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Eco-friendly and Safe Role of *Juniperus procera* in Controlling of Fungal Growth and Secondary Metabolites

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Abstract

Radial growth *Aspergillus flavus* and *Fusarium oxysporum* was determined on medium amended with *Juniperus procera* methanolic extract. The radial growth of A. flavus and F. oxysporum was significantly reduced at 150 and 200 mg. The precentage of reduction was 16.55%, 48.54% and 48.64%, 59.86% for A. flavus and F. oxysporum, respectively. However, addition of *J. procera* extract to Carbomar significantly reduced radial growth compared with using Carbomar alone. On the other hand, with addition of the *J. procera* extract, the productivity percentage of aflatoxins B2, aflatoxins B1, sterigmatocystin, cyclopiazonic acid and fusaric acid was reduced by100, 67.44, 96.28, 60.33 and 8.36%, respectively as a result of applied *J. procera* extract. Moreover, the extract of *J. procera* significantly reduced the F. oxysporum colony-forming units (cfu) in the cultivated soil with *Raphanus sativus* at 5 days. F. oxysporum populations at 100 and 200 mg of *J. procera* extract were 25.33×103 and 21.33×103 cfu g-1, respectively. However, when added with Carbomar, extract of *J. procera* strongly reduced F. oxysporum populations (9.33×10^3 cfu g-1). In addition, the *J. procera* extract reduced the mean disease rating of wilt disease of *R. sativus* caused by F. oxysporum. Less content of chlorophyll a and b (3.56 and 1.65 mg/g fresh weight, respectively at $P<0.01$) was detected in infected *R. sativus* than treated with *J. procera* extract or Carbomar.

Keywords: Eco-friendly role; Fungal growth; *Juniperus procera*; Secondary metabolites

Introduction

Public pressure to minimize the use of chemical fungicides in agriculture has increased in the recent years [1]. The extensive use of chemical fungicides in plant protection against fungal disease generates long-term residues in food and in the environment [2,3]. Pesticide residues and development of resistant strains of fungi from the continuous application of pestcides are the major constraints to their use [4]. Various studies have confirmed the efficacy of plant extracts in the control of fungal diseases [5-12], with the view to countering obvious pollution problems in the environment and avoiding the toxic effects of synthetic chemicals on non-target organisms. Plant derived compounds possess a high potential for pest management since most of them are not phytotoxic, easily biodegradable and sometimes stimulatory to the host metabolism [13,14], and thus may have inhibitory effects on fungi and other microorganisms [15,**16**] as well as mycotoxin production [17-19]. *Juniperus* is one of the major genera of Cupressaceae family. It is estimated that 70 species of *Juniperus* are distributed throughout the world [20]. *Juniperus* species have been extensively investigated as a source of natural products with potential antibacterial, antifungal and insecticidal activities [21-25]. *J. procera* is used locally to treat tuberculosis and jaundice [26], intestinal worms and eye infections (Klaus and Adala), its wood is resistant to termite and rot [27]. Pankaj et al. [28] investigated the different fractions of *Juniperus* leaves and bark, it inhibit the growth of aflatoxigenic *Aspergillus flavus* and *A. niger*. Extracts from the aerial parts of *J. lucayana* were assayed against phytopathogenic fungus *Botrytis cinerea*. The hexane extract showed to have a higher antifungal activity than ethanolic extract [29]. Pirzada et al. [30] studied the effect of ethanol, methanol, ethylacetate, chloroform and aqueous extracts of *Juniperus* against the human pathogenic fungi (*Aspergillus niger* and *A. flavus*). Fungal contamination is undesirable because of the potential for mycotoxin production. *Aspergillus* genera are the most important toxigenic fungi [31]. Aflatoxins are a potent toxic produced as secondary metabolites by the fungus *A. flavus* [32]. Furthermore several other extrolites are produced by *A. flavus*, including cyclopiazonic acid, aspergillic acid and β-nitropropionic acid [33]. Antifungal agents extracted from plants could be exploited in controlling the growth of fungi consequently inhibiting aflatoxin formation [34].

