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In vitro evaluation of commonly available fungicides against three fungal isolates

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Abstract

Three fungal plant pathogens were isolated from different host plants in District Mansehra, KP, Pakistan. *Fusarium oxysporum* f. sp. *capsici* from wilted chilies (*Capsicum frutescens*), *F. oxysporum* f. sp. *lentis* from wilted lentils (*Lens culinaris*) and *Rhizoctonia solani* from black scurf of potatoes (*Solanum tuberosum*). Five fungicides, Helonil (chlorothalonil), Clipper (copper oxychloride), Antracol (propineb), Ridomil gold (metalyxyl M + mancozeb) and Desomile platinum (cymoxanil + mancozeb) were evaluated for *in vitro* efficacy at concentrations of 100, 200, 300, 400, 500 and 1000 ppm. The poisoned food technique was used and radial colony growths were measured periodically. Helonil was found to be the most effective fungicide followed by Clipper and Antracol. Helonil and Clipper completely inhibited growth of *R. solani*. Antracol, Ridomil and Desomil were the least effective. The results of this investigation will be helpful for future research in agrochemical industries and for local growers in controlling different fungal diseases.

Key words – fungi – pathogens – chilies – mung beans – potatoes – lentils – control

Introduction

Plant diseases play a vital role in the decline of natural resources in agriculture. Their causal agents include biotic and abiotic factors, genetic disorders and living infectious agents such as fungi, bacteria, viruses, viroid's, phytoplasmas, nematodes, parasitic plants, and protozoan's (Agrios 2005). As compared to other plant pathogens, phytopathogenic fungi are the most prominent parasitic organisms, and source of serious diseases and important yield losses in crops (Gonzalez-Fernandez & Jorrin-Novo 2010). Approximately 20,000 species of fungal plant pathogens have been reported and about 85% of plant diseases are caused by these fungi (Cooper 2007, Ong 2011). Many fungal genera including *Fusarium*, *Alternaria*, *Botrytis*, *Helminthosporium*, *Penicillium*, and *Rhizoctonia* have proved harmful pathogenic fungi and cause huge loss of crop yield world-wide (Boyraz & Ozcan 2006).

In Pakistan, all major crops are frequently infected by fungal plant pathogens and cause loss of yield in quality and quantity. Among these diseases, *Rhizoctonia* black scurf and stem canker

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caused by the fungus *Rhizoctonia solani* Kühn, are a severe problem in all potato producing zones of the country (Sneh et al. 1991, Ahmad et al. 1995, Khan et al. 1995). Many other fungal pathogens cause serious diseases in chilies including root rot caused by *R. solani* and *Fusarium* wilt, incited by *F. oxysporum* f. sp. *capsici*. Likewise, lentils (*Lens culinaris*) are infected by many pathogens including the lethal *Fusarium* wilt caused by *F. oxysporum* f. sp. *lentis* (Eujayl et al. 1998, Taylor et al. 2007, Jaruhar & Prasad 2011).

Application of fungicides is the most convenient and predominant way for disease control. Their use has made it feasible to enhance crop yields and food production. The efficacy of fungicides is influenced by many biological and environmental factors that directly influence the metabolic activities of fungal cells (Reinprecht 2010). Sometimes critical concentrations are not effective long-term, as the fungus can become resistant to the fungicide (Neely 1969, Brent & Hollomon 2007).

District Mansehra is famous for the cultivation of various kinds of vegetables and pulses. However, fungal diseases are a problem and during the preliminary survey conducted for present study, it was observed that most farmers used locally available fungicides, but often the diseases were not well controlled. The ineffectiveness of some fungicides in the field not only causes a monetary loss but may also have side effects on crops and environment. In vitro evaluation of fungicides offers useful information regarding efficacy against pathogens. It can be carried out in a short time and provides guideline for future field testing. Thus, the discovery of agrochemicals, new product development, and in vitro evaluation of fungicides to measure and rank the fungitoxicity of fungicides against a particular pathogen must be sustained from time to time (Neely 1969, Brent & Hollomon 2007).

Materials and Methods

Specimens were collected in District Mansehra, Himalayan region of Pakistan. The climate of the district is moist temperate with seasonal periods of rainfall, snow, and drought (Mustafa 2003). Three fungal pathogens were isolated from different infected host plants. *Fusarium oxysporum* f. sp. *capsici* from wilted chilies, *F. oxysporum* f. sp. *lentis* from wilted lentils and *Rhizoctonia soloni* from potatoes. The microbiological procedures including isolation, identification and evaluation of fungicides were carried out in Phir Mazhar Ali Shah (PMAS) Arid Agriculture University Rawalpindi during 2011–2016.

