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STUDIES OF ASPERGILLUS BLACK SPOT DISEASE OF POMEGRANATE CAUSED BY *ASPERGILLUS NIGER* IN PUNJAB

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ABSTRACT

Twenty isolates of *Aspergillus niger* were recover from diseased fruits and morphologically characterized. All the 20 isolates were inoculated on five cultivars of pomegranate (Ganesh, Ruby, Bhagwa, Jyoti and Mridula) for pathological characterization and were clustered using UPGMA on basis of the incubation period. It was observe that four isolates namely As4, As5, As6 and As7 were highly virulent on almost all cultivars. Two cultivars namely Ruby and Mridula were found less susceptible as compared to other and both restricted infection of 45% isolates of *A. niger*. Six fungicides (Tilt, Folicur, Natio, SAAF, Bavistin and Dithane Z-78) tested against this pathogen under *in vitro* and *in vivo* condition. All the fungicides were able to reduced 50% pathogen growth (EC_{50}) at $<$1ppm. However, they varied in EC_{90} values. Less than 20ppm concentration of azole group fungicides (Tilt and Folicur) showed 90% reduction of pathogen mycelium. Natio fungicide showed 90% reduction at $<$25ppm, while others fungicides *viz*. SAAF, Bavistin and Dithane Z-78 required $>$25ppm dose to reduced that growth. In the field conditions, 90% and 85% disease reduction with yield increment of 22.7% and 20.9% was recorded via application of Tilt and Folicur fungicides respectively.

INTRODUCTION

Pomegranate (*Punica granatum* L.) is an earliest and vital fruit crop of sub-tropical and tropical regions of the world (Ashish and Arora, 2014). India is the world's leading pomegranate producer with nearly 50% of world's production. Pomegranate area under the country is 132 thousand hectare with production of 1357 thousand MT in 2014-2015 (Anon., 2015). Pomegranate black spot disease, known as "heart rot" or "black heart,"is a major pomegranate disease that impacts on production worldwide (Holland *et al*., 2009; Pala, *et al.,* 2009; Tziros, *et al.,* 2008). Many fungi are responsible for cosmetic black spot and heart rot such *Alternaria spp*., *Penicillium* sp., *Botrytis* sp., and *Rhizopus* sp., beside these fungi *Aspergillus niger* also have important role in black spot and heart rot development (Michailides, *et al.,* 2011). In Punjab, this disease invariably appears every year in the pomegranate orchards causing significant yield and quality loss. The disease is more sever in rainy season and fruit symptoms appeared in two forms; spherical depressed spots occurred in scattered form on the pericarp only and black rot restricted to internal fruit tissues. Fungicides had important role to control fungal plant pathogens. They inhibited the growth of the fungi under *in vitro* and *in vivo* condition (Archana and Jamadar, 2014; Mesterházy, *et al*., 2012; Nel, *et al*., 2007). Fungicides have different mode of action to control the fungal growth. Mista *et al*. (2011) used sterol demethylation inhibitors fungicides namely propiconazole and hexaconazole with conc 0.1% to control blight disease of Sunflower. Azoxystrobin fungicides have potential to inhibit mitochondrial respiration and blocking the cytochrome bc1 complex can be used to control *Alternaria solani* pathogen causing agent of potato early blight (Pasche *et al*. 2004). In India no comprehensive study is available on characterization and management of this disease. The present study was, therefore, undertaken to identify and characterize the pathogen with respect to its aggressiveness and also to evaluate different fungicides against this pathogen.

MATERIALS AND METHODS

Isolation and identification of the pathogen

Diseased fruits were collected from Punjab Agricultural University, Pomegranate Research Block, Fruit Research Station, Jallowal- Lesriwal Jalandhar, Punjab in the month of June-July, 2015 (Fig. 1A and B). After surface sterilization, the peel pieces were placed on potato dextrose agar separately and grown at 25 ^{o}C for 2-4 days. The rising fungal hyphal tips were transferred to PDA and grown for 4-5 days, and cultures were further purified by single spore isolation method. The morpho-logical characteristics of the fungi were analyzed using Leica microscope at 100X. Twenty isolates of the Aspergillus black spot pathogen were recovered and used for this study.