Plant essential oils and their components have been known to exhibit biological activities such as antifungal [35]. Juniper essential oil was evaluated by Pepeljnjak [36] for the antimicrobial activity against sixteen bacterial species, seven yeast-like fungi, three yeast and four dermatophyte strains. Juniper essential oil showed bactericidal activities as well as a strong fungicidal activity against yeasts, yeast-like fungi and dermatophytes. Recently, Mariana and Camelia, [37] reported that Juniper oil has inhibition action against *Aspergillus niger, Fusarium oxysporum, Monascus purpureus* and *Penicillium hirsutum*. Glisic et al. [38] stated that the oil of *J. communis* with a high content of α-pinene, and mixture of α-pinene and sabinene showed the highest antimicrobial activity, especially against fungi. The purpose of this study was to determine whether the natural control of *A. flavus* and *F. oxysporum* and their mycotoxins could be achieved by applying *J. procera* extract and to compare them with the effect of chemical fungicide Carbomar.

Materials and Methods

Plant material and preparation of extract

The leaves of *Juniperus procera* were collected in January 2012 from Fifa Mountains, Jazan, Kingdom of Saudi Arabia (KSA). The plant sample was kindly identified by Dr. Abduo Marie, Associate Professor of Plant Ecology, Biology department, Faculty of science, Jazan University, KSA according to Migahid, [39] and Chaudhary, [40]. Freash leaves (500 g) of juniper (*J. procera*) were air-dried at room temperature under shade, and ground into powder using an electric grinder. Then extracted with methanol in a Soxhlet apparatus. The solvent was removed using rotary evaporator under reduced pressure at temperature below 50°C. The resulting crude extracts were stored at 4°C in dark until used.

Chemical fungicide: Carbomar (Methyl Benzimacold-2-ylcarbamate) was used as chemical fungicide. It was prepared in Gyangsoetrnal, China.

Fungal strains: *Raphanus sativus* seedlings showing symptoms of wilt were collected from farm in Minufiya governorate, Egypt, and transferred in ployethylene bags to the laboratory. The fungal pathogen was isolated from root tissues on potato dextrose agar (PDA) medium according to the growth requirements of the isolate at 25°C for 5 days. The obtained Fusarium; *F. oxysporum*, was purified and identified using PDA media according to the morphological characteristics of the mycelia and spores as described by Domsch and Gams [41], Domsch et al. [42] and John and Brett as *F. oxysporum*. *Aspergillus flavus* strain was isolated from spoiled grains (*Zea mayse L.*) and identified according to Raper and Fennell [43].

Investigation of antimycotic activity

Inhibitory effect of *J. procera* extract on mycelial radial growth of test fungi was performed by placing 5 mm mycelia agar disks, cuts from the periphery of 5-day-old culture of the target fungi, in the centre of Petri dishes (9.0 cm diameter) containing PDA medium supplemented with 100,150 and 200 mg *J. procera* extract, 10 mg fungicide (Carbomar) and mixture of 200 mg *J. procera* extract with 10 mg Carbomar. Colony radius was measured after incubation period (8 days) at 27°C. The control sets were prepared subsequently using sterile distilled water instead of *J. procera* extract. The percent inhibition of the radial growth of the target fungi was calculated according to the following formula. Percent inhibition=(DC–DT)/DC×100, where DC is the colony diameter of the control sets and DT is the colony diameter of the treated sets. Diagnostic charitеria of *F. oxysporum* only were recorded at each treatment by using software for image analysis at Faculty of Science, Jazan university, Saudi Arabia.

Secondary metabolites analysis

After 10 days of incubation period, the growth medium (potato dextrose broth medium containing 200 mg of *J. procera*) of *A. flavus* and *F. oxysporum* containing extracellular metabolites was extracted twice with Chloroform/methanol (2:1 v/v), then concentrated and separated using High-Performance Thin-Layer Chromatography (HPTLC) techniques. Twenty microliters of the samples, with or without treatment, were applied to HPTLC plates (10 cm × 10 cm, 0.2 mm silica gel Merck 60 F 254 precoated plate; Merck Darmstadt, Germany) using CAMMAG LINOMAT 5 application system. The TLC plates were eluted for the detection of extracellular metabolites in toluene/ethyl-acetate/90% formic acid 5:4:1 (TEF). Once the runs had finished, the plates were observed under visible and ultraviolet at 254 & 365 nm illumination. Griseofulvin dissolved in chloroform/methanol 2:1 was used as standard in all cases and relative retention factor value (Rf value) to griseofulvin were calculated as Rfg. To identify the metabolites the absolute Rf and the relative Rf to griseofulvin (Rfg) were measured. Secondary metabolites were identified by descriptions in literature and comparison with the available standard was made [44-47]. Retention factor value (Rf value): The distance that the spot of a particular compound moved up on the TLC plate relative to the distance moved by the solvent front is called the retention factor or Rf value. The Rf values of individual secondary metabolites were calculated by following, Rf=Distance traveled by the compound/Distance travelled by the solvent. Quantification of secondary metabolites were done by comparing the Rf values and area % of secondary metabolite calculated using CAMMAG TLC scanner at Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University Cairo, Egypt. The percentage of Metabolite production inhibition was calculated using the following formula: (1 - Area of test sample/Area control)×100%.

