. ciceris and Alternaria alternata were isolated from the collected specimens and maintained in pure media of potato dextrose agar (PDA) to study the antifungal activity of the extracts in vitro ; Neem (A. indica) leaves, Argel (S. argel) shoot, Bitter leaf (V. amygdalina) leaves, Lemongrass (C. citratus) upper parts of the plant, Spearmint (M. spicata) shoots and Basil (O. gratissimum) shoots and a fungicide Benomyl (Bioneel R) at the standard recommended dose (50gm/100L) and untreated control (with sterilized distilled water) used for comparison were added to Potato Dextrose Agar (PDA) medium in different concentrations (10%; 20% and 30%) separately in petri plates. Each plate was inoculated with a mycelial disc (5mm diameter) taken from 7-days-old culture raised on PDA. The plates were arranged in a completely randomized design. Three replications were maintained for each treatment. The inoculated plates were incubated at 28 ± 2°C for 7 days and the diameter of the colony growth of each pathogen in each case was measured. The Percent inhibition of each fungus at the three concentrations over control was recorded by using the formula given below by Vincent (1947):

$I = ((C-T) \times 100)/C$ Where; I = Percent inhibition, C = Growth in control (mm), and T = Growth in treatment (mm). The data shown in tables 1, 2 and 3 were analyzed by6 ANOVA and treatment means were separated by Duncan's Multiple Range Test (DMRT) using MSTAT program.

Results and Discussion:

In vitro the present study revealed significant inhibition in mycelial growth of the soil borne pathogenic fungi i.e, F. oxysporum. f.sp. ciceri . F. solani .f.sp. ciceri , A. alternata by all the medicinal and aromatic plants essential oil and water extracts viz; Neem (A. indica) leaves, Argel (S. argel) shoot, Bitterleaf (V. amygdalina) leaves, Lemongrass (C. citratus) upper parts of the plant, Spearmint (M. spicata) shoots, and Basil (O. gratissimum) shoots, showed significant response in reducing the colony growth of the above mentioned fungi as compared to the standard used fungicide (Benomyl at 50gm/100L). Among the essential oil and water extracts, Argel at 10% concentration, significantly, exhibited the maximum mycelial inhibition against the above mentioned fungi (Table 1). At 20% concentration of the extracts, Argel recorded the maximum mycelial growth inhibition percentage against the fungus; A. alternata (73.8%), while it was statistically at par with Neem and Basil (81.82%, 78.79%, and 78.79%), Lemongrass (76.57%, 78.14%) and all extracts except the Bitterleaf extract which recorded the least inhibition percentage against; F. oxysporum. f.sp. ciceri, F. solani .f.sp. ciceri, and A. alternate, respectively (Table 2). However, at higher concentration (30%) Argel inhibited the three foresaid pathogens to the maximum extent (83.3%, 82.81% and 76.19 %) and it was at par with all extracts except Bitter leaf extract which exhibited low to moderate inhibition percentages (14.29%, 69.7 %), and with Lemongrass and Spearmint (82.81%, 79.69 % and 76.56 %) against F. oxysporum. f.sp. ciceri, F. solani .f.sp. ciceri and A. alternata, respectively (Table 3). The results of this study were similarly confirmed by Touba and Zakaria, (2011) whom reported that all the seven spices studied viz. cardamom, chilli, coriander, onion, garlic, ginger, and galangale were showed significant anti-fungal activity at three concentrations (10%, 20% and 30%) of the crude extracts used against three Roselle pathogens i.e. Phoma exigua, Fusarium nygamai and Rhizoctonia solani using the 'poisoned food technique'

Table 1: Effect of extracts from certain medicinal and aromatic plants on colony growth of ***Fusarium oxysporum* , *Fusarium solani*** and ***Alternaria alternata*** at 10% conc. after seven (7) days of incubation at 28 ± 2º C in vitro.

