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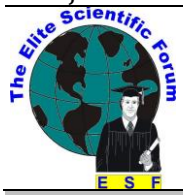

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RESEARCH ARTICLE

Emerging Resistance in *Alternaria solani* Against Different Fungicides in Southern Punjab, Pakistan

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ABSTRACT

Early blight of tomato caused by *Alternaria* (*A.*) *solani* is a devastating disease reducing not only quality but also the yield of tomato fruit. Fungicides application is the one of the most effective and rapid mean of managing fungal pathogens. However, several applications of a fungicide against same pathogen lead to the development of fungicide resistance. In this study, *A. solani* isolate obtained from farmers' fields located near Industrial State, Multan, Southern Punjab was tested to evaluate efficacies of different registered fungicides at different doses of 25, 50, 75, 100 µg mL^{-1} by using poison food technique as well as to check the resistance of tested pathogen against same set of fungicides. It is evident that Bravo, Score, Camelot and Cabrio Top not performed well and inhibited mycelial growth ranged between 6-35% at 100µg mL^{-1} concentration. While Bravo and Score at 25 µg mL^{-1} concentration completely failed in reducing mycelial growth of *A. solani*. Contrarily, Dithane Z-78, Dithane M-45, Cobox, Heritage and Maxim at 100 µg mL^{-1} concentration depicted significant reduction (56-85%) in mycelial growth of *A. solani* as compared to control. These findings revealed that field population of *A. solani* developed resistance against Score and Bravo that were previously considered best solution against same pathogen. It is suggested that Dithane Z-78, Dithane M-45 and Heritage can be replaced with Bravo and Score to manage this pathogen effectively. The experiment results revealed that *A. solani* developed resistance which reduced efficacy of fungicide applications as compared to earlier published data. The results provide information on the application and selection of fungicides in the overall disease management of tomato crop in Pakistan.

INTRODUCTION

Tomato (*Lycopersicum esculentum* Mill) belongs to family Solanaceae and is 2nd important processing crop all over the world after potato. Tomato fruit contains water, calcium and niacin which are of significant importance in humans metabolic activities as well as it is good source of vitamin A, C and E and antioxidant with high nutritional value to maintain human health (Olaniyi et al., 2010). In Pakistan, tomato covered 0.58 million hectares with 0.57 million tonnes production annually which is very low compared to other countries.
V arious biotic factors including fungi, viruses, bacteria nematodes and insects are responsible for its low production (Picó et al., 1996). Fungal pathogens are responsible for remarkable yield losses of various crops as biotropic pathogen throughout the world (Gonzalez-Fernandez et al., 2010; Akhtar, 2004; Chohan et al., 2015). About 85% plant diseases have been caused by more than 20, 000 fungal plant pathogens (Ong, 2011). One of the fungi, *Alternaria* (*A.*) *solani* cause early blight which is a devastating disease reducing not only quality but also the yield of tomato fruit each year (Tewariand Vishunavat, 2012; Abada et al., 2008; Abdel-Sayed, 2006). The *A. solani* is air borne soil inhabiting fungi which caused leaf blight, fruit and collar rot (Datar and Mayee, 1981). On lower side of infected leaves, bull eye shaped patterns having concentric rings of brown-black spots appeared (Chaerani and Voorrips, 2006) resulting these leaves exhibited pale yellow color and dry (Gleason and Edmunds, 2005). Pathogen causes infection on petiole, stem twigs, leaf and fruits as well as lead to drying of infected parts, defoliation and premature fruit drop

which reduce the yield and host plants are more susceptible during fruiting stage under high temperature, long period of leaf wetness and humidity (Momel and Pemezny, 2006).

