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ANTIFUNGAL ACTIVITY OF DIFFERENT SYSTEMIC FUNGICIDES AGAINST *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI* ASSOCIATED WITH TOMATO WILT AND EMERGENCE OF RESISTANCE IN THE PATHOGEN

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A B S T R A C T

Fusarium wilt in tomato (*Lycopersicon esculentum* L.) caused by *Fusarium oxysporum* f. sp. *lycopersici*. Pathogen is notorious because of its adverse effect on plant growth with causing significant yields losses. Further complication is developing by raising issue of resistance in *F. oxysporum* f. sp. *lycopersici* particularly in developing countries due to injudicious fungicides applications in the field of tomato plant infected by this pathogen. *F. oxysporum* f. sp. *lycopersici* was isolated from collected infected samples from tomato field in Multan, southern Punjab on Potato dextrose medium by adopting poisoned food technique. Antifungal activity of different systemic fungicides viz. Pyrimethanil, Fludioxonil, Benlate, Bromuconazole, Fosetyl-Al, Flumorph, Prochloraz, Myclobutanil, Epoxiconazole, Strobilurin, carbendazim, Fentin hydroxide, Streptomycin, Tebuconazole, Iprobenfos and azoxystrobin were tested against *F. oxysporum* f. sp. *lycopersici in vitro* by using poisoned food technique. All tested fungicides suppressed fungal mycelial growth with significantly high or low percent inhibition ranging from 1.48 to 85.92%. Among 16 tested fungicides, different concentrations of Bloom, Prochloraz and Bromuconazole significantly inhibited fungal growth ranging from 75 to 85.92 %. Carbendazim and epic reside in the bottom with reference to their efficacy in suppression of *F. oxysporum* f. sp. *lycopersici*. The results of present research expressed that isolates of *Fusarium oxysporum* f. sp. *lycopersici* develops resistance with time which causes reduced efficacy of fungicides as compared to previously published data. The results provided information about fungicides application and selection for the management of holistic disease in tomato crop in Pakistan.

Keywords: Tomato, Fungus, Fungicides, Resistance.

INTRODUCTION

Tomato crop is consumed as a food of significant nutritional value because it is a rich source of minerals and vitamins (Block *et al.*, 1985; Friedman, 2002). Pakistan had 150 thousand ha area and 57094 tons production of tomato in 2014-2015. Sindh is the highest tomato producing province with tomato grown on an area of 67.46 thousand hectares followed by Balochistan with 31.38 thousand hectare of area while Punjab had 18.29 thousand ha under tomato cultivation. Overall an increasing trend is observed in the acreage of tomato crop during the last two decades. Tomato crop faces yield losses due to adverse effects of both biotic and abiotic factors (Abdel-Sayed, 2006). Tomato crop is attacked with wide range of vicious fungal, bacterial, viral and nematode pathogens (Agrios, 2005). Fungal pathogens are considered as major contributor to reduced productions (Stone *et al.*, 2000). The fungal pathogens belonging to family *Ascomycota* order *Hypocreales* are most devastating and considered a serious threat to tomato crop (Mandal *et al.*, 2009). The *Fusarium oxysporum* f. sp. *lycopersici* belong to family Ascomycota causing major losses 10 to 50 % to tomato crop worldwide and 10 to 90% annually especially in the

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warmer regions of Pakistan (Lagopodi *et al.*, 2002). It destroys root vascular system and blocks transport system of affected plants resulting in reduced growth or death in severe cases (Haware *et al.*, 1978; Malhotra and Vashistha, 1993). *F. oxysporum* damages different growth stages of crops by inducing symptoms like yellowing of plant parts, dropping of leaves, wilting or root rot and brown-black discoloration in xylem vessels (Dubey and Singh, 2004). Different management strategies including crop rotation, cultural control, chemical control, resistant cultivars and biological control are employed to overcome this fungus (Abo-Elyousr and Mohamed, 2009). The effectiveness of conventional practices is limited due to saprophytic nature of *F. oxysporum*. Resistant cultivars are regarded as the most appropriate source in tackling this pathogen but their availability at wider scale and long term performance is a serious question (Marquina, 1988). In developing countries, chemical applications are applied as a principal control measure against pathogenic fungi (Rojo *et al.*, 2007). The injudicious Fungicides applications have raised some serious issues like pesticide residues, environmental pollution and development of resistance in target fungus species leading to failure of an antifungal property of the chemical (Waard *et al.*, 1993). In fact, lacking of latest efficacy reports data about fungicides against a particular species leads to injudicious fungicides applications against that target pathogen (Naqvi *et al.*, 2015). Importantly, evaluating the efficacy of different systemic fungicides is essential to develop a control strategy.