Pathological characterization and aggressiveness

All the 20 isolates were grown individually on PDA medium and PDA-plugs, 5 mm in diameter, with dynamically grown mycelium were transferred on fruit wounds made by a scalpel on earlier sterilized fruit surfaces. Full sized pomegranate fruits of all cultivars *viz*. Jyoti, Ganaesh, Ruby, Mridula and Bhagwa were inoculated with individual isolate (Tziros, *et al*. 2007). After inoculation fruits were covered with plastic bags for 5-10 days and moisture was maintained by hand sprayer in

plastic bags. The fruits inoculated with PDA medium only served as control. After inoculation, the plants were frequently irrigated to keep up high humidity and soil moisture which is vital for disease development. The disease symptoms were observed with varying day's interval. Pathogen virulence efficiency was recorded on the basis of incubation period. The pathogen was re-isolated from the infected inoculated fruits and reconfirmed as *A. niger*.

In vitro* efficacy of different fungicides against *A. niger

Fungicides (Table 1) were tested to determine their 50% effective concentration (EC_{50}) and EC_{90} val-ues for the inhibition of mycelial growth. To investigate the inhibi-tion of mycelial growth of *A. niger* five replicate PDA plates (90 mm in diameter) con-taining the fungicides were prepared at concentrations of 1, 10, 20, 25 and 50ppm. The control plates contained only PDA medium. Individual agar disks (6 mm in diameter) were removed from the edge of an actively growing culture (As 4) and placed at PDA plates that containing fungicides and incubated at 25ºC for 3-5 days. The mycelium growth of pathogen was measured by scale and compared with control growth. The concentration of each fungicide causing 50% (EC_{50}) or 90% (EC_{90}) reduction in mycelial growth compared to the absence of the fungicide was estimated referring to Matheron and Porchas (2000) and based on the estimated values.

The experiment was conducted at Punjab Agricultural University, Pomegranate Research Block, Fruit Research Station, Jallowal- Lesriwal Jalandhar, Punjab in the month of June-July, 2016.

Ten years old sick plants of pomegranate cv. Bhagwa were used for this experiment where disease occurred regularly. Each treatment spray was used two times, one in month of June (first week) and second in July (first week). The yield data was recorded at the time of harvesting in terms of kg/plant. Seven treatments were applied in this experiment viz; T1-Natio@1gram/lit; T2-Folicur@1ml/lit; T3-Tilt@1ml/lit; T4-Bavistin@1gram/lit; T5-Dithane- Z 78@2gram/lit; T6-SAAF@1gram/lit and T7-Control.

Statistical analysis

The pathotypic similarity between the various isolates was generated using unweighted paired group mean averages using software programme PAST ver. 2.1.5.

RESULTS

Isolation and identification of the pathogen

Twenty isolate were recovered from infected fruit samples. All the recovered isolates were showed specific morphological character of *A. niger*. The culture of A. *niger* initially showed white colonies which quickly turns black on Potato dextrose agar (PDA) at 25 ± 2ºC (Fig. 1D). The mycelium was septate and produced conidiophores. Conidiophores are long, smooth and hyaline becoming darker at the apex and

Table 1:Efficacy of different fungicides against *A. niger* under field condition"

Fungicides	Chemical group	Active ingredient	Concentration(%)	Formulation
Folicur	Azole	Tebuconazole	25.9	EC
Tilt	Azole	Propiconazole	25	EC
Natio	Mixture of Azole and Strobilurin	Tebuconazole + Trifloxystrobin	50 + 25	WG
SAAF	Mixture of benzimidazole and Carbamate	Carbendazim + Mancozeb	12 + 63	WP
Bavistin	benzimidazole	Carbendazim	50	WP
Dithane Z-78	Carbamate	Zineb	70	WP