Analysis of fungal fatty acids: Mycelium (5 g fresh weight) of *A. flavus* and *F. oxysporum* (cultivated on potato dextrose broth medium containing 200 mg of *J. procera*) was grinded in 10 ml Chloroform: Methanol (2:1 v/v), then filtered and concentrated into 1 ml. The concentrated extract was placed in Gas Chromatography (GC) auto-sampler vials until they were analyzed using Shimadzu GCMS-QP 5050 A. software class 5000. Searched library: Wiley229 LIB. Column: DB1, 30 m, 0.53 mm ID; 1.5 µm film. Carrier gas: Helium (flow rate 1 ml/min.). Ionization mode: El (70 ev). Temperature program: 70°C (static for 2 min) then gradually increasing (at a rate of 2°C /min) up to 220°C (static for 5 min). Detector temperature was set at 250°C and injector temperature at 250°C. The chromatographs were compared and individual peaks were identified by comparing mass spectra to the library references, at RCMB.

Pot experiment: Pots containing 750 g of autoclaved agricultural soil and cultivated with 15 seeds of radish (*Raphanus sativus*) surface-disinfected in 0.5% sodium hypochloride solution for 3 min, rinsed three times in sterile-distilled water prior to sowing, in an air conditioned glasshouse at 25-28°C, the soil was inoculated with 10 mm mycelia agar disk of *F. oxysporum* mycelia with their spores (1×104 /ml) and the soil was treated with various concentration of 100 to 200 mg *J. procera* extract, 10 mg Carbomar and mixture of 200 mg *J. procera* extract with 10 mg Carbomar. Control soil was treated separately with *F. oxysporum* and *J. procera* extract. After 10 to 20 days the growing and wilting seedlings were observed and quantitative determination of chlorophyll was estimated in the rest of seedlings.

Quantitative determination of chlorophyll content: Chlorophyll a and b were extracted from the leaves of *Raphanus sativus* and estimated by the method of Vernon and Seely [48] using the following equations:

Chlorophyll a (mg)/(g) fresh weight=11.63 (A665) - 2.39 (A649).

Chlorophyll b (mg)/(g) fresh weight=2.11 (A649) - 5.18 (A665).

Where (A), denotes the reading of the optical density.

Colony forming units of *Fusarium oxysporum*: After 1, 5, 10 and 15 days of *J. procera* extract and chemical fungicide (Carbomar) application in pot experiment with agricultural soil, cultivated with *R. sativus*, the total numbers of inoculated *F. oxysporum* was counted.

The numbers of colony forming units (CFU) in the selective media were determined by means of the serial dilution technique and the spread plate method. Analyses were performed in three replicates. Viable count of *F. oxysporum* was performed using rose bengal-streptomycin agar containing (per liter): 10 g glucose; 5 g peptone;1 g K2HPO4; 0.5 g MgSO4×7H2O; 0.033 g rose bengal; 15 g agar. The plates were incubated at 28°C and colonies were counted after 5 days.

All data were subjected to one-way analysis of variance (ANOVA) using a software package (SPSS ver. 15). The significance among treatments in each time interval was determined using the least significant difference test (LSD, $P<0.05$ and $P<0.01$).