Due to unavailability of basic data on efficacy of different systemic fungicides for the control of *F. oxysporum* f. sp. *lycopersici* in southern Punjab, Pakistan limits our understanding of developing control measures. To fill this major gap, current study was designed to investigate antifungal activity of broad range of systemic fungicides belonging to conventional and new chemical groups against *F. oxysporum* f. sp. *lycopersici* which arises various questions as a result of increasing resistance in pathogen against the fungicides in Pakistan.

MATERIALS AND METHODS

Diseases sample collection: Infected tomato plant parts showing typical symptoms of *Fusarium* wilt were collected from experimental fields of Bahauddin Zakariya University Multan. Samples were preserved in polythene bags and brought in to Mycology laboratory of Department of Plant Pathology, Faculty of Agriculture Sciences and Technology B.Z. University Multan (30.1984° N, 71.4687° E). Infected parts were used as a source of isolation of the pathogen on artificial growth medium i.e., Potato dextrose agar (PDA) (Chohan *et al.*, 2011). Infected parts were cut into small pieces (4-5 mm long) and were washed under tap water and disinfected with 3% solution of sodium hypochlorite for two minutes. After disinfection, these pieces were rinsed with sterilized distilled water and dried on filter paper. 10 ml of PDA was poured in sterilized petri dishes of 9 cm diameter in laminar flow chamber. After solidification of medium, these infected pieces of leaves were placed on PDA at 25±2°C for 4 days in an incubator. To obtain pure culture single spore culture technique was adopted. After incubation, petri plates were examined for fungal identification based on morphological characteristics (Nelson *et al.*, 1983). This purified fungal culture was stored at 4°C for further use.

Pathogenicity test: Tomato seed were grown in October in green house of Bahauddin Zakariya University. Tomato seedlings approximately size of 10 inches (Roma) of 30 days were transplanted to autoclaved soil infested with 10 ml of fungal conidial suspension inoculum with a concentration of 10^6spores/ml with the help of (Neubauer) haemocytometer prepared from 7 days old fungal culture in plastic pots of (12 cm diameter) in green house of Bahauddin Zakariya university. After inoculation pots were shifted in greenhouse. Symptoms development was recorded at regular intervals. After symptoms development pathogen was re-isolated from symptomatic parts on artificial medium. Morphological characters of isolated and source culture were compared for full filling requirements of Koch's postulates (Ignjatov *et al.*, 2012).

Fungicides: Antifungal activity of 16 different systemic fungicides (commercial formulations) belonging to different groups was evaluated against *F. oxysporum* f. sp. *lycopersici* at different doses (Table 1)

Antifungal Activity test: Poison Food technique was used for antifungal assay. Different concentrations including 20, 60, 80 and 100µg $ml^{-1}$of sixteen fungicides (Table 1) were added to PDA and poured into sterilized petri plates for solidification. An isolated culture of *Fusarium oxysporum* f. sp. *lycopersici* was inoculated using sterilized cork borer in petri dishes. An

experiment was conducted by using complete randomized design (CRD) with three replicates. Inoculated petri plates containing PDA medium free of tested fungicides were maintained as control. These petri plates were incubated at 25°C for one week until test fungus gained 9 cm radial mycelial growth in control treatments. Antifungal activities of various fungicides at different concentrations were examined against radial mycelial fungal pathogen growth. Reduction in growth in percent decrease was calculated by using this formula (Sahi *et al.*, 2012).

$$PD = \frac{(C - T)}{C} \times 100$$

Where PD is percent decrease, C is colony growth of *Fusarium* in control, Tis colony growth of *Fusarium* treated plate.