Plant extracts	10% conc. (10*3/100)	***Fusarium oxysporum***		***Fusarium solani***		***Alternaria alternata***	
		Colony* growth Diam.(cm)	% control	Colony* growth Diam.(cm)	% control	Colony* growth Diam.(m)	% control
Neem	0.3gm	1.50 d	77.27	3.40 c	46.88	3.20 b	23.81
Argel	0.3gm	1.40 d	78.79	1.50 e	76.56	1.20 c	71.43
Spearmint	0.3ml	2.30 c	65.15	2.50 d	60.94	1.90 c	54.76
Basil	0.3ml	3.90 b	40.91	4.40 b	31.25	1.80 c	57.14
Lemongrass	0.3ml	2.20c	66.67	1.70 e	73.44	1.90 c	54.76
Bitterleaf	0.3gm	3.80 b	42.42	3.40 c	46.88	3.90 ab	7.14
Benomyl	0.3gm	1.20 d	81.82	1.50 e	76.56	1.20 c	71.43
Control		6.6 a	0.00	6.40 a	0.00	4.20 a	0.00
SE±		0.22		0.256		0.261	
LSD		0.671		0.78		0.791	

*Means based on average of three replicates and separated according to DMRT (Duncan´s Multiple Range Test). Means on the same column having the same letters are not significantly different.

Table 2: Effect of extracts from certain medicinal and aromatic plants on colony growth of ***Fusarium oxysporum* , *Fusarium solani*** and ***Alternaria alternata*** at 20% conc. after seven (7) days of incubation at 28 ± 2º C *in vitro*.

Plant extracts	20% conc. (20*3/100)	***Fusariumoxysporum***		***Fusarium solani***		***Alternaria alternata***	
		Colony* growth Diam.(m)	% contro l	Colony* growth Diam.(m)	% contro l	Colony* growth Diam.(m)	% control
Neem	0.6gm	1.40 cde	78.79	2.40 bc	62.50	1.50 bc	64.29
Argel	0.6gm	1.20 de	81.82	1.50 d	76.56	1.10 bc	73.81
Spearmint	0.6ml	1.80 c	72.73	2.20 c	65.63	1.50 bc	64.29
Basil	0.6ml	1.40 cde	78.79	2.50 bc	60.94	1.30 bc	69.05
Lemongrass	0.6ml	1.70 cd	74.24	1.40 d	78.13	1.70 b	59.52
Bitterleaf	0.6gm	3.30 b	50.00	2.70 b	57.81	3.80 a	9.52
Benomyl	0.6gm	1.00 e	84.85	1.20 d	81.25	1.00 c	76.19
Control		6.6 a	0.00	6.40 a	0.00	4.20 a	0.00
SE±		0.178		0.16		0.227	
LSD		0.54		0.483		0.687	

* Legend as in table 1

Table 3: Effect of extracts from certain medicinal and aromatic plants on colony growth of ***Fusarium oxysporum* , *Fusarium solani*** **and *Alternaria alternata*** at 30% conc. after seven (7) days of incubation at 28 ± 2º C *in vitro*.

Plant extracts	30% conc. (30*3/100)	***Fusarium oxysporum***		***Fusarium solani***		***Alternaria alternata***	
		Colony* growth Diam.(cm)	% control l	Colony* growth Diam.(m)	% control l	Colony* growth Diam.(m)	% control
Neem	0.9gm	1.30 cd	80.30	1.70 cd	73.44	1.11 b	73.81
Argel	0.9gm	1.10 cde	83.33	1.10 e	82.81	1.00 b	76.19
Spearmint	0.9ml	1.50 c	77.27	1.50 cde	76.56	1.20 b	66.67
Basil	0.9ml	1.30 cd	80.30	2.50 b	60.94	1.20 b	71.43
Lemongrass	0.9ml	1.20 cde	81.82	1.30 de	79.69	1.00 b	76.19
Bitterleaf	0.9gm	2.00 b	69.70	1.90 c	70.31	3.60 a	14.29
Benomyl	0.9gm	0.90 e	86.36	1.10 e	82.81	0.80 b	80.95
Control		6.6 a	0.00	6.40 a	0.00	4.20 a	0.00
SE±		0.127		0.168		0.21	
LSD		0.384		0.511		0.624	

*Legend as in table 1.

Conclusion and recommendations:

The research carried out of this experiment indicates that five-out of the six medicinal and aromatic plant extracts studied viz. Neem (Azadirachta indica) leaves, Argel (Solanostemma argel) shoot, Lemongrass (cymbopogon citratus) upper parts of the plant, Spearmint (Mentha spicata) shoots, and Basil (Ocimum gratissimum) shoots were showed significant anti-fungal activity at the three concentrations (10%, 20% and 30%) of the crude extract in vitro.

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