Results and Discussion

In vitro antimycotic activity

Growth of *A. flavus* and *F. oxysporum* (Table 1 and Figure 1) indicated that both fungal species were sensitive to the *J. procera* extract with the severe inhibition of colony radius compared with the control. There was significant difference ($P<0.01$) among colony radius of A. flavus and F. oxysporum at different concentration of *J. procera* extract alone, *J. procera* extract with carbomar and carbomar alone and control. This confirms the antifungal activities exerted by juniper extracts against fungal mycelium [21,23,25]. Growth of A. flavus and F. oxysporum was decreased with increasing concentrations of *J. procera* extract (Figure 1B-D). On the other hand the antifungal activity of Carbomar was more pronounced (Figure 1E) against A. flavus and F. oxysporum. Furthermore, *J. procera* extract combined with Carbomar resulted in the best control of fungal species compared with using each of them alone, where the growth inhibition % was 82.1 and 88.42% for A. flavus and F. oxysporum and respectively (Table 1), similar findings were reported by Pirzada et al. [30]. Pankaj et al. [28] reported that the different fractions of Juniperus leaves and bark inhibit the growth of aflatoxigenic A. flavus and A. niger at concentrations ranged from 1000 to 4000 ppm of methanolic extract. The antifungal properties of *J. procera* may due to the presence of sandaracopimaric acid [29,49], diterpenes totarol, ferruginol, 7β-hydroxyabieta-8, 13-dien-11,12-dione, and 4-epiabetol [50].

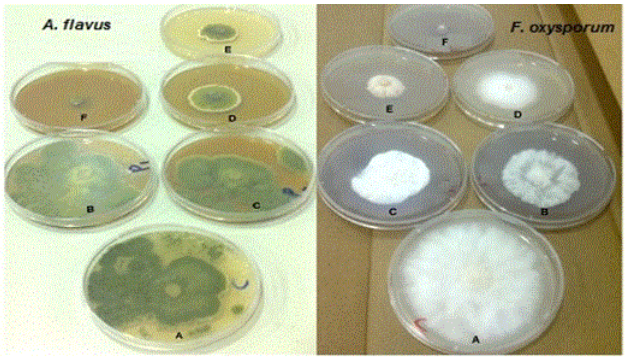

Figure 1: Growth of A. flavus and F.oxysporum on medium without treatment as control (A), with 100 mg of *J. procera* extract (B), 150 mg of *J. procera* extract (C), 200 mg of *J. procera* extract (D), 10 mg fungicide (E), 200 mg of *J. procera* extract and 10 mg chemical fungicide (F)

Concentration of *J. procera* extract (mg)	Growth and inhibition percentage (%) (mean ± SE)			
	A. flavus		F. oxysporum	
	Colony radius (mm)	Inhibition (%)	Colony radius (mm)	Inhibition (%)
Control	4.47 ± 0.15	0.00	7.40 ± 0.10	0.00
100	4.17 ± 0.15**	6.71	4.30 ± 0.10**	41.89
150	3.73 ± 0.06**	16.55	3.80 ± 0.10**	48.64
200	2.30 ± 0.10**	48.54	2.97 ± 0.12**	59.86
Carbomar (10 mg)	1.83 ± 0.06**	62.90	1.77 ± 0.15**	76.08
Carbomar (10 mg) + *J. procera* extract (200 mg)	0.80 ± 0.10**	82.10	0.87 ± 0.06**	88.42

Table 1: Growth of *A. flavus* and *F.oxysporum* on medium supplemented with different concentrations of *J. procera* extract and their mixture with chemical fungicide (Carbomar). Value is the mean ± SD of three replicates. **The mean difference is significant at the 0.01 level.

Extract of *J. procera* demonstrated good inhibitory effect on secondary metabolites production as well as mycotoxins of A. flavus and F. oxysporum (Tables 2 and 3). Production of all detected secondary metabolites (except aspergillic acid) of A. flavus including aflatoxins B1, sterigmatocystin, cyclopiazonic acid and ergosterol were reduced (Production inhibition was 67.44, 96.28, 60.33 and 42.75% respectively), while production of aflatoxins B2 was completely inhibited with the treatment of *J. procera* extract (Table 2). Fungitoxic effects indicate that *J. procera* extract block the biosynthesis pathway of aflatoxins B2. On the other hand, *J. procera* extract induced the production of 3-Nitropropionic acid. These results are partially supported by reports indicating that extracts of certain plants were able to inhibit production of aflatoxin, sterigmatocystin and cyclopiazonic acid [17,51].