STATISTICAL ANALYSIS

Experiment was conducted in complete randomized design and fungus radial growth data was subjected to analysis of variance (ANOVA) by using Statistix (8.1). All Treatment means compared by using Fisher's least significant differences (LSD) at (P= 0.05). Inhibition of fungus radial growth for all treatments was calculated as percent decrease.

RESULTS AND DISCUSSION

Antifungal affects of tested fungicides on mycelial growth of *F. oxysporum* f. sp*. Lycopersici*: Efficacy of sixteen systemic fungicides against *Fusarium oxysporum* f. sp*. lycopersici* is shown in (Table 2 & 3). Our results clearly showed fungicides have potential to reduce fungal pathogen growth which causes wilt disease to a large extent. In Table 2 and 3 shows detailed efficacies of tested fungicides in relation to inhibition of mycelial growth and percent decrease over control treatment of all the tested fungicides calculated statistically. Different concentrations of Bromuconazole showed significant control in comparison to other tested fungicides as it reduced growth of the fungal colony up to 1.83, 2.20 2.36, 2.80 cm at low concentration (LC) and high concentrations (HC) respectively. Result of defeater and wisdom in reduction of mycelial growth of *Fusarium* pathogen i.e., (3.76, 4.20, 3.60, 3.33), (3.16, 4.40, 3.60, 4.66) cm were closely matched at different tested concentration respectively as compared to control it was 9cm. In case of benomyl at LC and HC concentrations significantly reduced fungal mycelial growth of 3.26, 4.73, 3, 4.66 cm as compared to control. Fludioxonil and Pyranil on all similar concentrations significantly decreased fungal growth 3.20, 3.96, 3.20, 4.86 cm while in case of Pyranil inhibition zone of fungal growth 4.30, 5.66, 6.56, 5.90 cm (Table 2).

Fungicides Epic at both LC concentrations reduced fungal mycelial growth 7.96 and 8 cm as compared to control but HC concentrations were not found significant as compared to control. Picoxystrobin with similar concentrations reduced fungal mycelial growth 8.06, 8.36, 8.60, 8.50 cm as compared to control. Fungicides among carbendazim and Hiten, Hiten show better result at HC concentrations with inhibition zones of 5.76, 5.03 cm. while mycelial growth of *Fusarium* pathogen i.e. 7.93, 7.50, 8.86, 8.80 cm on all lower and high (LC and HC) concentrations of carbendazim respectively. Flare was found less effective with reducing mycelial growth and its growth is 7.06, 5.53, 5.40, 4.60 cm on 20, 50, 80, 100 (µg/mL) concentrations respectively. In case of Tebuconazole and benedict, there was decreased at LC concentrations in mycelial growth 7.70, 7.56, 7.60, 7.76 cm while on HC concentrations reduction in mycelial growth 8.36, 8.53, 8.70, 7.66 cm respectively. Azoxystrobin with LC concentrations shows more significant result as reduction in colony diameter i.e. 6.33, 6.06 cm while HC concentrations give less control with mycelial growth 7.60, 7.50 cm as compared to the control.

Percent inhibition of *F. oxysporum* f. sp. *lycopersici* colony growth was calculated over control at all tested LC and HC concentrations of fungicides. Bloom was found most significant showing decrease of 85.92, 81.10, 79.90% at HC80, LC 50, HC 100 respectively as compared to control as shown in Table 3. Prochloraz at LC50 and Bromuconazole at HC100 showed 2nd significant fungicides with inhibition of 79.62 %. Benomyl and Fludioxonil at LC20, HC80 concentrations showed better result with percent inhibition of 63.6, 64.40% as compared to control. Pyranil at LC 20 and flare at HC 100 concentrations give satisfactory decrease of 52.21, 48.88%. Carbendazim and epic at HC80 concentration were less significant with percent inhibition of 1.48 % as compared to all tested concentrations of fungicides shown in Table 3. Antifungal effects of sixteen systemic fungicides on all tested concentrations on fungal mycelial growth and percent inhibition are shown in Figure 1 & 2.