Different methods are used to control blight disease which include cultural practices, preventing wetness on surface of leaf, sanitation and resistance developed in host plant by application of different fungicides (Namanda et al., 2004). The disease may cause colossal losses if protective measures are not adopted well in time. Fungicides application is the one of the conventional, most effective and rapid measure of controlling fungal pathogens causing various notorious diseases in plants (da Silva Pereira, 2012). Various fungicides commercially available with different modes of action and effective against number of pathogens including *A. solani.* Many scientists focused on the comparative efficacy of different systemic and contact fungicides (Chourasiya et al., 2013; Patel and Choudhary, 2010; Mate et al., 2005).

Chlorothalonil has a multi-site activity and disrupts enzyme activity in the cell and considered as a low risk for resistance (Tillman et al., 1973). *A. solani* population has a history of developing resistance two years after introduction against few fungicides belongs to quinone outside inhibitors (Pasche et al., 2005; Rosenzweig et al., 2008). About 15 and 58% population of *A. solani* isolates was reported resistant, collected from Idaho during 2009 and 2010 respectively (Fairchild et al., 2013). Several fungicides inhibit spore penetration and germination, but pathogen spores can develop resistance against them. To avoid risk of fungicides resistance, fungicides belongs to different groups must be applied with proper intervals at recommended doses (Kirk et al., 2005). There is a dire need to adopt anti-resistance strategies upon introduction of new fungicides especially high-risk single site fungicides (Lucas, 2006). Development of resistance against single-site fungicides can be minimized by spraying with multi-site protectants like mancozeb and chlorothalonil (Miller and Miller, 2004; Zitter and Drennan, 2005).

Same chemistry fungicides under field conditions not work properly either due to regular application at same recommended doses or lead to development of resistance in fungi against specific applied fungicides. The systemic and contact fungicides belonging to different chemical group are commonly used by the farmers as a malpractice to control the fungal diseases in tomato crop, resulting to cause failure in antifungal property of fungicides in southern Punjab and thus leading to the development of resistance (Akram et al., 2018).

During survey of different tomato crop fields of the Southern Punjab it was observed that various fields were heavily infested with pathogen showing early blight disease symptoms in spite of application of different commercially available fungicides i.e. Cabrio top, Score, Cobox, Bravo, Maxim Pyranil, Epic etc. Keeping in view, present study was planned to check efficacies of different registered fungicides at different doses with the aim to evaluate most effective dose of fungicide as well as to check the resistance development in *A. solani*.

MATERIALS AND METHODS

Collection of diseased samples and isolation of pathogen

Infected leaves from fully grown tomato crops were collected from district Multan, Southern Punjab and isolate the fungi under *in vitro* conditions. The samples were put in zip lock plastic bags placed in cooler box and shifted within 5-6 h to the laboratory of Mycology, Department of Plant Pathology, Bahauddin Zakariya University Multan (30°12′ N, 71°25′ E) for further process.

Infected tomato leaves were cut into small pieces 4-5 mm along with healthy parts with sterilized scissors, disinfected in 1 % solution of sodium hypochlorite for 2 min followed by rinsing in sterile distilled water thrice. These samples were blot dried and aseptically placed on 90 mm petri plates containing 20 mL potato dextrose agar medium (Peeled potatoes = 250 g, Agar agar and Dextrose = 20 g each, , autoclaved at 121°C for 20 minutes at 103 kPa pressure) and incubated at 25±2 °C for seven days. Hyphal tip method and single spore sub-culture techniques were used to purify the obtained culture (Haggag and Farghaly, 2007). Isolated fungi was identified on basic of their specific characters e.g colony shape, color, mycelial character and spore shape with the help of optical microscope (Ellis, 1971; David, 1991).

Pathogenicity test

Seedlings of tomato variety (Moneymaker) were grown in sterilized soil by using earthen pots. Before inoculation one-month old seedlings were applied sterile distilled water and covered with polythene bags for one day. Conidial suspension (5×10^6 conidia/mL) prepared from 7-day-old culture was atomized @ 8 mL per plant and again covered with polythene bags for 2 days to ensure humidity for establishment of pathogen. Inoculated plants were then shifted to greenhouse and periodically observed for symptoms development. Pathogen was reisolated after 7 days and culture obtained was compared with the original one thus fulfilling Koch's postulates.