It was revealed from the results (Table 3) that *J. procera* extract inhibit the secondary metabolites production of F. oxysporum, including fusaric acid and ergosterol (production inhibition was 8.36% and 73.34% respectively). Many researchers focused on controlling of F. oxysporum mycotoxins [10,11,52]. Fusaric acid is probably one of the most widely distributed mycotoxins produced by strains in the genus Fusarium [53].

Secondary metabolites	Secondary metabolites of A. flavus cultivated on medium				
	Without *J. procera* extract		With *J. procera* extract		Inhibition (%) of secondary metabolite production (%)
	Area (AU)	Area%	Area (AU)	Area%	
Aflatoxin B2	5692.6	11.19	0.0	0.0	100
Aflatoxin B1	6494.4	12.76	2114.5	5.38	67.44
Sterigmatocystin	4527.3	8.90	168.4	0.43	96.28
Aspergillic acid	23824.4	46.81	29622.7	75.43	-ve
Cyclopiazonic acid	4078.5	8.01	1617.7	4.12	60.33
Ergosterol	6276.7	12.33	3592.8	9.15	42.75
3-Nitropropionic acid	0.0	0.0	2156.0	5.49	-ve

Table 2: Secondary metabolites of A. flavus cultivated on medium supplemented with *J. procera* extract (200 mg/100 ml). -ve, Secondary metabolite production increased.

Data in Table 3 show that *J. procera* extract have no suppressive effect on the productivity of other mycotoxins, where T2-Toxin, dihydrofusarbin, dihydroxyscripenol and butenolide productivity increased as compared with their productivity in case of control (without *J. procera* extract). Overall, the findings suggest that this extract has potential for use as antifungal agent (Figure 1), although it induce certain mycotoxin production and supress other. According to the explanation of Kiran et al. [54], it appears that there is no a relationship between the growth and mycotoxin production. Natural antifungal agents can be potential exploited in controlling the growth of fungi consequently inhibiting mycotoxin production [51,55,56].

Secondary metabolites	Secondary metabolites of F. oxysporum cultivated on medium				
	Without *J. procera* extract		With *J. procera* extract		Inhibition (%) of secondary metabolite production (%)
	Area (AU)	Area%	Area (AU)	Area%	
Dihydroxyscripenol	2422.3	17.41	8797.1	19.32	-ve
Fusaric acid	7808.8	48.94	7155.6	15.72	8.36
T2-Toxin	1428.3	10.27	2087.7	4.59	-ve
Butenolide	594.7	4.27	3701.0	8.13	-ve
Dihydrofusarbin	130.4	0.94	326.3	0.72	-ve
Ergosterol	893.5	6.42	238.2	0.41	73.34
Emodin	1634.5	11.75	2065.7	4.54	-ve
Unknown 1	0.00	0.00	12935.3	28.42	-ve
Unknown 2	0.00	0.00	8439.1	18.04	-ve

Table 3: Secondary metabolites of F. oxysporum cultivated on medium supplemented with *J. procera* extract (200 mg/100 ml). -ve, Secondary metabolite production increased.

The most fatty acids of A. flavus and F. oxysporum mycelia treated with *J. procera* extract were detected in lower concentration compared to the non-treated (Table 4). Overall results indicated that certain fatty acids including caprylic, tridecanoic and pentadecanoic was completely disappeared in A. flavus cultivated in medium amended with *J. procera* extract. On the other hand, palmitoleic, heptadecanoic, stearic and arachidic acid were decreased (0.24, 0.35, 2.42 and 0.56 mg/g) sharply in F. oxysporum mycelia treated with *J. procera* extract compared with their concentration (2.98, 6.41, 9.43 and1.82 mg/g) respectively in non-treated. Although the mode of action of *J. procera* extract is not completely explained, it has been suggested that they act over cytoplasmic membranes, producing changes in their fatty acids. Feng and Zheng [57] reported that the natural compounds from plants interfere in the electron transport, the nutrient absorption, the fatty acid synthesis, the adenosine triphosphatase activity, and other metabolic processes of the cell.

