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MYCOFLORA ASSOCIATED WITH SEEDS OF DIFFERENT SUNFLOWER CULTIVARS AND ITS MANAGEMENT

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Abstract

The present study was carried out to isolate fungi associated with seeds of seven cultivars of sunflower by using agar and blotter paper methods. A total of 13 phytopathogenic fungal species including *Alternaria alternata* and *A. helianthi, Aspergillus flavus, A. fumigatus* and *A. niger, Curvularia lunata, Drechslera tetramera, Fusarium solani* and *F. moniliforme, Macrophomina phaseolina, Mucor mucedo, Penicillium* and *Rhizopus* spp. were identified. The isolated fungi were found to reduce seed germination by 10-20% and seedling mortality by 10-12%. Two systemic fungicides viz., Topsin and Bayleton were found to be significantly effective in the elimination of seed-borne fungi. Among the plant material, best antifungal activity was achieved by extracts of *Azadirachta indica* (Neem), and *Allium sativum* (garlic) at the concentration of 0.015%.

Introduction

Fats and oils are important ingredients of human food. Vegetable oil is extracted from seeds and fruits of different crops and trees (Butt & Ali, 2005). Sunflower (*Helianthus annuus* L.), an important member of the family Asteraceae and is one of the major oilseed crops grown for edible oil in the world (Anon., 2007). In Pakistan, it is grown over an area of 316 thousand hectares with a total production of 569 thousand tons showing an average yield of 1803 kg per hectare (Anon., 2006). Seeds of different sunflower cultivars differ in their oil contents and properties. Sunflower seeds contain 40-50% oil and 23% protein and constitute excellent source of unsaturated fats, crude protein and fiber and important nutrients like vitamin E, selenium, copper, zinc and B-complex vitamin as well (Hatim & Abassi 1994; Relf 1997; Gonzalez *et al*., 2002).

Sunflower is attacked by a number of diseases caused by fungi, bacteria, nematodes and viruses. Of the fungal foliar diseases, leaf spot caused by *Alternaria helianthi, Septoria helianth, Albugo tragopogonis* and *Plasmopara halstedii,* inducing brown and grey spots, white rust and downy mildew, respectively, are relatively important (Masirevic & Jasnic, 2006a, Wyk *et al*., 1999, Achbani *et al*., 2000). Fusarium wilt is caused by many species of *Fusaria* as *Fusarium solani, F. oxysporum, F. helianthi, F. moniliforme, F. equestii,* and others (Masirevic & Jasnic, 2006b). *Sclerotina* wilt and head rot of sunflower are caused by S*clerotinia sclerotiorum* (El-Deeb *et al*., 2000). Several of these fungal species are reported to be seed borne. In addition, sunflower seeds are highly contaminated with fungi which attack the plants at different stages of development and subsequently during harvesting and storage (Vaidehi, 2002; Morar *et al*., 2004). In Pakistan, Ahmed *et al*., (1993) and Sharfun-Nahar *et al*., (2005) reported the association of large number of fungi with sunflower seeds and their list included: *Aspergillus flavus, A. niger, A. ocheraceus, Alternaria alternata, Fusarium solani, Penicillium digitatum, Rhizopus arrihizus, Acremonium fusidioides, Arthrobotrys oligospora, Bipolaris bisepta, Cephaliophora tropica, Chaetomium spinosu*m

,*Cladobotryum varium, Cladosporium cladosporioides, Emericella nidulans, Gonatobotrys simplex, Humicola grisea, Memnoniella echinata, Mucor mucedo, Myrothecium verrucaria , Phialophora verrucosa* and *Syncephalastrum racemosum*.

The deleterious effects of seed-borne fungi include: biodeterioration of sunflower seeds when used as feed, reduced seed viability and germination and seedling vigour, poor stand of the crop in the field and low yields. Therefore, control of seed-borne fungi is extremely important and the deleterious effects can be alleviated through integrated approaches (Vaidehi, 2002).The present study aimed to isolate and identify fungi from seeds of different cultivars of sunflower in Pakistan, to determine the frequency of their occurrence and to test the efficacy of different fungicides and plant extracts.

Materials and Methods

Collection of seed samples: Seeds of 7 sunflower cultivars viz., M-3255, Allsstarrm, G-2, Allium, Nusun-652, LG-5380-M and Nusun-5501 were obtained from the Coordinator, Oil Seed Program, National Agricultural Research Centre, Islamabad, Pakistan. The seeds were collected in sterilized polythene bags and stored in a refrigerator at 4-5^{0}C until used. At least 100 seeds were used in any treatment.