Table 2: Disease reaction of *A. niger* isolates on five pomegranate cultivars in the field condition

Isolates	Ruby	Ganesh	Jyoti	Mridula	Bhagwa
As1	0	22	21	0	19
As2	0	20	19	0	20
As3	0	19	20	0	21
As4	12	7	8	12	11
As5	13	8	9	13	11
As6	12	7	10	13	10
As7	11	9	12	12	9
As8	18	18	18	18	19
As9	17	17	18	17	17
As10	17	16	17	18	17
As11	18	17	17	17	18
As12	15	18	18	15	18
As13	17	18	17	18	17
As14	15	14	14	15	15
As15	0	21	21	0	21
As16	0	22	19	0	20
As17	0	20	20	0	19
As18	0	19	21	0	19
As19	0	23	23	0	20
As20	0	22	20	0	21

Table 3: Grouping of 20 isolates of *A. niger* on the basis of incubation period on five pomegranate cultivars

Group	Name of Isolates	No. of Isolates	Incubation Period (days) Mean	Range	Remark
A	As4, As5, As6 and As7	4	10.45	7-13	Highly Virulent
B	As8, As9, As10, As11, As12, As13 and As14	7	16.94	15-18	Moderately Virulent
C	As1, As2, As3, As15, As16, As17, As18, As19 and As20	9	20.44	19-23	Less Virulent even theses are not able to cause disease in cv. Ruby and Mridula

Table 4: *In vitro* efficacy of different fungicides against *A. niger*

Fungicides	Mycelial growth (mm) Concentration in ppm 1	5	10	20	25	50
Natio	25(.53)	18.5(.54)	19(.53)	12(.26)	0	0
Folicur	25(.43)	19.2(.56)	11.3(.33)	0	0	0
Tilt	20(.23)	13.2(.32)	9.5(.22)	0	0	0
Bavistin	36(.73)	31(.22)	29(.23)	16(.19)	12(.43)	5(.53)
Z-78	38.3(.32)	32.5(.22)	28.2(.73)	25.3(.28)	20.53(.53)	18.6(.53)
SAAF	35(.63)	30(.32)	28(.73)	15(.37)	10(.27)	4(.73)

Figures in parentheses are standard deviations from mean of five replications

Table 5: EC_{50} and EC_{90} values of mycelial growth of *A. niger* for the six fungicides

Fungicides	EC_{50}	EC_{90}
Natio	< 1ppm	< 25ppm
Folicur	< 1ppm	< 20ppm
Tilt	< 1ppm	< 20ppm
Bavistin	< 1ppm	< 50ppm
Z-78	< 1ppm	> 50ppm
SAAF	< 1ppm	< 50ppm

terminating in a globose vesicle. Metulae and phialides cover the entire vesicle and conidia are brown to black, globose and measure 4-5 μm in diameter.

Pathological characterization and aggressiveness

Response of different isolates with respect to aggressiveness was studied. The aggressive of the isolates were measured on the based incubation period. Disease symptoms were observed after seven day of inoculation with necrosis surrounded by water soaked area and superficially fruit rotting which was gone deep after some time. Many authors also widely acknowledged about heart rot type of symptom similarity within fungi *viz*. *Penicillium spp*., *Alternaria alternata* and *A. niger* (Tziros, *et al*., 2007; Bardas, *et al*., 2009; Ezra, *et al*., 2010; Gat, *et al*., 2012; Michailides, *et al*., 2011; Zhang and McCarthy, 2012). The data presented in Fig. 2 shows the presence of significantly diverse virulence spectrum in *A. niger* populations of Punjab. Disease response with respect to 20 isolates of *A. niger* clustered using UPGMA (Fig. 2). The data plotted in dendrogram (Fig. 2) showed four isolate *viz*. As4, As5, As6 and As7 to be clustered together and were classified in group A. These isolates were found to be highly virulent on almost all the cultivars tested producing disease with the minimum mean of incubation period 10.45 days (Table 2 and 3). Seven out of all the isolate showed more days for incubation period ranging from 15 to 18 days with mean 16.94 and were classified as moderately virulent (Table 2 and 3). Nine isolate were used more time as compared to group A and B for disease development and fall down in group C. These isolates were unable to produced disease in two cultivars *viz*. Ruby and Mridula (Table 2 and 3).