Table 1. List of systemic fungicides belongs to various chemical group tested against mycelial growth of *F.oxysporum* f. sp. *lycopersici* on PDA medium *in vitro*

Sr. No	Trade name	A.I	Action	A.I %	Formulation	Dose/(100 L)	FRAC group	Manufacturing	Chemical Family	A.I	Action
1	Pyranil	Pyrimethanil	Systemic	40	SC	300 ml	9	Yantai Keda Chemical Co., Ltd	Pyrimidine	Pyrimethanil	Systemic
2	Medallion	Fludioxonil	Systemic		SC	125 g/L	12	Syngenta	Phenylpyrrole	Fludioxonil	Systemic
3	Benomyl	Benlate	Systemic	50	WP	500 g/l	B1		Benzimidazole	Benlate	Systemic
4	Chipco	Bromuconazole	Systemic	20	SC	100 g/l)		Bayer Crop Science	Bromuconazole	Bromuconazole	Systemic
5	Aliette	Fosetyl-Al	Systemic	80	WDG	250 g	33	Syngenta	Ethyl phosphonates	Fosetyl-Al	Systemic
6	Defeater	Flumorph	Systemic	20	WDG	250 g	33	Bayer CropScience/Kanzo AG,	Morpholine	Flumorph	Systemic
7	Sporta	Prochloraz	Systemic		EC	20 mL	3	Bayer	Benzimidazole	Prochloraz	Systemic
8	Bloom	Myclobutanil	Systemic	25	EC	40 mL	3	Four Brothers Biologic AG Pakistan,	Triazoles	Myclobutanil	Systemic
9	Epic	Epoxiconazole	Systemic	12.5	SC	160 mL	3	Pak China Chemicals (Pvt.) Ltd.,	Triazoles	Epoxiconazole	Systemic
10	Picoxystrobin	Strobilurin	Systemic	25	SC	300 mL	11	Syngenta Pakistan Limited,	Methoxyacrylates	Strobilurin	Systemic
11	Bavistin	Carbendazim	Systemic	50	SC	200 g/l),	1	Syngenta		carbendazim	Systemic
12	Hiten	Fentin hydroxide	Systemic	50	SC	250 mL	30	Kanzo AG,	Tri phenyl tin compounds	Fentin hydroxide	Systemic
13	Flare	Streptomycin	Systemic	72	SP	100 g	18	Kanzo AG,	Cyanoimidazole	Streptomycin	Systemic
14	Folicur	Tebuconazole	Systemic	6	ME	750 mL	3	Pak China Chemicals (Pvt.) Ltd.,	Triazoles	Tebuconazole	Systemic
15	Benedict	Iprobenfos	Systemic	50	EC	200 mL	6	R. B. Avari Enterprises (Pvt.) Ltd.,	Organophosphate Esters	Iprobenfos	Systemic
16	Heritage	Azoxystrobin	Systemic	40	SC	180 ml	11	Syngenta	Strobilurins	azoxystrobin	Systemic

A.I: active ingredient, ** WDG: Water –dispersible Granule***WP: Wettable Powder ****SC: Suspension Concentrate*****EC: Emulsifiable concentrate,*****

Table 2. Effects of concentrations of different systemic fungicides on mycelia growth of *F. oxysporum* f. sp. *lycopersici* on PDA