In vitro screening of fungicides

Screening of three systemic; Cabrio Top, Heritage, Score and six contact fungicides; Maxim, Camelot, Bravo, Cobox, Dithane M-45, Dithane Z-78 belonging to different chemical groups was done under *in vitro* conditions (Table 1). These fungicides were tested at

different concentration of 25, 50, 75 and 100 µg mL^{-1} to check their efficacy on mycelial growth of *A. solan*i by using poison food technique (Dhingra and Sinclair, 1985). Different tested concentrations of fungicides were mixed in PDA medium after autoclaving 20 mL poisoned media was poured in 90 mm diameter petriplates and permitted to solidify. Mycelial disc of 5 mm from actively growing margins of *A. solani* culture was cut by using sterile cork borer and placed at the center of plates containing poisoned medium. Plates without fungicides were used as a control and the experiment was conducted in quadruplicate. These plates were incubated at 25±2 °C for seven days under 12 hours alternate light and dark conditions. Mycelial growth of fungus was measured to examine efficacy of different fungicides and percent decrease in treated replicates over control was calculated by using following formula suggested by Nene and Thapliyal, (1993).

$$I = \frac{C - T}{C} \times 100$$

I = percent inhibition of mycelial growth
T = radial growth of fungus in treatment
C = radial growth of fungus in control

Statistical analysis

Observed data of mycelial growth of *A. solani* fungus was analyzed through analysis of variance (ANOVA). Treatment means were compared by using Fisher's Least Significant Difference (LSD) test at $p \leq 0.05$ (Russel and Eisensmith, 1983).

RESULTS AND DISCUSSION

The fungus (*A. solani*) was identified from the diseased samples of tomato on the basis of morphological characters (Ellis, 1971). The size of conidia varies 150-300 µm in length and 15-20 µm in thickness in broadest part with 8-10 and 1-4 transverse and longitudinal septa respectively with uniform or straight tapering towards beak having pale or olivaceous brown color. These conidia are produced either singly or in the form of groups on conidiophores of pale or olivaceous brown color and this is similar to the given description (David, 1991).

Pathogenicity test of isolated fungus was done on moneymaker variety and the culture produced from fungi isolated from tested plants was compared with the pure culture of fungi hence morphological similarities confirmed the association of *A. solani* with collected diseased tomato samples.

Antifungal effects of six tested fungicides on mycelial growth of *A. solani given in table 1*.The detailed effect of testing fungicide to inhibit fungal mycelial growth and percent reduction as comparison to the control is shown in Table 2 and 3. Our results showed systemic and contact fungicides have ability to reduce fungal pathogen growth which causes blight disease in tomato.

Three systemic and six contact fungicides were tested at four different concentrations i.e. 25, 50, 75 and 100 µg mL^{-1} against mycelial growth of *A. solani* and showed statistically significant results. Out of three systemic fungicides; Cabrio Top, Heritage, Score, maximum average mycelial growth 9.00, 8.03, 7.23 and 7.16 cm was found in Score while average least growth 8.03, 5.86, 5.13 and 3.53 cm was observed in Heritage at 25, 50, 75 and 100 µg mL^{-1} compared to control (Table 2). All the tested contact fungicides; Maxim, Camelot, Cobox, Dithane M-45, Dithane Z-78 exhibited better results as compared to Bravo which showed 9.00, 8.93, 8.63 and 8.40 cm average mycelial growth at 25, 50, 75 and 100 µg mL^{-1} concentrations. Whereas, average mycelial growth reduced to 6.43, 6.36, 2.36, 1.3 and 4.56, 2.33, 1.80, 1.43 cm in case of Dithane Z-78 and Dithane M-45 compared to 8.50 and 8.90 cm growth in control respectively (Table 2).

