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Effect of Clethodim Herbicide on Acquired Tolerance of *Alternaria alternata* and *Fusarium solani* to Tetraconazole Fungicide

Hassan M.S. Khalifa and Wael M. Sameer

Department of Plant Protection, Faculty of Agriculture,
Al-Azhar University, Nasr City, Cairo, Egypt

Abstract: The side effect of excessive exposure of the tested fungi namely *Alternaria alternata* and *Fusarium solani* to the herbicide clethodim on the acquired tolerance to the fungicide tetraconazole was investigated. The obtained results indicated that the fungi, obtained from diseased tomato, were sensitive to fungicide tetraconazole, according to the EC_{50} values, i.e. 0.73 and 1.67 µg /ml for *A. alternata* and *F. solani*, respectively. Quite contrary, these fungi could grow on PDA containing 200 or 250 µg/ml of clethodim. Continuous exposure of these fungi to gradual increasing concentration of clethodim; 300, 400, 500 and 600 µg/ml resulted in tolerated isolates of these fungi to tetraconazole. The tolerance levels were 48.60- and 60.43- folds for *A. alternata* and *F. solani*, respectively. Image analysis showed that continued exposure of these fungi to the tested herbicide up to 500 µg/ml resulted in isolates differing morphologically from the parent isolate. Exposures of these isolates to the used fungicide exhibited levels of tolerance. The tolerance was lost by sub culturing the tolerant isolates on pesticide- free medium up to 10 sub culturing, indicating that the tolerance may be due to physiological adaptation.

Key words: Herbicide · Fungicide · Clethodim · Tetraconazole · Fungi · *Alternaria alternata* · *Fusarium solani*

INTRODUCTION

Infection of tomato plants (*Lycopersicon esculentum* mill) by several fungal diseases can cause reduction of the yield. *Alternaria alternata* (Fr.:Fr.) Keissl f. sp. *lycopersici* causes black mold on leaves and fruits which reduces the total leaves and fruit quality [1]. *Fusarium solani* is a common pathogen of tomato where it causes a wilt, pre- and post- emergence damping off [2] and a fruit rot [3]. It has also been recorded causing a foot rot of tomato [4-7]. Fungicides application is the effective means to control pathogen populations and their effect on yield. At the same time, cultivators routinely apply the herbicide clethodim to control annual and perennial grasses affecting tomato plants [8].

When the herbicide is introduced into the plant environment, several types of interaction are possible, some of the common herbicides increase the incidence of the diseases, while others could control them [9]. It has been reported that the incidence of root rot in pea and corn, caused by *Fusarium solani* f. sp. *Pisi* and *F. roseum* f. sp. *Cerealis*, was increased in soil treated with atrazine [10]. Several studies reported rhizosphere and soil *Fusarium* spp. increase in response to glyphosate addition [11, 12] including infection and disease severity by *F. solani* f. sp. *glycines*, which increases with glyphosate resistant GR soybean treated with glyphosate compared to no herbicide treatment [13]. Root of GR soybean and maize treated with glyphosate are heavily colonized by *Fusarium* compared to non-GR or GR cultivars not treated with glyphosate [14]. Cole and Batson [15] found that diphenamid reduces growth of *Rhizoctonia solani* and *Pythium aphamidermatum* on artificial media and decreases the incidence of pre-emergence damping off of tomato seedlings. Sharma *et al*. [16] suggested that glyphosate prevents the formation of ascocarps of *Pyrenophora tritici-repentis* in infested wheat straw. Bauske and Kirby [17] showed that no significant interactions are observed between herbicides; trifluralin, pendimethalin and ethalfluralin and inoculum treatments of *R. solani* on soybean. Mahmoud and Khalifa [18] and Ali [19] evaluated

Corresponding Author: Wael M. Sameer, Plant Protection Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt.

the herbicide, tralkoxydim, against the incidence of leaf blight, caused by *Alternaria alternata* and tan spot caused by *Pyrenophora tritici-repentis*, of wheat leaves. They found that the herbicide somewhat reduces the incidence of the diseases, especially when it is applied as post-inoculation treatment. Olajire and Fawole [20] suggested that application of herbicide Galex (metalachlor + metobromurom) can be effectively used to control some important pathogenic fungi of legumes. Tanney and Hutchison [21] suggested the possibility of a shift towards tolerant species of fungi when they are exposed to herbicide glyphosate. Pasaribu *et al*. [22] determined the growth and development of mycorrhizal fungi (*Glomus mossea*) in soils treated with herbicides alachlor and glyphosate. They found that germination of spores and its hyphal growth are not significantly affected in soil treated with herbicides at the recommended field application rates or less. Alachlor reduced the spore germination and hyphal growth significantly at treatments higher than recommended rates, but non- significant effect was caused by glyphosate.