Isolation and identification of fungi: Two standard methods i.e. Blotter and Agar plate method (ISTA, 1976) were used for the isolation of fungi from sunflower seeds. In the blotter paper method, surface-sterilized seeds, using 2.5% bleach-NaOCl for one minute, and untreated seeds, were placed on moistened blotter paper. In Agar plate method, 20 ml of PDA was poured in glass Petri plates of 9 cm diameter. In each case, five seeds, treated and untreated, were used and replicated four times. The Petri plates were incubated at 25-27^{o}C±2 under 12h alternating cycles of fluorescent light and darkness for a week. Fungi were identified on the basis of their typical structure and basic characters as suggested by Barnett (1960) and Melone & Masket (1964).

Multiplication of fungi and pathogenicity tests: The pure cultures of the fungi isolated were maintained on PDA in culture tubes which were stored in the refrigerator at 4C and used frequently. These were multiplied on 2% PDA for two-three weeks. The inoculum potential of each isolate was prepared by taking 1gm culture in 20 ml distilled water and mixed with soil. Pathogenicity tests were carried out in plastic pots replicated five times. Each pot with 10 cm diameter contained 200 gm sterilized soil mixture (I part field soil: 1 part sand: 1 part manure) and planted with 20 seeds of a sunflower cultivar, both sterilized and untreated lots. Similar protocol was followed for other experiments keeping appropriate controls.

Management of Seed-borne fungi: Efficacy of fungicides: Four seed-dressing fungicides were evaluated for their inhibitory effect on growth of the fungi using "Poisoned Food Technique" (Dhingra & Sinclair, 1993). The chemicals were: Bayleton (Triadimefon), Topsin (Dimethyl 4,4-o.phenylenebis 3-thioallphanate), Captan (N-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide) and Vitavax (Carboxin). One mg of each of the fungicides was dissolved in 20 ml of Potato Dextrose Agar in Petri plates, and one set of agar plates without fungicide was included as control. All the plates were inoculated at the centre with small quantity of seven day old cultures of each of the isolated fungi keeping five replications. The plates were incubated at 25^{o}C for seven days and fungal growth was measured in cm. Growth inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{\text{Diameter of colony in control} - \text{diameter of colony of fungi in fungicide}}{\text{Diameter of colony of control}} \times 100$$

Plant extracts: Extracts of four plants were assessed for their inhibitory effect on the growth of fungi isolated from sunflower seeds, according to the method used by Bhatti (1986). Leaves of Neem (*Azadirachta indica*) and Jimson weed (*Datura stramonium*), rhizomes of ginger (*Zingiber officinale)* and garlic *(Allium sativum*) were air-dried at room temperature (25-28^{o}C) for 30 days, surface sterilized with 90% ethanol and used for preparing aqueous solutions using 5, 10 and 15 g material of each source. It was finely macerated in 2 ml water and diluted to 0.005, 0.01 and 0.015% concentrations. The macerate was filtered through Whatmann No 1 filter paper. Two ml of the filtrate was mixed with 20 ml of agar in Petri plates, keeping one set without extract to serve as control. Small quantity of 7-day old culture of each fungus was transferred at the centre of Petri plates which were incubated at 25^{o}C for seven days. Growth of fungi and percentage inhibition was calculated as above.

Statistical analysis: Data were analyzed statistically by applying ANOVA and comparing means by using Least Significant Difference test (Steel & Torrie, 1990).

Results and Discussion

Isolation of fungi: Blotter and Agar plate methods were employed for this study and two sets of seeds were analyzed i.e., unsterilized and surface sterilized seeds. A total of 13 different fungi belonging to 9 distinct genera in 12 families were isolated (Table 1). All fungi were identified on the basis of their cultural and morphological characteristics. These were identified as *Alternaria alternata* Nees, *A. helianthi* (Hansf.). Tubaki & Nishihara, *Aspergillus flavus* Link ex Gray, *A. fumigatus* Fre., *A. niger* van Teighem, *Curvularia lunata* (Wakker) Boed, *Drechslera tetramera* (Mchinney) Sub & Jain, F*usarium solani* (Mart) App. & WR, *F. moniliforme* Sheldon, *Macrophomina phaseolina* (Tasssi) Goid, M*ucor mucedo, Penicillium* and *Rhizopus* spp. It was observed that treated seeds yielded less population of seed-borne fungi than the untreated seeds indicating partial elimination of some contaminating fungi. The results obtained are in close conformity with those of Limnord (1968) who reported that chloral disinfection effectively reduced the microbial contamination. Reduction of frequency rate of fungi from sterilized sunflower seeds was also found by Bhutta *et al*., (1998), Sharfan Nahar *et al*., (2005). Similar results have also been reported from seeds other than sunflower eg., groundnut seeds by Rasheed *et al*., (2004) and legume seeds by Embaby & Abdel-Galil (2006).