Fusarium oxysporum f. sp. *lentis* and *F. oxysporum* f. sp. *capsici* were isolated from diseased parts of *Lens culinaris* and *Capsicum frutescens*, respectively. Infected parts showing typical symptoms of *Fusarium* wilt were collected from Shinai Bala, Upper parts of Dhodial, Dharyal and Bugarhmang of District Mansehra. Standard tissue isolation technique was followed to obtain *F. oxysporum* f. sp. *capsici* and *F. oxysporum* f. sp. *lentis.* The infected roots, stems and leaves were cut in to pieces of 1–2 mm and surface sterilized with 0.1% sodium hypochlorite solution (NaOCL) and washed three times in sterile distilled water.

The sterilized tissue was dried on sterile filter paper on a clean bench, plated on potato dextrose agar (PDA; potatoes 20 g, glucose 20 g, agar 20 g) and incubated at 25°C. Mycelium growth was observed and transferred to a new plate containing sterilized malt extract agar (MEA). Pure cultures obtained through hyphal tip method and single spore subculture techniques. Mycelia from pure cultures were examined using a microscope and pathogens were identified with reference to published descriptions on the basis of colony morphology and microscopic features such as shape of apical and basal cells, length of phialides, macroconidia, microconidia, chlamydospores, etc. (Khare 1980, Nelson et al. 1983, Leslie & Summerell 2006, Taylor et al. 2007).

For the isolation of *R. solani* potato tubers contaminated with black scurf and sclerotia was collected from a field near campus area of Hazara University Mansehra. The infected tubers washed to remove surface contamination and then dried near a heater. The dried potato samples were bathed in a spirit container for 2 seconds and the process was repeated three times. A piece from outer layer of the tubers was placed on PDA medium in a Petri dish. After 24 hours whitish threads appeared around the piece and mycelium was transferred to PDA. Further purification was

carried out through hyphal tip method and single spore subculture. The pathogen was identified with reference to a published description (Woodhall & Peters 2011). Pure cultures were maintained on PDA slants at 5°C and refreshed every 10 days.

The following commonly available fungicides were selected from the local market. The detail of these fungicides is given in Table 1.

Table 1 Fungicides used for in vitro evaluation.

Common name	Trade Name	Chemical name	Formul-ation	Manufacturer
Chlorothalonil	Helonil	2,4,5,6-tetrachloro isophthalonitrile	75%WP	United Distributors Pakistan
Copper oxychloride	Clipper	Copper oxychloride	50%WP	Ali Akbar Enterprises Pakistan
Propineb	Antracole	Zinc-1-methylethylenbisdithiocarbamat	70%WP	Bayer Crop Science
Metalyxyl M + Mancozeb	Ridomil gold	N-(methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate + Ethylene bisdithiocarbamate	68%WP	Syngenta
Cymoxanil + Mancozeb	Disomil Platinum	2-cyano-N-[(ethylamino)carbonyl]-2 (methoxyimino) Acetamide + Ethylene bisdithiocarbamate	72%WP	Ali Akbar Enterprises Pakistan

The five fungicides were tested in vitro to evaluate their efficacy on colony growth of the three fungal isolates using the poison food technique (Hawamdeh & Ahmad 2001). All fungicides were used at 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm and 1000 ppm. The different fungicide concentrations were prepared in flasks by dissolving requisite quantities of each fungicide in warm media (50ºC). The fungicides were added after the media had been autoclaved (Shovan et al. 2008). Flasks without fungicide served as control. About 20 ml of sterilized medium was poured in each 9 cm petri dish. After solidification, the plates were inoculated with a 5 mm disk of 10-days-old cultures. Three replicate plates were used for each concentration of fungicide. Tests were conducted with MEA medium for *Fusarium* spp. while PDA was used for *Rhizoctonia*.

The inoculated plates were incubated at 25°C for *Fusarium* species and at 28°C for *Rhizoctonia* isolates. For *Fusarium* radial colony diameter was measured after 5 days of incubation, when growth on the control plates covered the plate. For *R. solani* measurements were made after 7 days of incubation. Colony growth was measured in two directions from the underneath side, perpendicular to each other, taking growth as the mean of the two measures. Percent inhibition of radial growth was computed based on colony diameter on control plate using the following formula (Sundar et al. 1995) and data were analyzed using MSTAT-C program (Khan et al. 2007).

$$\% \text{ Inhibition} = \frac{X-Y}{X} \times 100$$

Where,
X= Growth of control plate
Y= Growth of fungicide treated plate

Results

Fusarium oxysporum* f. sp. *lentis Figs 1, 2

The top leaves of infected host plants were yellow, resembling water deficiency. Sometimes the entire plant was severely damaged with yellow leaves and stunted growth. Shrinking and curling of leaves began from the lower branch of the plant and gradually moved up the stems. Root growth was reduced with marked brown discoloration however, no rot symptoms were found. The root was brown-black and compact. The disease was mostly observed near or at reproductive stage.