Fatty Acids		Fatty acids concentration (mg/g fresh weight)			
		A. flavus cultivated		F. oxysporum cultivated	
		Without *J. procera* extract	With *J. procera* extract	Without *J. procera* extract	With *J. procera* extract
Caprylic	C8	0.31	0.00	0.04	1.89
Capric	C10	0.79	0.17	0.05	0.06
Lauric	C12	1.48	0.36	1.83	1.12
Tridecanoic	C13	0.63	0.00	0.20	0.47
Myristic	C14	1.84	1.16	1.58	1.87
Pentadecanoic	C15	0.12	0.00	0.32	0.14
Palmitic	C16	25.64	33.8	28.89	27.6
Palmitoleic	C16:1	0.66	0.21	2.98	0.24
Heptadecanoic	C17	14.92	15.19	6.41	0.35
Oleic	C18:1	26.59	12.74	29.54	49.81
Stearic	C18	9.78	9.17	9.43	2.42
Linoleic	C18:2	7.46	22.06	16.61	11.36
Arachidic	C20	7.31	5.51	1.82	0.56
Behenic	C22	3.26	0.15	0.12	0.08

Table 4: Fatty acids content of A. flavus and F. oxysporum cultivated in medium supplemented with *J. procera* extract (200 mg/100 ml).

Carbomar at concentration 10 mg had clear toxicity to F. oxysporum growth as well as their morphology and sporogenisis (Figure 2E and F), where hyphae were deformed with appearance of large vesicles inside hyphae. *J. procera* extract had a rather similar effect on the F. oxysporum morphology (Figure 2D) at high concentration (200 mg) but had a slight effect at low concentratin 100 and 150 mg (Figure 2A-C). Synergistic effect between Carbomar and *J. procera* extract was observed on diagnostic charaiteria of F. oxysporum (Figure 2G and H) compared with all treatments and control. Different degrees of antifungal activities represented by deformations in the diagnostic charaiteria of F. oxysporum can probably be explained by the varied concentration of the tested *J. procera* extract. Similar results have been reported, showing less number of conidiospores of F. oxysporum as a result of exposure to 20 µL natural mint extract [37].

In vivo antimycotic activity

The in vivo experiment demonstrated the *J. procera* extract showed efficient control of F. oxysporum, where the disease symptoms were suppressed when *J. procera* extract was added in *R. sativus* grown in fungus-infested soil. F. oxysporum suppression was based on observations of symptoms of their disease in the wilting and number of growing seedlings (Figure 3). Furthermore, the severity of symptoms decreased with increasing of *J. procera* extract concentration (Figure 3D and E) compared to the fungus-inoculated without any treatments (Figure 3C). Development of safer anti-fungal agents such as plant extracts to control phytopathogens in agriculture were reported in recent years [25,58].

Carbomar had the highest inhibitory effect on F. oxysporum wilt disease (Figure 3F) compared with using *J. procera* extract. Weitang et al. [59] indicated that carbendazim were the most effective fungicides in inhibiting mycelial growth of F. oxysporum f. sp. Furthermore, the disease severity of *R. sativus* was reduced after an integrated treatment of Carbomar combined with *J. procera* extract (Figure 3G). Surprisingly Nguefack et al. [60] found that the antifungal activity of chemical fungicide (Carbendazim 100 mg/ml + chlorothalonil 550 mg/ml) was lower than that of plant extract (Ocimum gratissimum and Callistemon citrinus) against Bipolaris oryzae. On the other hand no negative impact was observed on *R. sativus* grown in non fungus-infested soil amended with *J. procera* extract (Figure 3B). According to Bansal and Gupta [61] many plant extracts have been reported to increase seed germination through decreasing F. oxysporum incidence.