Virulence frequency of *A. niger* isolates on five pomegranate cultivars

In present inadequate work might be available regarding screening of pomegranate germplasm against this disease. In the present study five pomegranate cultivars were screened (Fig. 3) which showed different reaction to Punjab population of *A. niger*. Twenty percent isolates showed highly virulent reaction on the basis of incubation on all cultivars Jyoti, Ganesh, Bhagwa, Ruby and Mridula. Thirty five per cents isolates caused moderately virulence in all cultivars. However, forty five per cents isolates which showed less virulent reaction on cv. Ganesh, Jyoti and Bhagwa were found non virulent on Ruby and Mridula (Fig. 3). It was observed that both cultivars Ruby and Mridula showed resistance to less virulent isolates and both were comparatively less susceptible to Punjab population of *A. niger*.

In vitro* efficacy of different fungicides against *A. niger

The study revealed the efficacy of several fungicides *viz*. Natio, Folicur (tebuconazole), Tilt (propiconazole), Dithane Z-78, Bavistin and SAAF against pathogen (Table 4 and 5). The EC_{50}/ EC_{90} values of all fungi-cides against *A. niger* were observed (Table 4 and 5). The EC_{50} values of *A. niger* for the all fungicides were < 1ppm, these reduced the fungi growth more than half at this concentration (Table 4, 5 and Fig. 4). Azole group fungicides (Tilt and Folicur) were inhibited the mycelial growth of *A. niger* most effectively even fungi not able to start growth at concentration of 20 ppm com-pared to the others fungicides (Natio, Bavistin, Dithane Z-78 and SAAF) (Table 4, 5 and Fig. 4). The EC_{90} values of *A. niger* for Natio was 25ppm while SAAF and Bavistin fungicides stand on 50ppm. In this concentration growth of fungi reduced > 90% (Table 4, 5 and Fig. 4). Dithane Z-78 was show less effective with EC_{90} > 50

Table 6: Evaluation of different fungicides against *A. niger* under field conditions

Treatments	Mean No. of infected Fruits per plant	Maximum no. of black spot on individual fruit	Mean No. of fruit rot	Yield (kg) per plant
T1-Natio@1gram/lit	7	5	0	21
T2-Folicur@1ml/lit	6	6	0	21.5
T3-Tilt@1ml/lit	4	4	0	22
T4-Bavistin@1gram/lit	9	7	0	21
T5-Dithane- Z 78@2gram/lit	15	6	2	20
T6-SAAF@1gram/lit	10	6	0	21
T7-Control	40	>10	7	17

Figure 1: A and B show natural symptom of Aspergillus black spot disease, C- Disease free fruit after Tilt spray and D- Culture of *A. niger*