Colonies of *F. oxysporum* f. sp. *lentis* were white cotton cloudy to pinkish white. Mycelium growth was abundant, thick and dense feathery. The mycelium was hyaline, septate, much branched and the colonies were violet to dark violet. Growth on PDA was slower than on MEA, and no conidia or chlamydospores were formed. On MEA, *F. oxysporum* f. sp. *lentis* grew vigorously with well-developed conidia. This is the reason that we used MEA for in vitro tests.

The isolates had short monophialides and three kinds of spores: microconidia, macroconidia and chlamydospores. All isolates had branched, hyaline and septate conidiophores. The distance between the septa of conidiophores ranged from 8 to 28 µm. The macroconidia were long and thin-walled, usually 3–4-celled, and 9–27 × 2–4 µm in size. The apical end cell was long and pointed while the basal cell was foot-shaped or notched. Microconidia were ovoid or kidney-shaped, hyaline and usually one-celled. In some isolates two-celled microconidia were observed (Fig. 1). The microconidia measured 4.5–8.5 × 1.5–3.5 µm. Chlamydospores were oval or spherical, one-celled, thick-walled, and formed singly in macroconidia or apically or intercalary in the hyphae or terminal in the aerial mycelium. They were smooth to rough-walled, singly or in pairs to short chains in the hyphae and produced after 2–4 weeks. The characteristics of this fungus agree with the descriptions given for *F. oxysporum* f. sp. *lentis* (Khare 1980, Nelson et al. 1983, Leslie & Summerell 2006, Taylor 2007).