The interaction between the herbicides and the efficiency of fungicides against plant pathogenic fungi had been observed. The herbicides, pendimethalin, dinitramine, fluometuron and prometryne affected the antifungal activity of toloclofos-methyl, pencycuron, carboxin and carbendazim as indicated by the mycelia growth of *R. solani*. Similar effects were also obtained in soil experiment [23]. Additionally, Mahmoud [24] found that atrazin- alachlor and metolachlor reduce the inhibitory effect of carboxin and captan fungicides toward the mycelial growth of *Fusarium moniliforme*. The herbicides applied as pre- emergence markedly reduced the efficacy of the fungicides applied as seed dressing to control blighted seedlings of maize.

It has been reported that continued exposure of *R. solani* to herbicides trifluralin and pendimethalin resulted in benomyl- and carboxin + captan- tolerant isolates. The level of tolerance depends upon the type of the herbicide used and the tolerance was not stable after 12 repeated subculturing on herbicide untreated medium [25]. Additionally, Mahmoud and Khalifa [26] reported that excessive exposure of *Fusarium graminarum*, *Rhizoctonia solani* and *Pyrenophora tritici-repentis* to herbicide tralkoxydim resulted in acquired resistance to the fungicides thiabendazole and toloclofos-methyl. Also, excessive exposure of *Fusarium oxysporum* f. sp. *lycopersici* and *Alternaria alternata* to herbicide metribuzin resulted in acquired resistance to the fungicide difenoconazole [27].

The objective of this study was conducted to determine the effect of continuous exposure of *Alternaria alternata* and *Fusarium solani* to herbicide clethodim on the acquired resistance to the fungicide tetraconazole.

MATERIALS AND METHODS

Identified isolates of *Alternaria alternata* and *Fusarium solani* were obtained from leaves and root of tomato in Plant Protection Department, Faculty of Agriculture, Al- Azhar University.

The herbicide clethodim (Select super 12.5 % E.C) was used and the tested fungicide was tetraconazole (Domark 10 % E.C).

***In vitro* Sensitivity Test of the Fungi to the Compounds:** The sensitivity of the tested fungi to the herbicide and fungicide was assayed according to Carling *et al*. [28]. Aqueous stocks of each compound were prepared and added to autoclaved PDA cooled to 50°C to obtain final concentrations of 0.01 to 75 µg tetraconazole / ml and 1.0 to 250 µg clethodim / ml. The zero- concentration treatment received a quantity of water equivalent to that used in the fungicide or herbicide treatments. After through mixing, pesticide- amended PDA medium was dispensed into 9-cm plates and allowed to solidify. Disks of mycelium 4 mm in diameter cut from the growing edge of 7- day- old cultures of the tested fungi were placed in the center of plates. Each fungus- pesticide concentration was replicated three times. Radial growth was recorded after 7 days of incubation at 25°C. Percent inhibition of pesticide for each concentration was calculated based on mycelia growth of the control treatment. The concentration of fungicide required to give 50% inhibition of growth (EC_{50}) was estimated according to Finney [29].

Stepwise Exposure to Clethodim: The parent isolate of each fungus was exposed to clethodim in which mycelia inocula grown on 250 µg clethodim / ml medium were exposed to gradually increasing concentrations of clethodim. The mycelia inoculum (4 mm diameter) taken from this concentration was seeded on PDA containing 300 µg clethodim / ml (1^{st} exposure), then the plates were incubated at 25°C for 7 days. The growing mycelium was transferred to gradually increasing concentrations of clethodim up to 600 µg / ml. This trial was performed for each fungus for 4 continued exposures and the redial growth was measured (cm diameter) after the end of incubation period.