Similarly, average mycelial inhibition was less in Score, followed by Cabrio Top and Heritage. Heritage inhibited mycelial growth 10.73, 34.81, 42.96 and 60.73% compared to Score 0.00, 10.73, 19.62, 20.36 and Cabrio Top 11.48, 14.07, 14.81, 35.18% at 25, 50, 75 and 100 µg mL^{-1} concentrations (Table 3). Moreover, contact fungicides exhibited better results while some of them inhibited mycelial growth up to 85.00 %. Bravo and Camelot showed poor average mycelial inhibition i.e. 0.00, 0.74, 4.07, 6.66 and 11.48, 12.21, 12.22 and 17.03 % at 25, 50, 75 and 100 µg mL^{-1} concentrations. When the results of bravo and Camelot were compared with least performed systemic fungicides Score then later due to systemic nature exhibited better results compared to earlier contact fungicides. Furthermore, Cobox and Dithane Z-78 inhibited average mycelail inhibition efficiently with 15.18, 29.62, 66.29, 83.29 and 28.51, 29.25, 73.70, 85.55 % at 25, 50, 75 and 100 µgmL^{-1} concentrations. While Dithane M-45 exhibited maximum average mycelial inhibition 49.25, 74.07, 79.99 and 84.07% at 25, 50, 75 and 100 µg mL^{-1} concentrations respectively compared to all tested systemic and contact fungicides (Table 3).

It is evident that Bravo, Score, Camelot and Cabrio Top not performed well and inhibited mycelial growth ranged between 6-35% at 100µg mL^{-1}concentration. While Bravo and Score at 25 µg mL^{-1} concentration completely failed in reducing mycelial growth of *A. solani*. Dithane Z-78, Dithane M-45, Cobox, Heritage and Maxim at 100µg mL^{-1} concentration depicted significant 56-85% reduction in growth of *A. solani* as compared to control.

Use of fungicides to combat plant diseases is very common and immediate measure to control diseases but its uncontrolled use leads towards the development of

resistance among pathogens. Therefore, it is necessary to check the efficacy of fungicides against different pathogens at regular intervals to minimize the chances of resistance development. In this study, a comprehensive survey was carried out to collect early blight diseased samples from farmers' field on the basis of symptomatology. The main objective was to obtain *A. solani* isolate which is exposed to different commercially available fungicides and to check the efficacy of various fungicides at different doses under laboratory conditions against same isolate. The infectious fungus *A. solani* has been isolated from fruits, twigs, leaf and petioles causing severe losses in tomato production up to 50-86% (Mathur and Shekhawat, 1986).

We found that Dithane Z-78, Dithane M-45, Cobox, Heritage and Maxim at 100µg mL^{-1}concentration depicted significant reduction (56-85%) in growth of *A. solani* as compared to control. Many scientists evaluated various systemic and contact fungicides against mycelial inhibition of *A. solani*. Various fungicides i.e. benomyl, captafol, carbendazim, copper oxychloride and mancozeb showed promising results in reducing tomato early blight disease (Mate et al., 2005). Similarly, effectiveness of Dodine and Mancozeb with Alachor and Acephate was studied against *A. solani* on tomato (Sawant and Desai, 2001). Control of early blight of tomato was obtained by foliar applications of 3 consecutive sprays at fortnightly intervals (Naveenkumar et al., 2001). Tomato plants treated with Mancozeb resulting into higher yield and lower incidence of disease along with highest cost-benefit ratio compared to non-treated plants (Parsad and Naik, 2003). Furthermore, Abhinandan et al. (2004) confirmed that Dithane M- 45 and Kavach at 0.25% concentration effectively managed the disease up to 50%. Chourasiya et al. (2013) reported that mancozeb, zineb, copper oxychloride effectively reduced mycelial growth of *A. solani*. Out of nine fungicides evaluated Prochloraz followed by Saaf and Mancozeb performed

Table 1: List of fungicides belongs to different chemical group tested against mycelial growth of *A. solani in vitro*