In the present study, it was found that both the agar and blotter paper methods of fungal isolation are effective, routinely and consistently applicable and provide reliable results (Table 1). A total of 12 fungi were isolated by agar plate method and 11 fungi by blotting paper method under unsterilized conditions. Out of 7 seed samples, *Aspergillus flavus* was isolated from all sample, showed 100% seed infection in both the methods and as such it appeared as the most predominant fungus of sunflower seeds. *Penicillium* and *Rhizopus* species were found in four seed samples in blotter paper and three seed samples in agar plate method. *Alternaria helianthi* was found in two seed samples in agar plate method under both sterilized and unsterilized conditions. *Alternaria alternata* appeared in a range of 42-71%, *Fusarium solani , Mucor mucedo* and *Curvularia lunata* to the extents ranging between 14-20% in both methods.. The results agree at large with many of the investigators working on seed pathology. Wagan *et al*., (2006) isolated *Alternaria helianthi* from sunflower seeds. Godika *et al*., (1999) found that the agar plate method was more suitable for isolation of *Macrophomina phaseolina* but they also detected *A. helianthi* by the blotter method. Saulastiano *et al*., (2006) found efficacy of blotter method in detecting *A. helianthi.* Gowder *et al*., (2007) observed that standard blotter method was better for isolation of large number of fungal species.

Table 1. Percentage frequency of fungi in Blotter and Agar plate methods.

S#	Isolated fungi	NSI	Mean ± S.E	NSI	Mean ± S.E	NSI	Mean ± S.E	NSI	Mean ± S.E
1.	*Alternaria alternate*	3	16.67 ± 4.41	5	23 ± 2.00	3	15 ± 2.88	4	21.25 ± 1.25
2.	*Alternaria helianthi*	0	0	2	15 ± 0.00	0	0	2	12.5 ± 2.50
3.	*Aspergillus flavus*	7	34.28 ± 2.97	7	33.57 ± 2.10	7	27.14 ± 1.48	6	25 ± 1.83
4.	*Aspergillus fumigatus*	0	0	1	10 ± 0.00	0	0	0	0
5.	*Aspergillus niger.*	3	25 ± 2.88	3	18.33 ± 4.41	1	15 ± 0.00	1	5 ± 0.00
6.	*Curvularia lunata*	1	10 ± 0.00	1	10 ± 0.00	0	0	1	10 ± 0.00
7.	*Drechslera tetramera*	2	12.5 ± 2.50	2	12.5 ± 2.50	2	10 ± 0.00	1	10 ± 0.00
8.	*Fusarium moniliforme*	1	15 ± 0.00	2	17.5 ± 2.5	1	10 ± 0.00	1	15 ± 0.00
9.	*Fusarium solani*	2	20 ± 0.00	2	17.5 ± 2.5	1	5 ± 0.00	1	10 ± 0.00
10.	*Macrophomina phaseolina*	1	10 ± 0.00	0	0	0	0	0	0
11.	*Mucor mucedo*	2	12.5 ± 2.50	2	12.5 ± 2.50	1	10 ± 0.00	1	15 ± 0.00
12.	*Penicillium* sp.,	4	6.25 ± 1.25	3	16.67 ± 1.67	0	0	0	0
13.	*Rhizopus sp.,*	4	11.25 ± 1.25	3	21.67± 1.67	1	10 ± 0.00	2	7.5 ± 2.5

NSI = No. of samples infected out of 7 seeds samples
SE = ± Standard error

Table 2. Effect of fungicides on mean diameter of fungal colonies (cm) on agar medium at 25^{0}C.