Resistance Measurement to Fungicide: Both the parent isolates (P.I.) and clethodim- exposed isolates (C.E.I.) of *Alternaria alternata* and *Fusarium solani* were tested for their sensitivities to tetraconazole according to Carling *et al*. [28]. Tetraconazole was emulsified in sterile distilled water, then added to cooled (50°C) PDA media at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0, 125.0 and 150.0 µg tetraconazole / ml. Fungicide- free medium check was also prepared for each isolate. Mycelial plugs (4 mm diameter) cut from the margins of 7- day- old cultures of each isolate, were placed into 3 replicates of fungicide- amended medium at each concentration. Radial growth of the replicate was measured after 7 days at 25°C. The percent of inhibition and EC_{50} values were estimated as described before. The level of tolerance was determined according to the following equation:

$$\text{Level of tolerance} = \frac{EC_{50} \text{ of clethodim- exposed isolate}}{EC_{50} \text{ of the parent isolate}}$$

Morphological features of the clethodim- exposed and tetraconazole tolerant isolates were studied using asoftware for image analysis (SIS Docu Software) at the Regional Center of Mycology and Biotechnology, Al- Azhar University.

Persistence of Tolerance: Tetraconazole- tolerant isolate (T.T.I.) or clethodim- exposed isolate (C.E.I.) of both fungi were sub cultured weekly on pesticide- free PDA medium, over 10 transfers. At each one, the sensitivity of the isolates to tetraconazole was measured by using different concentrations of the fungicide, ranged from 0.01 to 150.0 µg / ml. The EC_{50} values of each step were determined.

RESULTS AND DISCUSSION

***In vitro* Sensitivity Test of the Fungi to the Pesticides:** Results in table 1 show the sensitivity of *Alternaria alternata* and *Fusarim solani* to tetraconazole. It was found that increasing the concentration of tetraconazole decreased gradually the growth of the tested fungi. For example, the fungicide at 0.5 µg / ml caused inhibition of mycelia growth of both fungi represented by 44.44 and 38.88 % for *A. alternata* and *F. solani*, respectively. Tetraconazole at 50 µg / ml induced inhibition by 88.88 and 83.33 % for *A. alternata* and *F. solani*, respectively. Indeed, tetraconazole at 75 µg / ml exhibited inhibition by 100 % for *A. alternata*, but caused 92.66 % inhibition of growth of *F. solani*. The estimated EC_{50} values of tetraconazole were 0.73 and 1.67 µg / ml for *A. alternata* and *F. solani*, respectively. Based on these values, *A. alternata* is considered to be more sensitive to tetraconazole than *F. solani*. This result is confirmed by Ali [30] who found that the EC_{50} values of tetraconazole on *A. solani* and *F. oxysporum* f. sp. *Lycopersici* are 1.35 and 1.15 µg / ml, respectively. El- Khawaga – Maii [31] found that the EC_{50} value of tetraconazole for *F. oxysporum* is 0.5 µg / ml. The mode of action of the sterol biosynthesis inhibitors, including tetraconazole, was extensively investigated. These compounds are inhibitors of the C-14 demethylation of lanosterol or 24-methylendihydrolanosterol, a biosynthesis step that occurs during the conversion of lanosterol to ergosterol, the final product of fungal sterol synthesis [32]. The primary effect of ergosterol biosynthesis inhibitor is the inhibition of C-14 demethylation via the inhibition of cytochrome P-450 or the interference with many isomerases [33-36]. This could be the reason for the high sensitivity of mycelial growth of *A. alternata* and *F. solani* grown on PDA amended with tetraconazole.

Concerning the sensitivity of the tested fungi to clethodim, results in table (2) revealed that the tested fungi could grow on PDA medium containing different concentrations of clethodim. In view of *A. alternata*, the fungus could grow normally (9 cm) up to 75 µg / ml, but at higher concentrations of clethodim, i.e. 100, 150, 200 and 250 µg / ml, slight inhibition of the fungal growth was detected being 5.55, 7.44, 11.11 and 33.33 %, respectively. Also, the mycelium growth of *F. solani* was not affected by clethodim, whereas the fungus grew normally up to 150 µg / ml, but at higher concentrations of clethodim, i.e. 200 and 250 µg / ml, slight inhibition of the fungal growth was detected being 5.55 and 11.11%, respectively. Generally, it could be mentioned that the herbicide clethodim did not has an inhibitor effect to the growth of tested fungi.