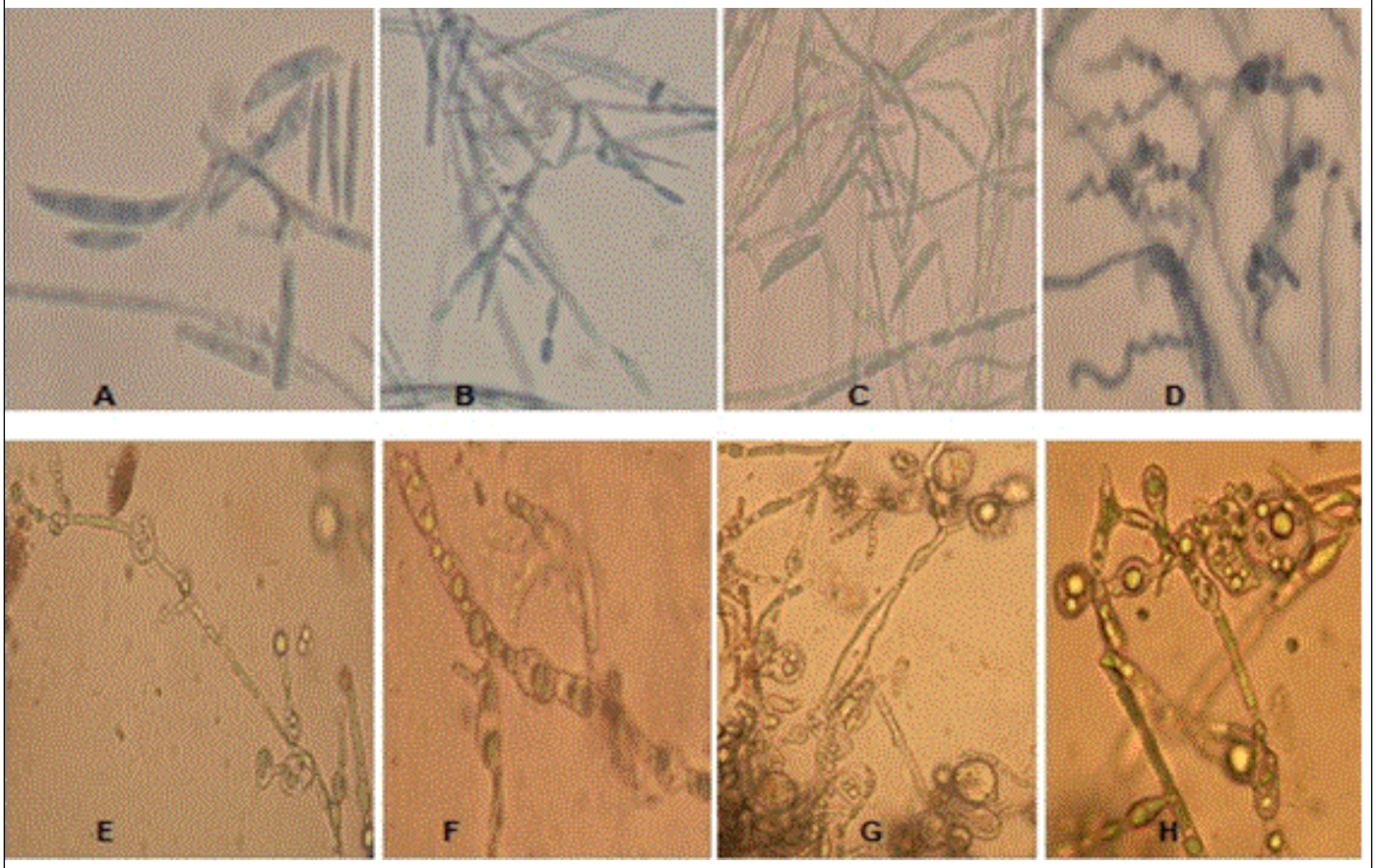

Figure 2: Morphological charaiteria of F. oxysporum in medium without treatment as control (A), 100 mg of *J. procera* extract (B), 150 mg of *J. procera* extract (C), 200 mg of *J. procera* extract (D), 10 mg Carbomar (E and F), 200 mg of *J. procera* extract and 10 mg Carbomar (G and H)

Anyway, the results suggest that the *J. procera* extract not only suppress F. oxysporum growth in vitro, but also in vivo. This is not the first study which used the plant extract to control plant pathogens under in vivo conditions. Application of Dittrichia viscosa extract [62], Ocimum basilicum extract [63] neem seed powder [64], J. oxycedrus [65] plant essential oils [66] gave efficient protection against phytopathogens under in vivo conditions. Data presented in Table 5 showed that chlorophyll a and b were significantly decreased in *R. sativus* at certain treatment. Minimum content of chlorophyll a and b (3.56 and 1.65 mg/g fresh weight for chlorophyll a and b respectively at $P<0.01$) was observed in *R. sativus* cultivated in soil inoculated with pathogen alone compared with other treatments. Chlorophyll a of *R. sativus* cultivated in soil inoculated with pathogen showed no significant decrease at treatment with 100 mg *J. procera* extract and Carbomar, where it was 4.86 and 4.93 mg/g fresh weight respectively.