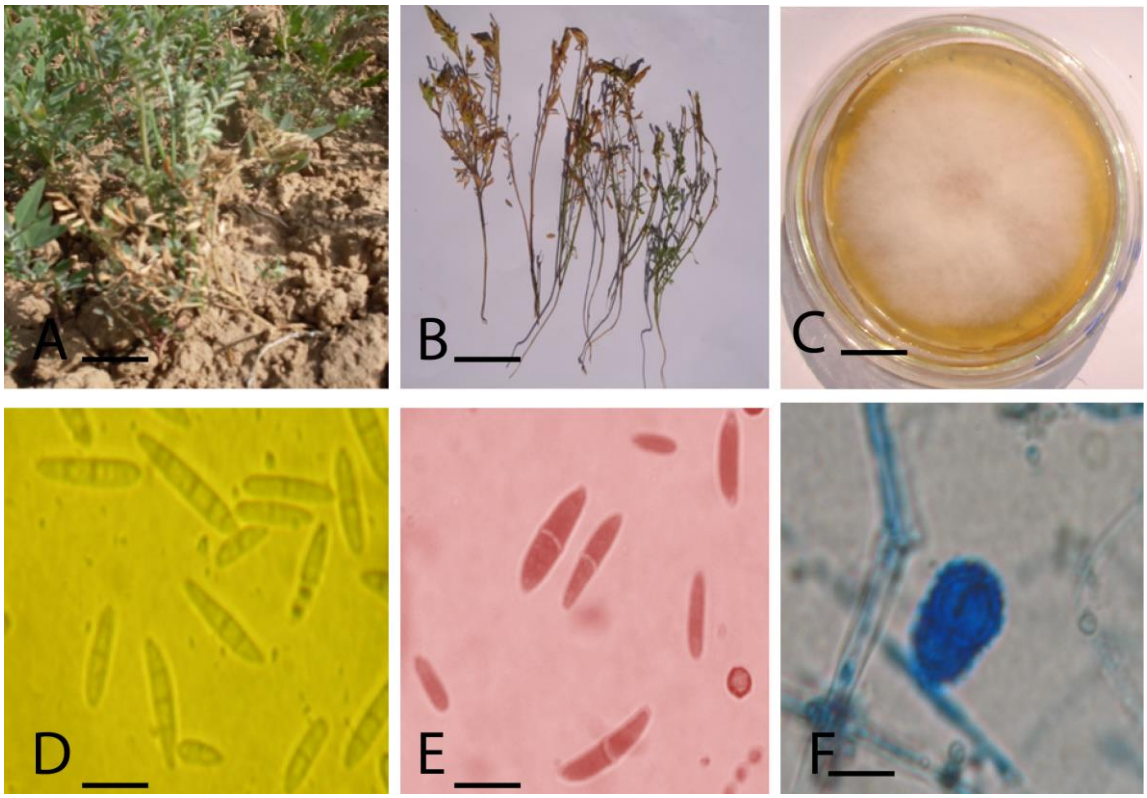

Fig. 1 – Morphology of *Fusarium oxysporum* f. sp. *lentis* and infected host plants: A–B Symptoms of *Fusarium* wilt in lentils (*Lens culinaris*). C Pure culture on MEA medium. D Macroconidia. E Microconidia. F Chlamydospores. Scale bars: A–B = 7.5 cm, C = 1.5 cm, D = 16 µm, E–F = 6 µm.

Radial colony growth along with percentage inhibition of *F. oxysporum* f. sp. *lentis* treated with different fungicides are in Table 2.

Table 2 *Fusarium oxysporum* f. sp. *lentis* radial colony growth and percentage growth inhibition with fungicides.

Concentration (ppm)	Fungicides									
	Helonil		**Clipper**		**Antracol**		**Ridomile**		**Desomil**	
	Radial growth (mm)	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**
100	50.3 bc	44.0	79.0 b	12.2	85.0 b	5.55	79.5 b	11.6	75.3 b	16.3
200	48.5 c	46.1	80.6 b	10.6	81.0 c	10.00	77.8 b	13.5	73.3 c	18.5
300	52.1 b	42.0	65.3 d	27.4	76.0 d	15.55	65.6 c	27.0	72.8 c	19.0
400	42.5 d	52.7	80.6 b	10.3	49.0 g	45.55	57.3 e	36.3	74.1 bc	17.5
500	34.8 e	61.3	60.0 e	33.3	50.0 f	44.44	62.6 d	30.3	73.6 bc	14.8
1000	27.3 f	69.6	70.5 c	21.6	54.0 e	40.00	58.5 e	35.0	72.5 c	19.4
Control	90	0.0	90.0	0.0	90.0	0.00	90.0	0.0	90	0.0
LSD (P=0.05)	3.1		2.4		0.9		2.1		1.9	

[a]Mean values followed by different letters within the same column are significantly different at *P*=0.05

Helonil was the superior fungicide at all concentration. The most effective dose of Helonil was 1000 ppm with inhibition of (69.6%) followed by Antracol (45.6%) at 400 ppm and clipper (46.6%) at 500 ppm. Least inhibition was observed with Antracol (5.6%) at 100 ppm (Fig. 2).

Fusarium oxysporum* f. sp. *capsici Figs 3, 4A–E

Capsicum frutescens infected by *Fusarium oxysporum* f. sp. *capsici* showing typical wilt symptoms and yellowing of leaves, stunted growth, discoloured vascular tissues, vein clearing, downward drooping and detaching of leaves.

Colonies of *F. oxysporum* f. sp. *capsici* were pale white or pale orange, with profuse mycelial growth. From below the colony was transparent purple in colour. The hyphae were septate and produced short monophialides. Three types of spores were produced. Macroconidia were 8.5–28.2 × 2.2–4.2 µm, straight to slightly curved, thin-walled, and usually 3–4-celled. The apical cell was tapered and curved, while the basal cell was foot-shaped or pointed. Microconidia, which were borne on short monophialides, and also in false heads were oval, elliptical or kidney shaped, asseptate or with one septum, 3.8–9 × 1.4–3.6 µm. Chlamydospores were abundantly produced after 2–4 weeks, smooth to rough-walled, mostly produced singly, rarely in pairs and either intercalary or terminal in the aerial mycelium.

Radial colony growth along with percentage inhibition of *F. oxysporum* f. sp. *capsici* with fungicides are given in Table 3. There was a significant difference among fungicides at different concentrations in inhibiting the growth of *F. oxysporum* f. sp. *capsici.* Helonil was the best fungicide at all concentrations. The most effective dose of Helonil was 1000 ppm with inhibition of (61.1%) followed by Antracol (32.0%) at 300 ppm. Ridomil, Desomil and Clipper were least effective at all concentration (Fig. 4A–E).

Rhizoctonia solani Figs 4F-I, 5

There was branching near the distal septum of cells in young vegetative hyphae, constriction of hyphae and formation of septa, at short distance from the point of origin of hyphal branches, the presence of a dolipore septa and absence of clamp connection and conidia.