Group Name	Chemical Group	Trade name	A.I*	Action†	Formulation	Manufacturer
Quinone Outside Inhibitor (QoI) + Dithiocarbamate	Methoxy-carbamate + Dithiocarbamate	Cabrio Top®	Pyraclostrobin + Metiram	Systemic	WP**	Arysta life science
Quinone Outside Inhibitor (QoI)	Methoxy-acrylate	Heritage®	Azoxystrobin	Systemic	WG***	Syngenta
Demethylation Inhibitor (DMI): SBI Class I	Triazole	Score®	Difenoconazole	Systemic	WP	Syngenta
Phenylpyrrole (PP)	Phenylpyrrole	Maxim®	Fludioxonil	Contact	SC****	Syngenta
Inorganic	Inorganic	Camelot®	Copper Sulphate	Contact	WP	SePRO Corp
Chloronitrile	Chloronitrile	Bravo®	Chlorothalonil	Contact	WP	Syngenta
Inorganic	Inorganic	Cobox®	Copper Oxychloride	Contact	WP	Sygenta
Dithiocarbamate& relatives	Dithiocarbamate & relatives	Dithane M-45®	Mancozeb	Contact	WP	Arysta life science
Dithiocarbamate& relatives	Dithiocarbamate & relatives	Dithane Z-78®	Zineb	Contact	WP	FMC Corp

*A.I: active ingredient, ** WP: Wettable Powder, *** WG: Water –dispersible Granule, ****SC: Suspension Concentrate

Table 2: Effect of different concentrations of systemic and contact fungicides on mycelial growth of *A. solani*

Concentrations	Average mycelial growth (cm ± Standard Error)								
	Cabrio Top	Heritage	Score	Maxim	Camelot	Bravo	Cobox	Dithane M-45	Dithane Z-78
Control	9.00±0.00^{a}	9.00±0.00^{a}	9.00±0.00^{a}	8.90±0.08^{a}	8.80±0.09^{a}	9.00±0.00^{a}	9.00±0.00^{a}	8.90±0.08^{a}	8.50±0.24^{a}
25µg mL^{-1}	7.96±0.07^{b}	8.03±0.12^{b}	9.00±0.00^{a}	8.60±0.05^{a}	7.96±0.07^{b}	9.00±0.00^{a}	7.63±0.07^{b}	4.56±0.30^{b}	6.43±0.18^{b}
50µg mL^{-1}	7.73±0.11^{b}	5.86±0.18^{c}	8.03±0.14^{b}	7.73±0.10^{b}	7.90±0.12^{b}	8.93±0.05^{a}	6.33±0.14^{c}	2.33±0.10^{c}	6.36±0.15^{b}
75µg mL^{-1}	7.67±0.19^{b}	5.13±0.10^{d}	7.23±0.10^{c}	5.20±0.22^{c}	7.90±0.09^{b}	8.63±0.07^{b}	3.03±0.10^{d}	1.80±0.09^{cd}	2.36±0.15^{c}
100 µg mL^{-1}	5.83±0.20^{c}	3.53±0.10^{e}	7.16±0.19^{c}	3.93±0.38^{d}	7.46±0.15^{c}	8.40±0.05^{c}	1.50±0.04^{e}	1.43±0.15^{d}	1.30±0.05^{d}

Means followed by the same letters in each column are not statistically significant ($P \leq 0.05$)

Table 3: Effect of different concentrations of systemic and contact fungicides on percent mycelial inhibition of *A. solani*