It has been reported that some herbicides decrease or increase the diseases incidence caused by fungi, other have no effect [9]. In early screening tests, Altman [37] found that 25 conventional herbicides recommended in crop production stimulate growth of *Rhizoctonia solani in vitro* at 100 ppm. Also, James and Lockwood [10] found that the incidence of root rot in pea and corn, caused by *F. solani* f. sp. *Pisi* and *F. roseum* f. sp. *Cerealis,* is increased in soil treated with atrazine. Metosulam and isoproturon could not control or give any satisfactory control of *A. alternata* and *Pyrenophora tritici – repentis* [19].

Stepwise Exposure to Clethodim: Continued and excessive exposure of the fungi to high concentrations of clethodim resulted in decreasing the mycelium growth (Table 3). Such decreases were varied according to the interactions between fungus species, herbicide

Table 1: Mycelium growth , inhibition percent and EC_{50} of tested fungi on PDA medium treated with different concentrations of tetraconazole.

Tetraconazole concentrations (µg/ml)	*Alternaria alternata*		*Fusarium solani*	
	Mycelium growth (cm)	Inhibition %	Mycelium growth (cm)	Inhibition %
0.00	9.00	-	9.00	-
0.01	8.00	11.11	7.66	14.88
0.05	7.33	18.55	7.66	14.88
0.1	6.33	29.66	6.66	26.00
0.5	5.00	44.44	5.50	38.88
1.0	4.50	50.00	5.00	44.44
5.0	4.00	55.55	4.16	53.77
10.0	2.50	72.22	3.16	64.88
25.0	2.33	74.11	2.16	76.00
50.0	1.00	88.88	1.50	83.33
75.0	0.00	100.0	0.66	92.66
EC_{50}*	0.73		1.67	

*EC_{50}: The concentration of tetraconazole required to give 50 % inhibition of mycelium growth.

Table 2: Mycelium growth and inhibition percent of tested fungi on PDA medium treated with different concentrations of clethodim.

Clethodim concentrations (µg / ml)	*Alternaria alternata*		*Fusarium solani*	
	Mycelium growth (cm)	Inhibition %	Mycelium growth (cm)	Inhibition %
0.00	9.00	-	9.00	-
1.00	9.00	0.00	9.00	0.00
5.00	9.00	0.00	9.00	0.00
10.00	9.00	0.00	9.00	0.00
25.00	9.00	0.00	9.00	0.00
50.00	9.00	0.00	9.00	0.00
75.00	9.00	0.00	9.00	0.00
100.0	8.50	5.55	9.00	0.00
150.0	8.33	7.44	9.00	0.00
200.0	8.00	11.11	8.50	5.55
250.0	6.00	33.33	8.00	11.11

Table 3: Mycelium growth and inhibition percent of tested fungi on PDA medium after subsequent exposure to different concentrations of clethodim.

Clethodim concentrations (µg /ml)	*Alternaria alternata*		*Fusarium solani*	
	Mycelium growth (cm)	Inhibition %	Mycelium growth (cm)	Inhibition %
1st exposure 300	6.00	33.33	8.5	5.55
2nd exposure 400	5.50	38.88	8.0	11.11
3rd exposure 500	5.16	42.66	7.5	16.66
4th exposure 600	4.50	50.00	7.0	22.22

concentrations and number of exposures. At 300 µg / ml (1st exposure) the mycelia growth of *A. alternata* was 6.0 cm diameter, which was inhibited by 33.33 %. The second exposure (at 400 µg / ml) reduced the fungal growth being 38.88 %, but 42.66 % reduction of the growth occurred by transferring the fungus to 500 µg / ml of clethodim (3rd exposure). The lowest mycelia growth (4.5 cm) occurred after the 4th exposure to 600 µg clethodim / ml, which was inhibited by 50 %. Concerning *F. solani*, the results showed that the mycelial growth of the fungus was 8.5 cm diameter, which was inhibited by 5.55 % after the 1st exposure to 300 µg clethodim / ml. Interestingly, the fungus gave colony with 8.0 cm diameter after the 2nd exposure to clethodim. However, subsequent exposures caused a reduction of the fungal growth with increasing clethodim concentration.

Table 4: Mycelium growth , inhibition percent and EC_{50} of clethodim-exposed isolates of the tested fungi on PDA medium treated with different concentrations of tetraconazole.