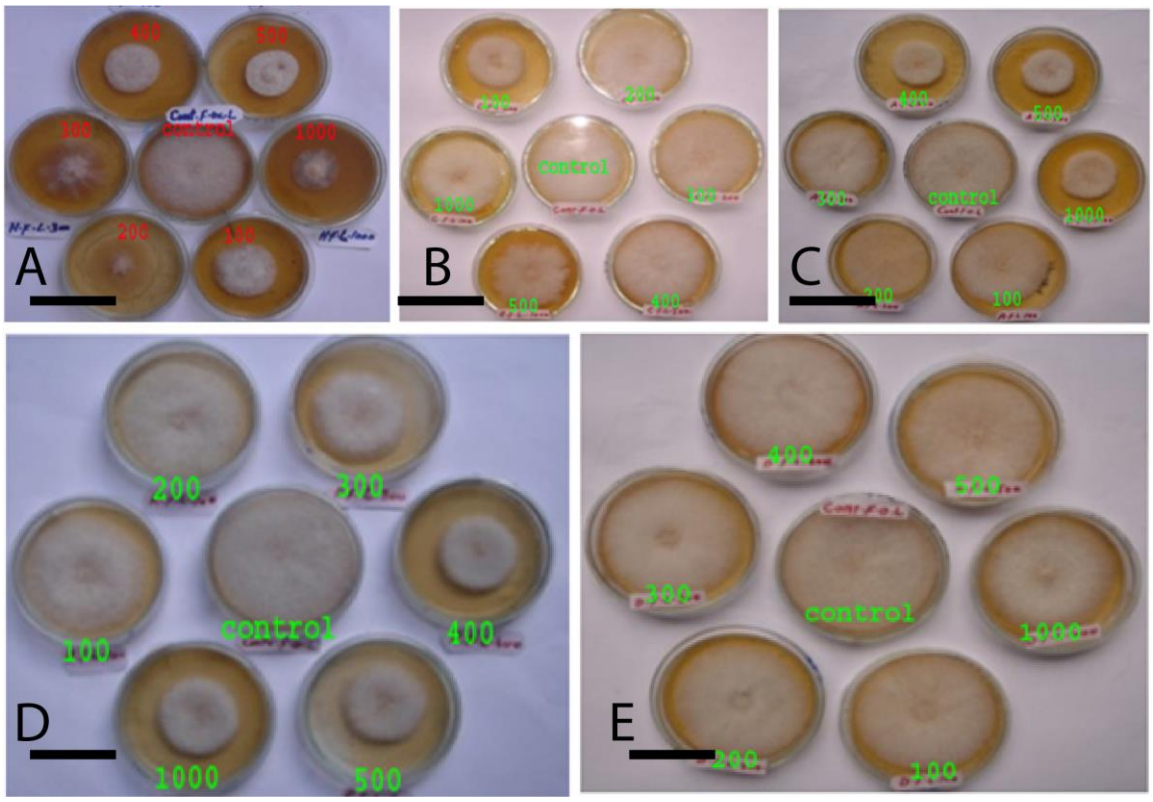

Fig. 2 – Effect of different fungicides on radial colony growth of *Fusarium oxysporum* f. sp. *lentis* at 100, 200, 300, 400 and 500 ppm. A Helonil. B Clipper. C Antracol. D Ridomil. E Desomil. Scale bars: A–C = 5.5 cm, D–E = 4 cm.