Treatment	*Alternaria alternata*	*A. flavus*	*A. niger*	*Curvularia lunata*	*D. tetramera*	*Rhizopus* sp.	*Penicillium* sp.
Control	3.550 A	4.400 A	4.375 A	3.175 A	2.925 B	2.675 A	3.125 A
Bayleton	1.475C	1.975 B	1.675 B	1.950 B	1.475 B	0.55 D	1.500 B
Vitavax	2.150 B	1.900 C	2.325 B	1.925 B	1.325 B	2.125 AB	1.600 B
Topsin	1.275 C	2.025 B	1.975 B	1.125 C	0.875 D	1.250 C	0.825 C
Captan	3.100 A	2.800 A	2.375 B	2.825 A	2.800 A	1.625 BC	2.050 B
LSD value	0.57	0.72	0.56	0.69	0.52	0.43	0.45

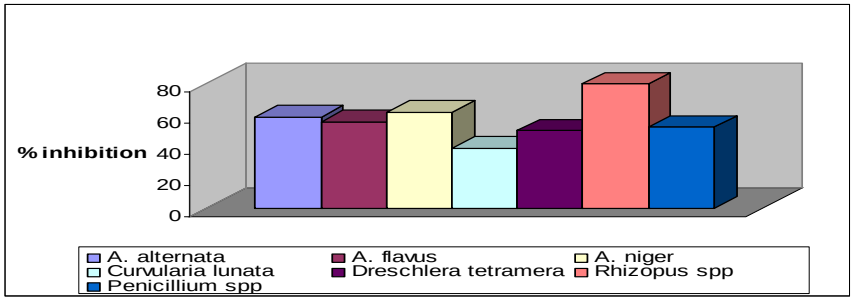
$p<0.05$ values within the same column show the same letters are not significantly different from each other

Pathogenicity tests with the fungal isolates carried out in soil mixtures, using sterilized and unsterilized seeds of sunflower cv. M-3225,.indicated that the treated seeds germinated to an extent of 85% with subsequent seedling viability of 60%, as against 75% and 50% values, respectively, in the untreated seeds. The germination range in the sterilized seed was 70-75% and seedling survivability between 45-70% by all the fungal isolates except *Curvularia lunata* and *Penicillium* spp., which showed little deleterious effect on seed germination and seedling viability for some unknown reasons. .

Management of seed-borne fungi: Mycoflora associated with sunflower seeds can be managed to a great extent and the use of pathogen free seed is of paramount importance. Efficacy of seed treatment is one of several factors that influence the cost, risk and benefits of seed treatment. Some chemicals, thermotherapy and plant extracts were evaluated against seed- borne fungi and the results are presented and discussed as under.

Treatment with seed dressing fungicides: Effect of four fungicides viz., Bayleton, Topsin, Vitavax and Captan, was assessed against 7 fungi viz., *Alternaria alternata, Aspergillus flavus, A . niger, Dreschlera tetramera Rhizopus* sp., isolated from sunflower seeds. *Curvularia lunata* and *Penicillium* sp. Fungicides in general significantly reduced the colony growth of the fungi as compared to control (Table 2). Best results were achieved with two systemic fungicides viz., Bayleton and Topsin; which inhibited mycelial growth as compared to other fungicides. Bayleton gave 79.93% inhibition of *Rhizopus* sp. 61.7% of *A. niger* and 58% of *A.alternata* (Fig. 1a). Antifungal property of Topsin was also recorded against *A. alternata (*64.8%), *Curvularia lunata (*64.56%), and *D. tetramera* (70.08%) (Fig. 1c). Vitavax did not show its effectiveness against these fungs as compared to Topsin and Bayleton but it was effective against *A. flavus, D. tetramera* and *A. alternata* (Fig. 1b). Inhibitory effect of Captan was observed only on *A. flavus* (36.36%) and *A. niger* (45.7) (Fig. 1d). It was concluded from this study that the fungicides can vary greatly in their effectiveness against different fungi. Bayleton and Topsin were relatively more effective and their efficacy may be attributed to quick entry of their molecules into the fungal mycelia, interaction within a short time and possibly disintegration of the fungal protoplasm. Chemical ingredients present in these two fungicides may be inhibitory to germination of fungal propagules. This observation gets ample support from Bhutta *et al*., (2001b) who evaluated the effect of Topsin on fungal population and observed no increase in seed germination. However, promising effect of Topsin has been reported against mycoflora associated with watermelon seeds (Bharath *et al*., 2005). Similarly, efficacy of Captan at 500 ppm against *Rhizoctonia bataticola* was observed by Hussain *et al*., (2000). It can be safely concluded and recommended that the fungicidal seed treatment is highly effective, economical and easily applicable as it can reduce the seed-borne mycoflora, improve seed germination and protect seedlings for sufficient time. However, it should be followed with great care and caution because it can produce serious problems leading to toxicity, phytotoxicity, environmental and soil pollution and bioaccumulation etc.