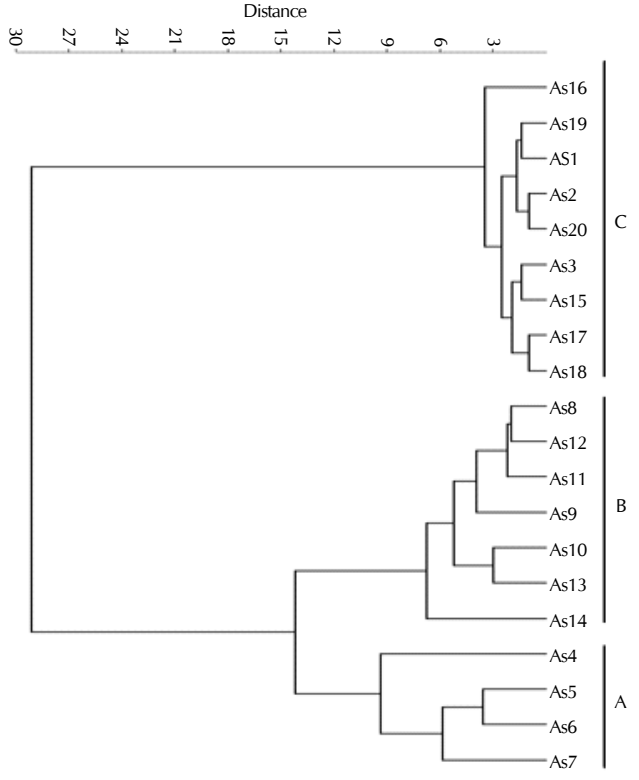

Figure 2: Clustering of different isolates of *A. niger* on the basis of incubation period character

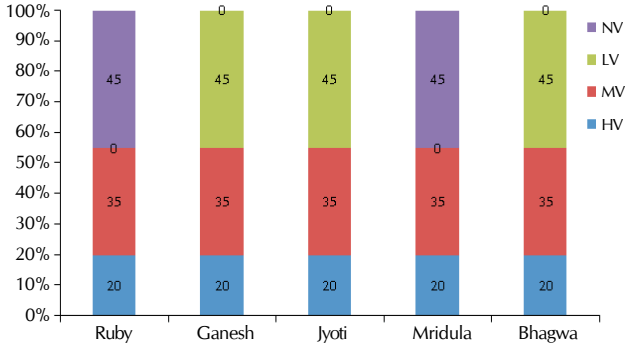

Figure 3: Virulence frequency of 20 isolates of *A. niger* on five pomegranate cultivars

ppm. Natio was the third best fungicide which reduced the mycelia growth efficiently with 25ppm. Present study revealed that azole fungicides (Tilt and Folicur) were more effective to inhibiting the mycelial growth of *A. niger* than other fungicides based on EC_{50} and EC_{90} values (Table 5).

Efficacy of different fungicides against *A. niger* under field condition

In the field condition azole also dominant to reduced the *Aspergillus* black spot disease. It also observed that the fungi like *A. alternata*, *Penicillium spp*. which caused approximately same black spot symptom on pomegranate also reduced (Labuda *et al.*, 2004; Tziros *et al.*, 2007 and Pala et al., 2009). The trails were revealed that application of azole fungicides (Tilt and Folicur) was very effective in controlling the disease (Table 6, Fig. 1C and Fig. 5). Tilt fungicide was most effective where only four infected fruits with maximum four black spot observed. The disease reduction % of Tilt was 90% with increase yield % 22.7 (Fig. 5). It was at par with the treatment -2 where Folicur was used. Natio was the third best fungicide where disease reduction was 82.5 with yield increase % 19.05. SAAF and Bavistin showed approximately similar disease reduction % with same yield increase % 19. It was also