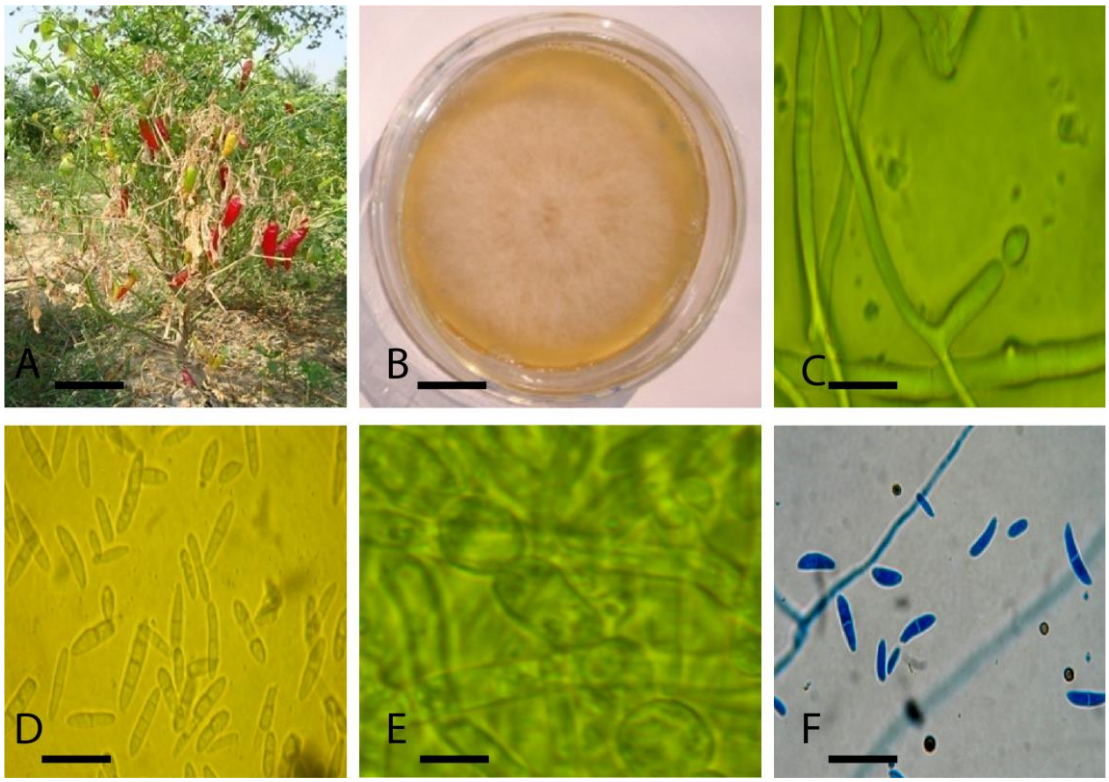

Fig. 3 – Morphology of *Fusarium oxysporum* f. sp. *capsici* and infected host plants: A Symptoms of *Fusarium* wilt in chili (*Capsicum frutescens*). B Pure culture on MEA medium. C Short monophilide. D Macroconidia. E Chlamydospores. F Microconidia. Scale bars: A = 7.5 cm, B = 1.7 cm, C, D = 25 µm, E–F = 11 µm.

Table 3 Radial colony growth along with %inhibition of *Fusarium oxysporum* f. sp. *capsici* with fungicides.

Concentration (ppm)	Fungicides									
	Helonil		**Clipper**		**Antracol**		**Ridomile**		**Desomil**	
	Radial growth (mm)	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**
100	57.1 b	36.4	83.6 b	7.0	66.3 c	26.3	84.0 bc	6.6	78.3 cd	12.9
200	55.0 b	38.8	82.6 b	8.1	65.0 cd	27.7	82.5 bc	8.3	77.6 cd	13.7
300	56.0 b	37.7	73.3 d	18.5	61.1 c	32.0	85.3 b	5.1	82.3 b	8.5
400	44.1 c	50.9	74.3 d	17.4	71.3 b	20.7	81.3 c	9.6	80.1 bc	10.9
500	35.8 d	60.1	80.6 c	10.3	64.0 d	28.8	63.3 e	29.6	80.3 bc	10.7
1000	35.0 d	61.1	79.3 c	11.8	66.6 c	25.9	77.6 d	13.7	76.1 d	15.3
Control (Untreated)	90	0.0	90	0.0	90	0.0	90	0.0	90	0.0
LSD (P=0.05)	3.2		1.6		2.2		3.1		2.7	

[a]Mean values followed by different letters in the same are different at *P*=0.05

Radial colony growth along with percentage inhibition of *R. solani* with fungicides are given in Table 4. Among the tested fungicides Helonil was found the most effective with 100 % inhibition at all concentration followed by Clipper with 100% inhibition at 1000 ppm and then Antracol with 73.8% inhibitions at 1000 ppm. Least inhibition was found with Ridomil (8.0%) at 100 ppm (Fig. 4 F–I).

Discussion

In Pakistan, application of fungicides is the most convenient and predominant way to control plant diseases. Most formers use locally available fungicides for all type of fungal diseases. However, the present results indicate that there is a significant difference among the five fungicides tested. None of the fungicides is uniformly effective with a single concentration. Helonil (Chlorothalonil) was recorded as the best fungicide at all concentration over other fungicides, followed by clipper and Antracol. Helonil was most effective at a concentration of 1000 ppm against *Fusarium oxysporum* f. sp. *lentis* and *F. oxysporum* f. sp. *capsici* while it was highly effective at all concentration against *Rhizoctonia solani*. Clipper was ineffective against *Fusarium* species with less than 35% inhibition, however, it was highly effective at concentration of 1000 ppm against *R. solani* with 100% inhibition. Antracol was effective at a concentration of 1000 ppm against *R. solani.* Ridomil was effective against *R. solani* at a concentration of 1000 ppm. Disomil was ineffective with less than 30% inhibition of growth.