Treatment with plant extracts: The plant extracts can be used as alternative sources due of presence of some bioactive compounds which are known to control seed mycoflora. Efficacy of Datura, Ginger, Garlic and Neem was determined at different concentrations against seven isolated fungi viz., *Alternaria alternata, Aspergillus flavus, A. niger, Drechslera tetramera, Rhizopus* spp., *Curvularia lunata* and *Penicillium* spp.



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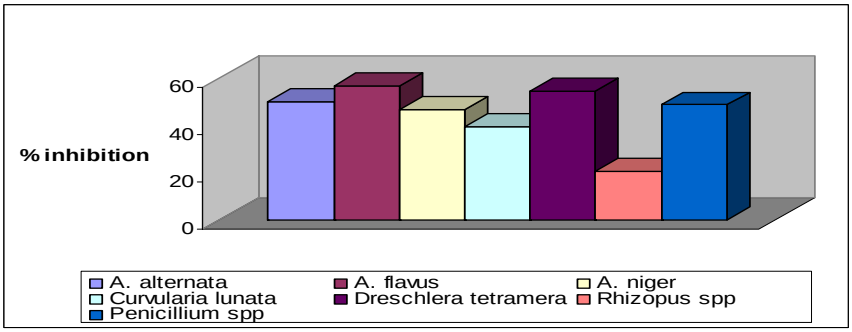

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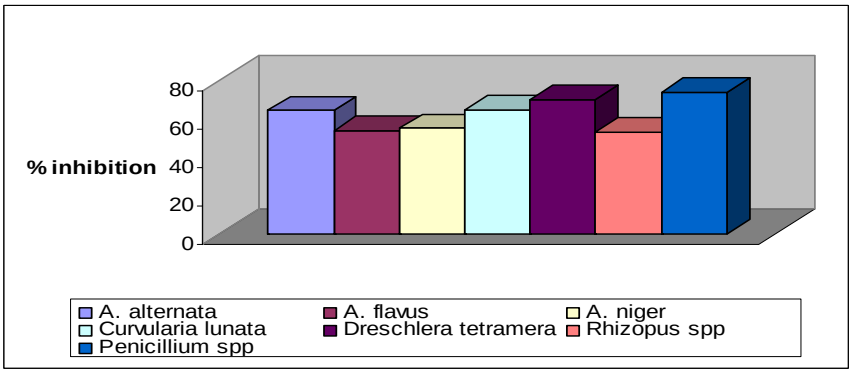

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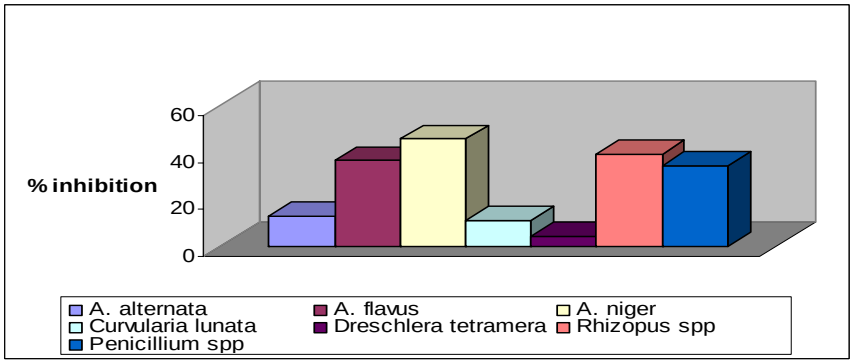
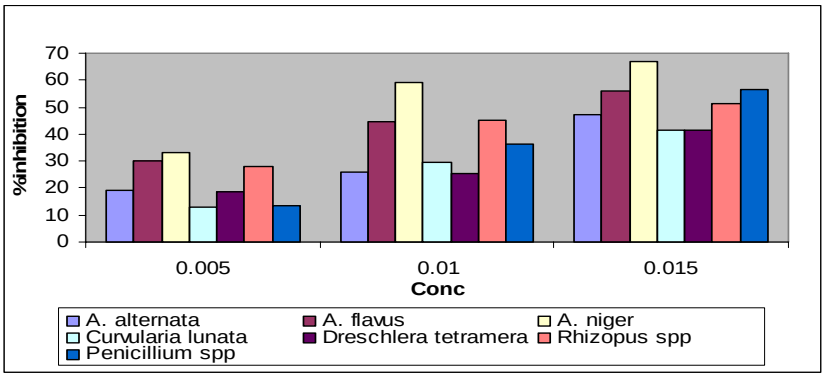


D

Fig. 1. Inhibitory effect of Seed Dressing Fungicides on the growth of seven fungi of sunflower. Bayleton (A), Vitavax (B), Topsin (C) and Captan (D).