The agricultural soil treated with *J. procera* extract and Carbomar showed significant ($P<0.01$) alterations in F. oxysporum population (Table 6). The population of F. oxysporum increased with increasing application time in the fungus-inoculated soil, where the mean of CFU/g was 28.67, 46.33, 56 and 57.33 at 1,5,10 and 15 days respectively. On the other hand, *J. procera* extract at 100 and 200 mg suppress F. oxysporum at 5 days of application (CFU was 25.33 and 21.33 respectively). At 10 and 15 days of plant extract application, the population of F. oxysporum increased. This may be due to degradation of active ingredient of *J. procera*. On the other hand the chemical fungicide was more effective than natural extract of *J. procera*. At the same time, the addition of *J. procera* extract (200 mg) to carbomar increased their antifungal activity on CFU of F. oxysporum. Alkhail [67] showed antifungal activity of some plant extracts when tested against fungi viz., F. oxysporum and Botrytis cinerea. Mogle and Maske [68] reported that chemical fungicide Dithane M-45 decreased the mycoflora and enhanced the seeds of cowpea germination percentage [69]. Screening for antimicrobial activity has been the subject of many investigations and plant extracts with very potent antimicrobial activity could be promising agents for in vivo examination. Among such plant extracts is the extract from *J. procera* and it has been emerged as alternative to replace chemical fungicides.

Figure 3: Seedlings growth of *R. sativus* cultivated in control soil without treatment (A), soil treated with 100 mg of *J. procera* extract (B), soil inoculated with F. oxysporum (C), soil inoculated with F. oxysporum and treated with 100 mg of *J. procera* extract (D), soil inoculated with F. oxysporum and treated with 200 mg of *J. procera* extract (E), soil inoculated with F. oxysporum and treated with 10 mg Carbomar (F), soil inoculated with F. oxysporum, treated with 200 mg of *J. procera* extract and 10 mg Carbomar (G).

Treatments	Chlorophyll contents (mg/g fresh weight)	
	Chlorophyll "a"	Chlorophyll "b"
Control	4.96 ± 0.17	2.31 ± 0.05
J. procera extract (100 mg)	4.38 ± 0.10**	1.94 ± 0.06*
F. oxysporum (FO)	3.56 ± 0.03**	1.65 ± 0.10**
J. procera extract (100 mg) and FO	4.86 ± 0.06	2.28 ± 0.06**
J. procera extract (200 mg) and FO	3.65 ± 0.11**	1.76 ± 0.06**
J. procera extract (200 mg), Carbomar (10 mg) and FO	4.23 ± 0.07**	1.93 ± 0.03**
Carbomar (10 mg) and FO	4.93 ± 0.05	2.30 ± 0.10**

Table 5: Chlorophyll contents (mg/g fresh weight) of *Raphanus sativus* seedlings cultivated in soil treated with *J. procera* extract, F. oxysporum and Carbomar. Control, autoclaved soil and without treatment. *The mean difference is significant at the 0.05 level. **The mean difference is significant at the 0.01 level.

Soil treatments	Colony forming units (CFU) of F. oxysporum at different days			
	1	5	10	15
Control	0.00 ± 0.00	0.00 ± 0.00	1.33 ± 0.58	4.67 ± 1.15
F. oxysporum (FO)	28.67 ± 1.53**	46.33 ± 2.65**	56.00 ± 2.65**	57.33 ± 1.53**
J. procera extract (100 mg) and FO	26.00 ± 1.00**	25.33 ± 1.15**	57.67 ± 1.15**	61.33 ± 1.53**
J. procera extract (200 mg) and FO	23.67 ± 1.15**	21.33 ± 1.53**	36.67 ± 1.15**	42.67 ± 1.53**
J. procera extract (200 mg), Carbomar (10 mg) and FO	13.33 ± 0.58**	9.33 ± 0.58**	8.67 ± 1.15**	17.33 ± 0.58**

Carbomar (10 mg) and FO	14.33 ± 0.58**	11.67 ± 1.15**	11.67 ± 1.53**	16.67 ± 1.15**

Table 6: Colony forming units (CFU) of F. oxysporum in soil inoculated with F. oxysporum and treated with *J. procera* extract and Carbomar at different days of application. Autoclaved soil without treatment was used as control. **The mean difference is significant at the 0.01 level.

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