Thus, commonly available fungicides are effective against fungal pathogens but not at all concentrations. It is recommended that Chlorothalonil be utilized in the field instead of other fungicides. It is also recommended for the agrochemicals to formulate such effective concentration of fungicides against different fungal isolates. The present investigation will be very useful for the local growers in controlling different fungal diseases.

The above results agree with the finding of Hawamdeh & Ahmad (2001). Braithwaite et al. (1996) found that chlorothalonil was the best fungicide against *Alternaria* species in vivo. Rehman (2011) evaluated Chlorothalonil against *Botryodiplodia theobromae in vitro* and reported that it is the second most effective fungicide after Carbendazim. Ashoka (2005) also listed chlorothalonil among the most effective fungicides. Begum et al. (2010) reported that Ridomil was the most Ridomil was ineffective against mycelial growth. Mamza et al. (2008) reported that Mancozeb gave the least inhibitory effect on mycelial growth but there was no significant difference between 1.0x and 0.5x concentrations. Iqbal et al. (2010) reported Carbindazim, hexacanazole, and Benlate, among the superior fungicides with 100% inhibition against *Fusarium* spp. He further listed copper

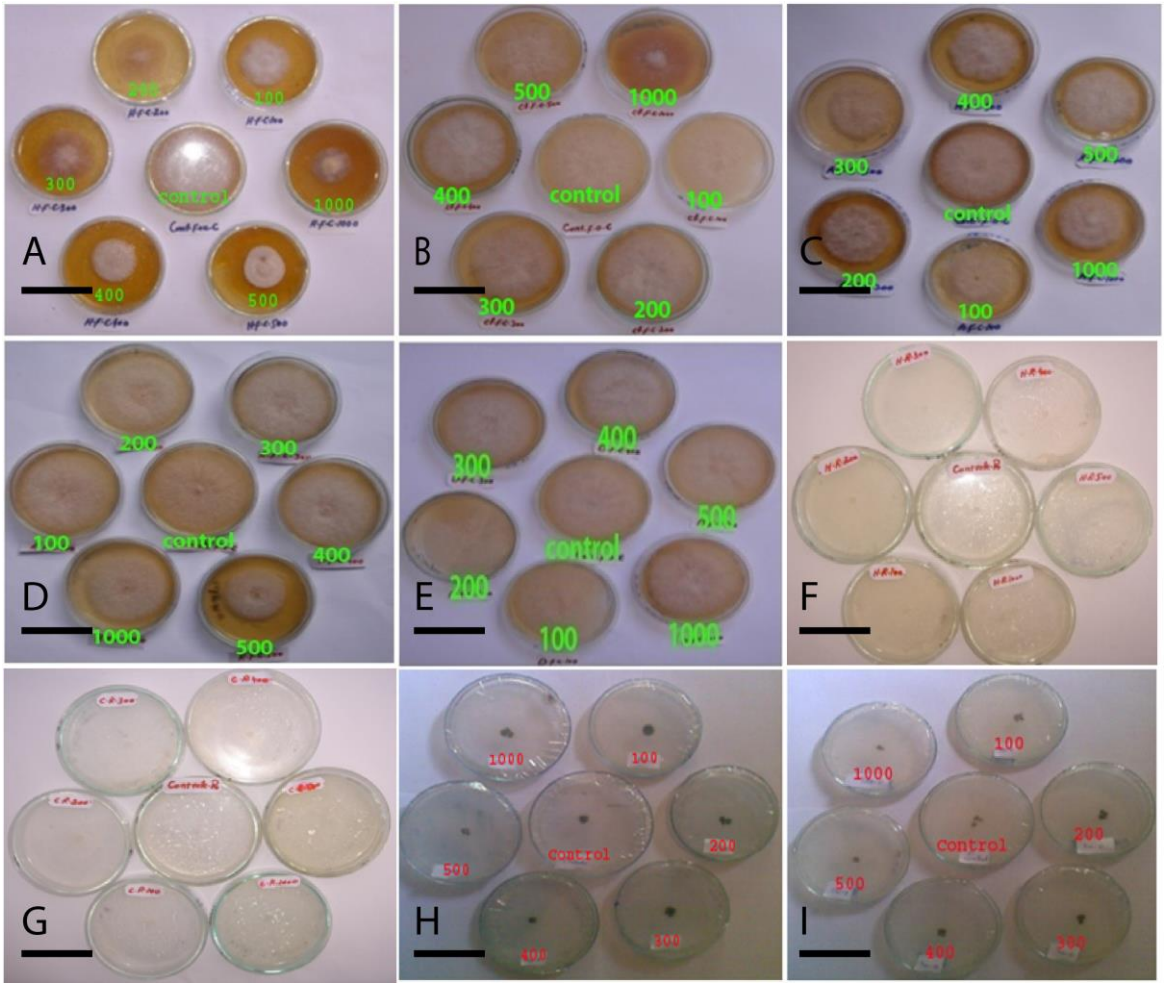

Fig. 4 – Inhibitory effects of different fungicides on radial growth of *Fusarium oxysporum* f. sp. *capsici* and *Rhizoctonia solani* at different concentration A, F Helonil against *F. oxysporum* f. sp. *capsici R. solani*. B, G Clipper against *F. oxysporum* f. sp. *capsici* and *R. solani*. C, H Antracol against *F. oxysporum* f. sp. *capsici* and *R. solani*. D, H Ridomil against *F. oxysporum* f. sp. *capsici* and *R. solani*. E Desomil against *F. oxysporum* f. sp. *capsici.* Scale bars: A–E = 6.5 cm, F–I = 5.5 cm.

Fig. 5 – Morphology of *Rhizoctonia solani* A Symptoms of *Rhizoctonia* black scurf on potatoes. B Hyphae of *R. solani.* Scale bars: A = 1.6 cm, B = 16 µm.

Table 4 Radial colony growth along with percentage growth inhibition of *Rhizoctonia solani* with fungicides.

Concentration (ppm)	Fungicides									
	helonil		**clipper**		**antracol**		**ridomile**		**desomil**	
	Radial growth (mm)	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**	**Radial growth (mm)**	**Inhibition (%)**
100	0.0 b	100	35.0 d	61.1	64.0 b	14.6	69.0 b	8.0	55.3 c	24.5
200	0.0 b	100	34.5 d	61.6	59.0 d	21.3	68.0 c	9.3	58.0 b	20.9