Concentrations	Average mycelial inhibition (% ± Standard Error)								
	Cabrio Top	Heritage	Score	Maxim	Camelot	Bravo	Cobox	Dithane M-45	Dithane Z-78
Control	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{d}
25µg mL^{-1}	11.48±0.80^{b}	10.73±1.32^{d}	0.00±0.00^{c}	4.44±0.52^{d}	11.48±0.80^{b}	0.00±0.00^{c}	15.18±0.80^{d}	49.25±3.37^{c}	28.51±1.98^{c}
50 µg mL^{-1}	14.07±1.21^{b}	34.81±1.98^{c}	10.73±1.60^{b}	14.07±1.09^{c}	12.21±1.39^{b}	0.74±0.60^{c}	29.62±1.60^{c}	74.07±1.09^{b}	29.25±1.68^{c}
75 µg mL^{-1}	14.81±2.12^{b}	42.96±1.09^{b}	19.62±1.09^{a}	42.21±2.40^{b}	12.22±1.05^{b}	4.07±0.80^{b}	66.29±1.09^{b}	79.99±1.05^{ab}	73.70±1.68^{b}
100 µg mL^{-1}	35.18±2.18^{b}	60.73±1.09^{a}	20.36±2.12^{a}	56.29±4.20^{a}	17.03±1.68^{a}	6.66±0.52^{a}	83.29±0.44^{a}	84.07±1.68^{a}	85.55±0.52^{a}

Means followed by the same letters in each column are not statistically significant ($P \leq 0.05$).

best against *A. alternata* under *in vitro* conditions (Sharma and Gaur, 2009). Kapsa and Osowski (2003) sprayed mancozeb, chlorothalonil and mixture of Zoxamide + Mancozeb against late blight of potato and concluded that the treatment with mixture of Zoxamide + Mancozeb effectively reduced the disease and resulting in increased tuber yield. Alternate sprays of Mancozeb and Tebuconazole were found effective in controlling early blight and powdery mildew of tomato as well as increase yield of tomato (Ilhe et al., 2008;). The findings of above-mentioned scientists are in agreement with the outcome of this study and support our experiment result.

Inourfindings Bravo, Score, Camelot and Cabrio Top not performed well and inhibited mycelial growth ranged between 6-35 % at maximum concentration used. While Bravo and Score at 25 µg mL^{-1} concentration completely failed in reducing mycelial growth of *A. solani*. At one time Score and Bravo were considered best fungicides against many fungal pathogens resultantly their frequent use allows the pathogens to develop resistance and this study revealed that *A. solani* isolates present in the various tomato fields are now showing resistance against Score and Bravo in the Southern Punjab. Previous findings revealed that resistant is developing in *A. solani* isolates against widely used fungicides (Latha, 2009). Resistance against fungicides is stable inherent adjustment by fungus to specific fungicide(s) that reduced or increased sensitivity (Ma and Michailides, 2005). Difenoconzole was found least effective in reducing mycelial growth of pathogen compared to hexaconazole and propiconazole (Roopa, 2012). Contrary to this, Patel and Choudhary (2010) revealed that Difenoconazole effectively reduced early blight disease and helps to improve yield. *A. solani* isolates collected from farmers' fields of Idaho were found resistant to chlorothalonil (Fairchild et al., 2013). Several applications of a fungicide against same pathogen during a growing season lead towards the development of fungicide resistance (Gudmestad et al., 2013; Rosenzweig et al., 2008). Malpractice in application of chlorothalonil and difenoconazole decreased its performance due to severe infection pressure of *A. solani* and development of resistance in pathogen. Development of resistance in pathogens is due to prolong application of commonly fungicides with single mode of action and this resistance can accelerate resistance development against multi-site inhibitor (Latorre and Torres, 2012). Our findings are cognizant with the results of these scientists.

It is concluded that application of Score and Bravo must be discouraged and it may be replaced by Heritage and Dithane M-45 to manage early blight of tomato in field. Moreover, alternate application of fungicides with different mode of actions will be helpful to minimize the chances of resistance development among pathogen isolates. Our results indicate that farmers must be aware of the importance of rotational practice to infer the choice of fungicides with single chemical nature, which significantly contributes to the development of resistance to target pathogens. This is the first work in Pakistan on the evolution of resistance to *A. solani* caused by unjudicial use of fungicides in tomato crop. The development of resistance in a pathogen is a serious problem in tomato crops. Our findings are to question the sustainability of today's management strategy for *A. solani* is completely dependent on the application of fungicides and requires the development of new control strategies that can satisfactorily control diseases and pathogens.

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