Reduction in Fungal mycelial growth (cm ± Standard .Error)								
Concentration (µg /mL)	Pyranil	Fludioxonil	Benomyl	Bromuconazole	Wisdom	Defeater	Prochloraz	Bloom
LC20	4.30±0.17 d	3.20±0.16 c	3.26±0.22 b	2.36±0.10 c	3.16±0.14 c	3.76±1.18 bc	2.63±0.22 b	2.03±0.12 b
LC50	5.66±0.27 c	3.96±0.12 c	4.73±0.52 b	2.80±0.09 b	4.40±0.25 b	4.20±0.34 b	1.83±0.22 c	1.70±0.17 c
HC80	6.56±0.24 b	3.20±0.34 c	3.00±0.33 c	2.20±0.12 c	3.60±0.25 c	3.60±1.14 bc	1.90±0.05 c	1.27±0.07 c
HC100	5.90±0.22 bc	4.86±0.31 b	4.66±0.17 b	1.83±0.07 d	4.66±0.17 b	3.33±0.14 c	2.16±0.03 bc	1.80±0.12 b
Control	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a
*LC: Lower concentration, ** HC: High concentration								
Concentration	Epic	Picoxystrobin	Carbendazim	Hiten	Flare	Tebuconazole	Benedict	Azoxystrobin
LC 20	7.96±0.07 b	8.06±0.10 c	7.93±0.10 b	6.80±0.22 b	7.06±0.10 b	7.70±0.17 c	7.60±0.08 b	6.33±0.17 c
LC50	8.00±0.19 b	8.36±0.12 bc	7.50±0.21 b	6.70±0.09 b	5.53±0.24 c	7.56±0.10 c	7.76±0.15 b	6.06±1.10 c
HC80	8.86±0.11 a	8.60±0.09 b	8.86±0.11 a	5.76±0.07 c	5.40±0.19 c	8.36±0.12 b	8.70±0.14 a	7.60±0.17 b
HC100	8.70±0.12 a	8.50±0.05 b	8.80±0.16 a	5.03±0.07 d	4.60±0.17 d	8.53±0.20 ab	7.66±0.14 b	7.50±0.24 b
Control	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a	9.00±0.00 a

*LC: Lower concentration, ** HC: High concentration

Means followed by the same letters in each column are not statistically different (P ≤ 0.05)

Table 3. Percentage decrease in fungal growth of *F. oxysporum* f. sp. *lycopersici* on PDA mixed with systemic fungicides at different concentrations.

Decrease in Fungal mycelial growth (% ± Standard. Error)								
Concentration (µg /mL)	Pyranil	Fludioxonil	Benomyl	Bromuconazole	Wisdom	Defeater	Prochloraz	Bloom
LC 20	52.21±1.89 a	64.40±1.81 a	63.69±2.42 a	73.70±1.09 b	64.81±1.51 a	58.14±1.98 ab	70.73±2.48 b	77.40±1.32 b
LC50	37.04±3.02 b	55.92±.1.32 a	47.40±5.77 b	68.88±1.05 c	51.18±2.77 b	53.33±3.78 b	79.62±2.48 a	81.10±1.89 ab
HC80	27.03±2.69 c	64.44±3.78 a	66.66±3.67 a	75.55±1.39 b	59.99±2.77 a	59.99±2.77 ab	78.88±0.52 a	85.92±0.80 a
HC100	34.44±2.40 bc	45.92±3.49 b	48.14±1.84 b	79.62±0.80 a	48.14±1.84 b	62.96±1.60 a	75.92±0.30 ab	79.90±1.39 b
Control	0.00±0.00 d	0.00±0.00 c	0.00±0.00 b	0.00±0.00 d	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c	0.00±0.00 c
*LC: Lower concentration, ** HC: High concentration								
Concentration	Epic	Picoxystrobin	carbendazim	Hiten	Flare	Tebuconazole	Benedict	Azoxystrobin
LC 20	11.80±0.80 a	10.36±1.09 a	11.85±1.09 a	24.43±2.40 c	21.48±1.09 c	14.44±1.89 a	15.55±0.91a	29.63±0.17 a
LC50	11.10±2.10 a	7.036±1.32 ab	15.92±2.36 a	25.55±1.05 c	38.52±2.64 b	15.92±1.09 a	13.70±1.68 a	32.59±1.09 a
HC80	1.480±1.21 b	4.40±1.05 b	1.48±1.21 b	35.92±0.80 b	39.99±2.10 b	7.03±1.32 b	3.33±1.57 b	15.55±1.89 b
HC100	3.330±1.39 b	5.55±0.52 b	2.22±1.81 b	44.07±0.80a	48.88±1.89 a	5.18±2.18 bc	14.81±1.60 a	16.66±2.62 b
Control	0.00±0.00 b	0.00±0.00 c	0.00±0.00 b	0.00±0.00 d	0.00±0.00 d	0.00±0.00 c	0.00±0.00 b	0.00±0.00 c

Means followed by the same letters in each column are not statistically different (P ≤ 0.05)

Figure 1 & 2 *In-vitro* antifungal activity of systemic Fungicides at different fungicides against *Fusarium oxysporum* f. sp. *lycopersici*