300	0.0 b	100	33.8 d	62.4	56.0 e	25.3	44.0 d	41.3	59.1 b	19.3
400	0.0 b	100	41.3 b	54.0	48.0 f	36	40.0 e	46.6	52.0 d	29.0
500	0.0 b	100	36.8 c	59.0	60.0 c	20	39.0 f	48.0	54.1 cd	26.1
1000	0.0 b	100	0.0 e	100	19.6 g	73.7	37.0 g	50.6	54.0 cd	26.3
Control (Untreated)	90.0	0.0	90	0.0	75	0.0	75.0	0.0	73.3	0.0
LSD (P=0.05)	0.0		1.270		0.9		0.6		2.3	

Mean values followed by different letters in the same are different at *P*=0.05

oxychloride among the best category but we placed it at 3rd with least inhibition. The same authors listed chlorothalonil with 91% of inhibition which is congruent with our findings. It should be noted that in the present experiments no one fungicide was able to give 100% inhibition against mycelia growth of *F. oxysporum*. As we selected available fungicides, which are commonly used by farmers, we can only recommend chlorothalonil against *Fusarium.* Most fungicides have some inhibitory effect but few gave us 100% inhibition.

It should be noted that most researcher used PDA media during in vitro evaluation tests and culturing of *F. oxysporum*. The use of MEA in current research is due to the fact that *F. oxysporum* showed best growth on this medium. The growth of *F. oxysporum* on PDA was checked on 20th day and compared to the growth on MEA on 4th day. It seems clear that MEA is the best media for culturing of *F. oxysporum.*

Among the five tested fungicides, Helonil (chlorothalonil) was found to be the most effective fungicide against all tested fungal isolates. The results of present investigation indicated that the fungicides used for evaluation have inhibitory effects on selected strains but at different concentrations. Due to local environmental and edaphic conditions the use of some fungicides for broad spectrum use is questionable. The present study clearly indicates that none of fungicides used in Mansehra has the same inhibitory effect on all local pathogens in the same concentration, so the formulation of these fungicides should be re-evaluated in the future. It has been observed that many fungicides used in fields do not show the efficacy in vitro against the same fungal pathogens and vice versa. Ridomil, Desomil and Antracol are not highly effective fungicides against selected fungal pathogens. Therefore, farmers should use those fungicides in district Mansehra, KP, Pakistan and adjacent countries which have already been evaluated and found to give fruitful results.

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