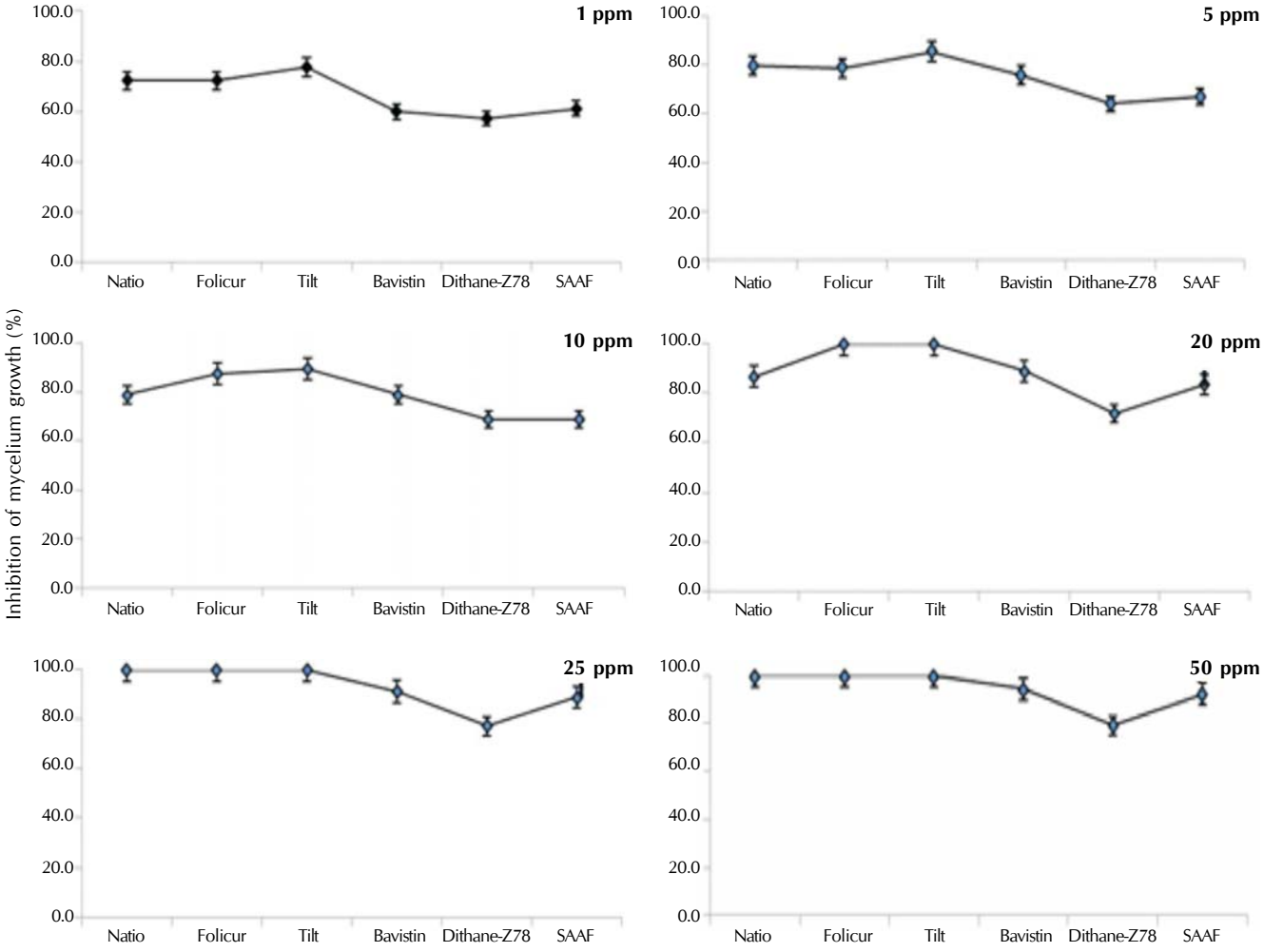

Figure 4: Effect of different fungicides *i.e.* Natio, Folicur, Tilt, Bavistin, Dithane Z-78 and SAAF on mycelial growth of *A. niger* at different concentrations

observed that Dithane- Z-78 did not show significant control over the disease in field conditions. Several authors have also reported the control of black spot complex disease of pomegranate.

DISCUSSION

In the current studies, twenty isolate of *A. niger* were recovered from diseased fruit. All the recovered isolates were showed specific morphological character of *A. niger* with long conidiophores, becoming darker at the apex and terminating in a globose vesicle. Metulae and phialides cover the entire vesicle and conidia are brown to black, globose and measure 4-5 μm in diameter. Similar characters of *A. niger* also described by Wani and Taskeen-Un-Nisa (2011) after isolation of the pathogen from onion.