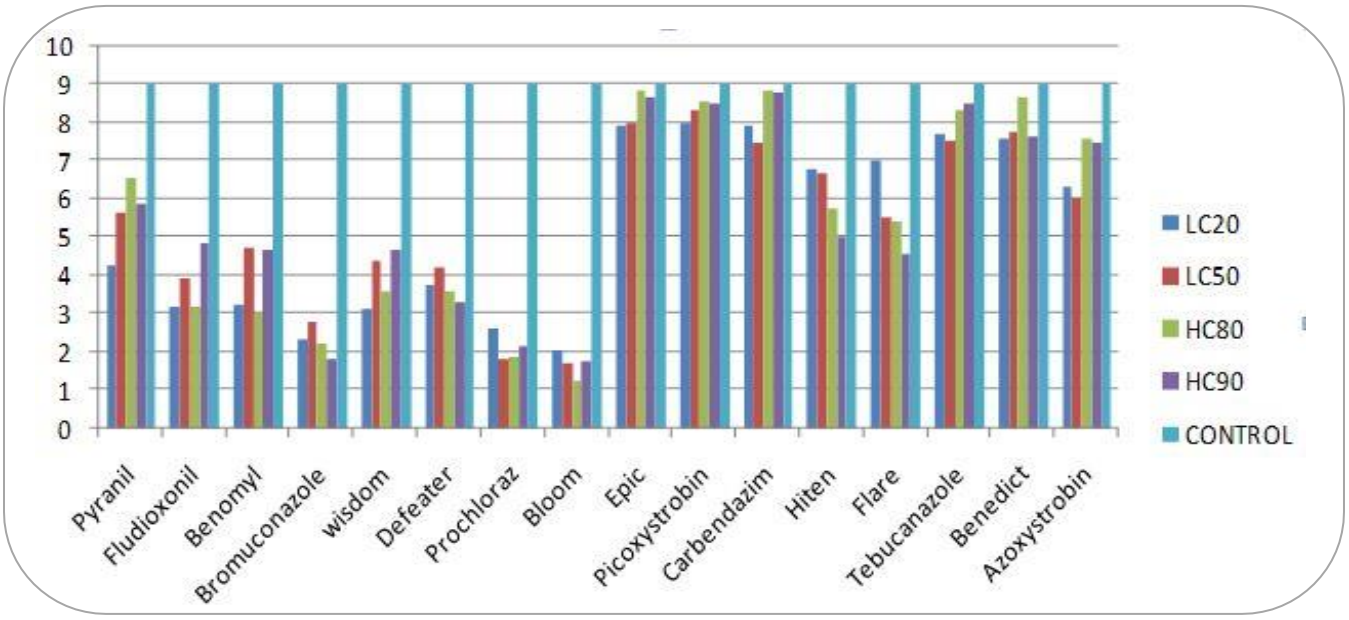

Figure 1. Reduction in colony diameter of *Fusarium oxysporum* f. sp*. lycopersici* at different concentrations of fungicides.