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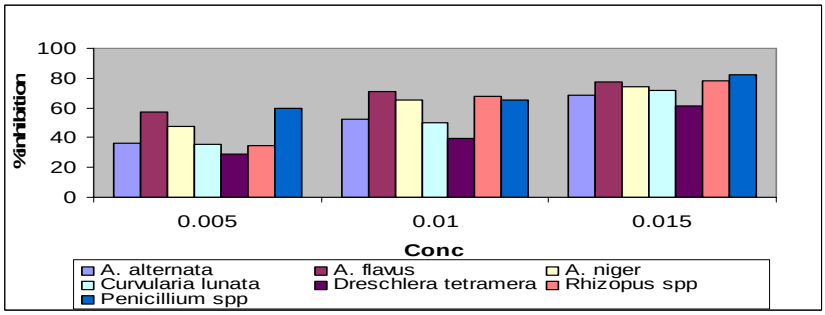

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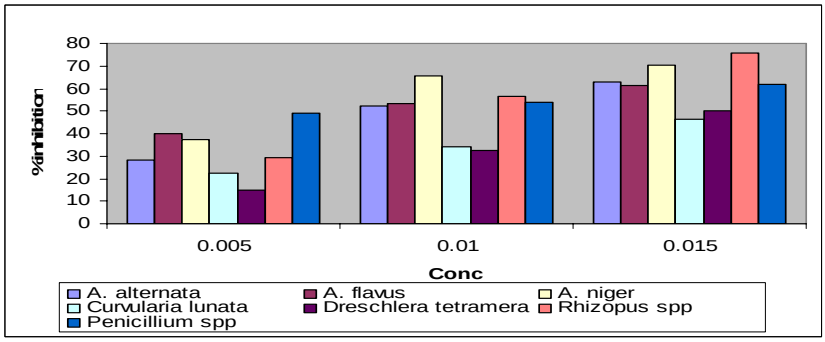

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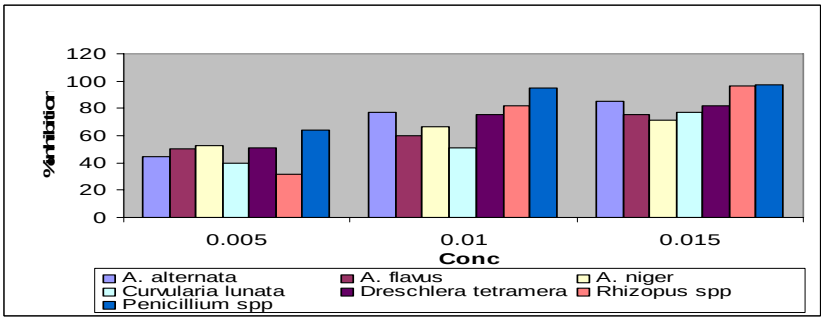

D

Fig. 2. Inhibitory effect of Plant extracts on the growth of seven fungi of sunflower. Datura (A), Garlic (B), Ginger (C) and Neem (D).

Table 3. Effect of plant extracts on mean diameter of fungal colonies (cm) on agar medium at 25^{0}C.