In present inadequate work might be available regarding screening of pomegranate germplasm against this disease. The pomegranate cultivars showed diverse disease reactions to pathogen based on incubation period and pathogen isolates were categorized into four groups, highly virulent (HV) moderately virulent (MV), less virulent (LS) and non virulent

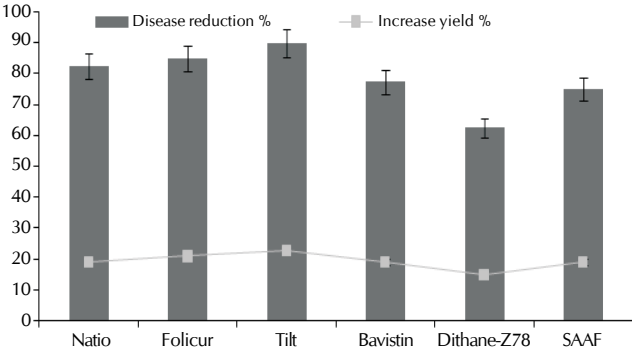

Figure 5: Effect of different chemicals on percent reduction in Aspergillus black spot disease and increase in yield

(NV). The pathogen used virulence factors such as mycotoxins (oxalic acid, malformin A and malformin C) which helped in disease development (Anderegg *et al*., 1976). In the current study we were able to discover that two cultivars namely Ruby and Mridula showed less susceptible reaction as compared to other three cultivars *viz*. Ganesh Jyoti and Bhagwa. Less susceptibility might be happening due to the highest

concentration of antioxidant enzymes in the studied pomegranate cultivars (Arfaoui, *et al.,* 2007; Santigo, *et al.,* 2009; Pal, *et al.* 2011; Parihar, *et al.,* 2012).

Antifungal activity of six fungicides acquired in present study is in conformity with several previous findings (Tofali, *et al.* 2003; Wani and Taskeen-Un-Nisa, 2011; Gondal *et al.,* 2012; McKay, *et al.* 2012; Saxena, *et al.*, 2014). In current study all the fungicides showed fifty % reduction of *A. niger* with same concentration while varied in 90% fungal growth reduction under *in vitro* condition. Azole group of fungicide was found most effective under *in vitro* and field condition to reduce the disease. These are sterol demethylation inhibitors (DMIs) that inhibit the C-14 á-demethylation of 24-methylenedi hydrolano sterol, a pre-cursor of ergosterol in fungi (Yin, *et al.*, 2009). Many authors also widely acknowledged role of tebuconazole and propiconazole under *in vivo* and *in vitro* condition to control plant pathogenic fungi in different crops (Koller and Scheinpflug, 1987; Islam, *et al.*, 2007; Iviæ, *et al.,* 2011). Natio, SAAF and Bavistin also found effective respectively. However Dithane Z-78 fungicide was less effective according to all. Similarly, severity of black mold rot of onion reduced significantly after dip treatment with azole, carbamate and benzimidazole fungicides (Wani and Taskeen-Un-Nisa 2011). Formenti *et al.* (2012) also used azoles fungicides to control *Aspergillus* sp. and *Fusarium* sp. and they found significant results. Jamadar and Patil (2007) found efficacy of difenconazole (Score 25EC) and prochloraz 45EC @1.00 ml/l each in the management of pomegranate leaf spot and fruit spot achieving more than 74.0 to 86.7 per cent reduction of the disease respectively over unsprayed control. Many studies have shown that role of these fungicides to disease reduction (Nel *et al.*, 2007; Iviæ, *et al.*, 2011; Mesterházy, *et al.*, 2012; Shin, *et al.* 2014; Joshi and Gohe, 2015; Bandyopadhyay, *et al.,* 2015; Gurudatte, 2015).

From the current study it is evident that diversity exist in Punjab isolates of *A. niger* with respect to incubation period they are differentially aggressive on five pomegranate cultivars. It is also concludes that azole group of fungicide found more effective under *in vitro* and field condition as compared to other used fungicides.

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