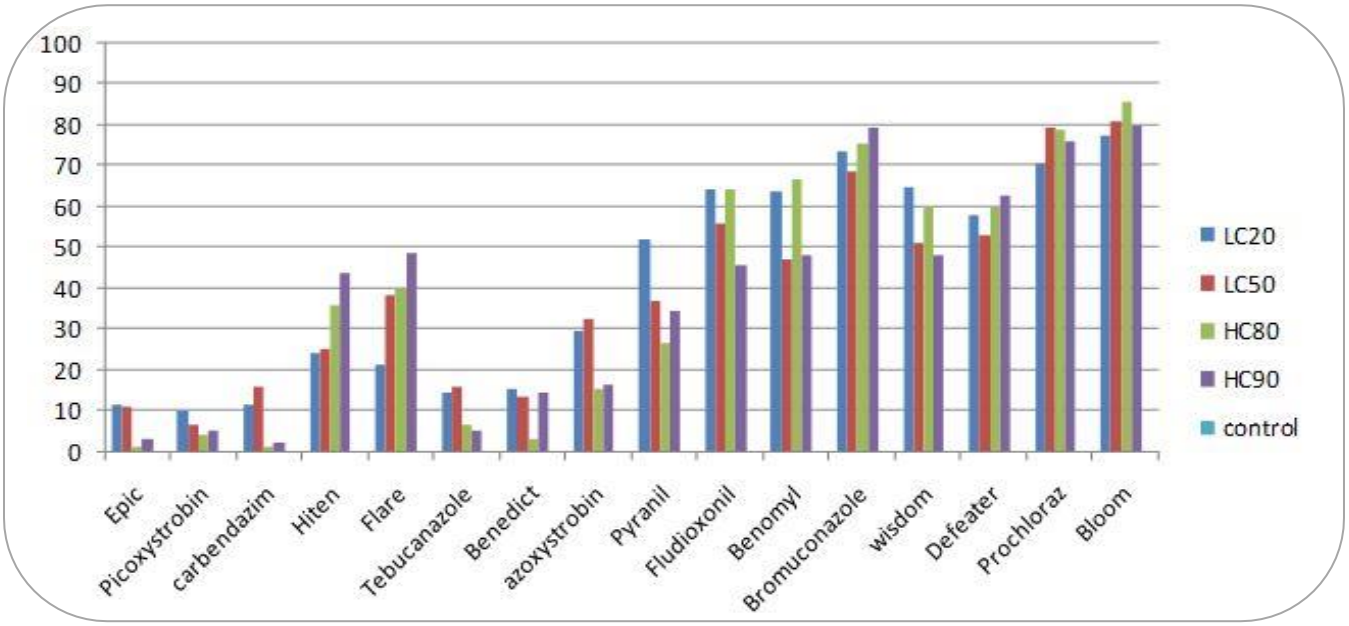

Figure 2. Percent inhibition on different concentrations of fungicides against *Fusarium oxysporum* f. sp. *Lycopersici.*

Evaluation of different systemic fungicides for selecting most efficient compounds is main step for pathogen control. Our result showed Bloom, Prochlorazol, Bromuconazole, defeater sand wisdom was most affective *in vitro* among all tested 16 systemic fungicides by suppressing 75 to 85 % fungal mycelial growth (Amini and Sidovich, 2010; Khan, 2013).The systemic fungicides Benomyl, Pyranil belong to chemical family benzimidazole and Pyrimidine had better curative and preventive effects on *Fusarium* tomato wilt(Allen *et al.*, 2004).Azoxystrobin was found less effective as fallowed by Fludioxonil (Amini and Sidovich, 2010). Tebuconazole bloom and Epic belong to triazole group caused structural destruction in *F. oxysporum* f. sp. *lycopersici* by unavailability of ergo sterol to fungi that lead to suppression in growth (Han *et al.*, 2006).

Tebucanazole and Epic both fungicides significantly less effective as compared to bloom (Khan, 2013).

Antifungal property of Carbendazim, Azoxystrobin and Picoxystrobin may be lost due to resistant development in fungal isolates by heavy pressure created by fungicides application (Pappas, 1997; Reimann and Deising, 2005). Benedict, Hiten and Flare fungicides on different concentrations showed less percent inhibition in fungal mycelial growth as compared to previous work (Akhtar *et al.*, 2014).

Our experiment result showed tested fungicides slowly losing their antifungal affect for controlling wilt disease of tomato crops due to pathogen sensitivity reduction. Various literature stated resistance development problem in *Fusarium oxysporum* f. sp. *lycopersici to* fungicides can be solved by formulating new fungicides with advance chemistries to avoid a variety of resistant fungal isolates for longer period of time.

Our result demonstrates that farmers must be aware of significance of rotation practices to dispirit the selection of single chemistry fungicides which significantly lead to resistance development in target pathogens. It is first work in Pakistan on evolution of resistance in *F. oxysporum* f. sp. *lycopersici* due to malpractice application of systemic fungicides application in tomato groves. Our result reinforcing the fungicides resistance is severe problem of tomato crop in Pakistan.

Our finding is questioning on sustainability of today management strategies against *F. oxysporum* f. sp. *lycopersici* which exclusively depends on fungicides application and there is need to develop new control strategies which satisfactory control to disease and pathogen.

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