Treatment	Conc (%)	*Alternaria alternata*	*A. flavus*	*A. niger*	*Curvularia lunata*	*D. tetramera*	*Rhizopus* sp.	*Penicillium* sp.
Datura stramonium L. (Jimson weed)	0	3.775 A	4.425 A	4.200 A	3.425 A	2.825 A	2.775 A	3.900 A
	0.005	3.050 B	3.050 B	2.550 B	3.050 B	2.300 A-C	2.000 B	3.375 A
	0.01	2.800 C	2.450 CD	1.72C E	2.475 CD	2.075 B-D	1.525 B-D	2.525 B
	0.015	2.000 EF	1.950 EF	1.400 EF	2.050 DE	1.650 D-F	1.350 B-E	1.950 BC
Allium sativum L. (Garlic)	0	3.775 A	4.425 A	4.200 A	3.425 A	2.825 A	2.775 A	3.900 A
	0.005	2.650 C	2.650 C	2.700 B	2.725 BC	2.400 AB	1.950 BC	2.000 BC
	0.01	1.800 FG	2.075 D-F	1.550 DEF	2.250 C-E	1.900 B-E	1.200 C-F	1.800 C
	0.015	1.650 H	1.700 F	1.250 EF	1.875 D-F	1.400 E-G	0.6750 E-G	1.525 C
Zingiber officinale Roscoe (Ginger)	0	3.775 A	4.425 A	4.200 A	3.425 A	2.825 A	2.775 A	3.900 A
	0.005	2.375 D	1.950 EF	2.200 BC	2.250 C-E	1.975 B-E	1.750 BC	1.575 C
	0.01	1.800 FG	1.275 G	1.500 EF	1.750 EF	1.675 C-F	0.8500 D-G	1.350 C
	0.015	1.150 H	1.025 G	1.050 F	0.9500 G	1.100 FG	0.5750 FG	0.700 D
Azadirachta indica (L.) A. Juss. (Neem)	0	3.775 A	4.425 A	4.200 A	3.425 A	2.825 A	2.775 A	3.900 A
	0.005	2.050 E	2.225 DE	2.025 CD	2.125 C-E	1.375 E-G	1.900 BC	1.425 C
	0.01	0.8750 I	1.775 F	1.400 EF	1.675 EF	0.8250 GH	0.525 F-G	0.5250 D
	0.015	0.5500 J	1.125 G	1.150 F	1.375 FG	0.4500 H	0.3750 G	0.4250 D
LSD value		**0.457**	**0.729**	**0.754**	**0.52**	**0.67**	**0.48**	**0.43**

$p<0.05$ values within the same column show the same letters are not significantly different from each other

(Table 3). This study revealed a significant decrease in the growth of fungal colonies by all plant extracts at all concentrations as compared to control having large colony diameter. It was observed that the growth reduction and the concentration of material in the medium were directly proportional to each other. Results showed significant reduction of fungal growth by different plant extracts at concentrations of 0.005%, 0.01% and 0.015%. *Azadiracthta indica* and *Allium sativum* showed the best antifungal property against all the fungi tested and thus represent useful substitutes for the control of hazardous fungicides. *Datura stramonium* manifested an inhibition of growth of *A. niger (*66.66%), *A. flavus (*55.80%), *Penicillium* spp. (56.63%) at 0.015 (Fig. 2A), but little reduction at other concentrations, whereas ginger showed inhibitory effect on *A. niger*, *Rhizopus* and *Penicillium* spp., at 0.015 and moderate effect at 0.005 and 0.01 (Fig. 2C). *Allium sativum* showed a wide antifungal spectrum almost at every concentration, and it was maximum at 0.015 which inhibited 60-82% of the growth in all the fungi tested (Fig. 2B). Extract of *Azadiracta indica* completely controlled the fungal growth due to the presence of some strong inhibitory factor or an active constituent (Fig. 2D). It means Neem has a potential and ability to minimize fungal population. Efficacy of Neem was also observed by Bhutta *et al*., (1999) against five seed borne fungi viz., *Alternaria alternata, Emericellopsis terricola, Fusarium solani, Macrophomina phaseolina and Stemphylium helianthi*. Bhutta *et al*., *(*2001a) also reported the effectiveness of seed diffusates of neem in controlling several other fungi as *Alternaria alternata, Cochliobolus specifer, Emericellopsis terricola, Gibberella fujikuroi, Fusarium semitectum, Macrophomina phaseolina* and *Phoma oleracea Leptosphaeria maculans* and there was a significant increase in seed germination after elimination of fungi. Hussain *et al*., (2000) found that Neem was more effective in controlling *C. lunata* and *Rhizoctonia bataticola* with inhibition rate 98.87 and 88.83%. The efficacy of Neem has recently been reported and reviewed by Rao *et al*., (2007) and similar results were found by Ravishankar & Mamatha (2005) on forest seeds. Present study suggests to assess more number of indigenous plant species along with their exudates and to identify active compounds, toxic to plant pathogens which can be used in the integrated disease management programme for obtaining high yield of crops.

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