Tetraconazole	*Alternaria alternata*		*Fusarium solani*	
concentrations (µg /ml)	Mycelium growth (cm)	Inhibition %	Mycelium growth (cm)	Inhibition %
0.00	9.00	-	9.00	-
0.01	8.50	5.55	9.00	0.00
0.05	8.50	5.55	9.00	0.00
0.1	8.00	11.11	8.66	3.77
0.5	8.00	11.11	8.50	5.55
1.0	7.66	14.88	8.00	11.11
5.0	7.50	16.66	8.00	11.11
10.0	5.33	40.77	7.50	16.66
25.0	5.00	44.44	6.66	26.00
50.0	3.66	59.33	5.50	38.88
75.0	3.50	61.11	4.66	48.22
100.0	3.50	61.11	4.00	55.55
125.0	3.00	66.66	3.50	61.11
150.0	2.66	70.44	3.50	61.11
EC_{50}*	35.48		100.93	

*EC_{50}: The concentration of tetraconazole required to give 50 % inhibition of mycelium growth.

Table 5: Conidiophora and mycelium diameter and width and length of conidia of clethodim- exposed isolates C.E.I. and tetraconazole- tolerant isolates T.T.I. of *Alternaria alternata* and *Fusarium solani*.

Isolate	Conidiophora diameter (um)	Mycelium diameter (um)	Width of conidia (um)	length of conidia (um)
Alternaria alternata				
PI*	4.89	5.17	7.68	14.84
C.E.I.**	3.24	3.85	13.92	22.39
T.T.I.***	4.63	4.10	10.02	15.68
Fusarium solani				
PI*	3.37	4.38	3.12	8.43
C.E.I.**	4.41	5.76	3.32	8.52
T.T.I.***	4.61	7.09	4.32	13.15

PI*= Parent isolate

C.E.I.**= Clethodim- exposed isolate (500 ppm)

T.T.I.***= Tetraconazole- tolerant isolate (150 ppm)

Resistance Measurement to Fungicide: Results in Table (4) indicated that excessive exposure of the fungi to clethodim resulted in acquired resistance to tetraconazole. It was found that the C.E.Is of *A. alternata* and *F. solani* grew well on medium treated with 150 µg / ml of tetraconazole with 2.66 and 3.5 cm colony diameter, respectively. On the other hand, 75 µg / ml of tetraconazole was required to give inhibition by 100 and 92.66 % for parent isolates of *A. alternata* and *F. solani*, respectively, as mentioned before (Table 1). The previous results were more obvious when the level of tolerance was calculated.

The end of experiment showed that the EC_{50} values of tetraconazole to clethodim exposed isolate (C.E.I.) of both fungi were 35.48 and 100.93 µg / ml for *A. alternata* and *F. solani*, respectively. Thus, *A. alternata* tolerated tetraconazole by 48.60- fold, while tolerance level was 60.43- fold for *F. solani*.

Generally, the excessive exposure of the tested fungi to clethodim exhibited isolates resistant to tetraconazole. These results agree with that obtained by Mahmoud [25] who found that continued exposure of *Rhizoctonia solani* to the herbicides trifluralin and pendimethalin either in PDA or soil results in isolates tolerated the fungicides benomyl and carboxin captan. Mahmoud and Khalifa [26] found that the continued exposure of *F. graminarum*, *R. solani* and *Pyrenophora tritici-repentis* to the herbicide tralkoxydim can build up resistance to the fungicides thiabendazole and toloclofos-methyl. Also, Mahmoud and Khalifa [27] found that the excessive

Table 6:Persistence of tolerance in *Alternaria alternata* and *Fusarium solani* to tetraconazole after 10 transfers on clethodim- or tetraconazole- free PDA medium

	EC_{50} values (µg / ml) of tetraconazole			
Number of transfer	*Alternaria alternata* *	*Alternaria alternata* **	*Fusarium solani**	*Fusarium solani***
1st	28.82	10.46	72.29	13.56
2nd	23.450	8.23	53.12	9.66
3rd	14.96	6.72	22.82	7.19
4th	12.29	3.23	16.65	3.27
5th	8.24	1.16	9.42	1.86
6th	3.65	0.80	4.61	1.18
7th	2.74	-	3.42	-
8th	2.48	-	2.98	-
9th	1.96	-	2.18	-
10th	0.83	-	1.61	-

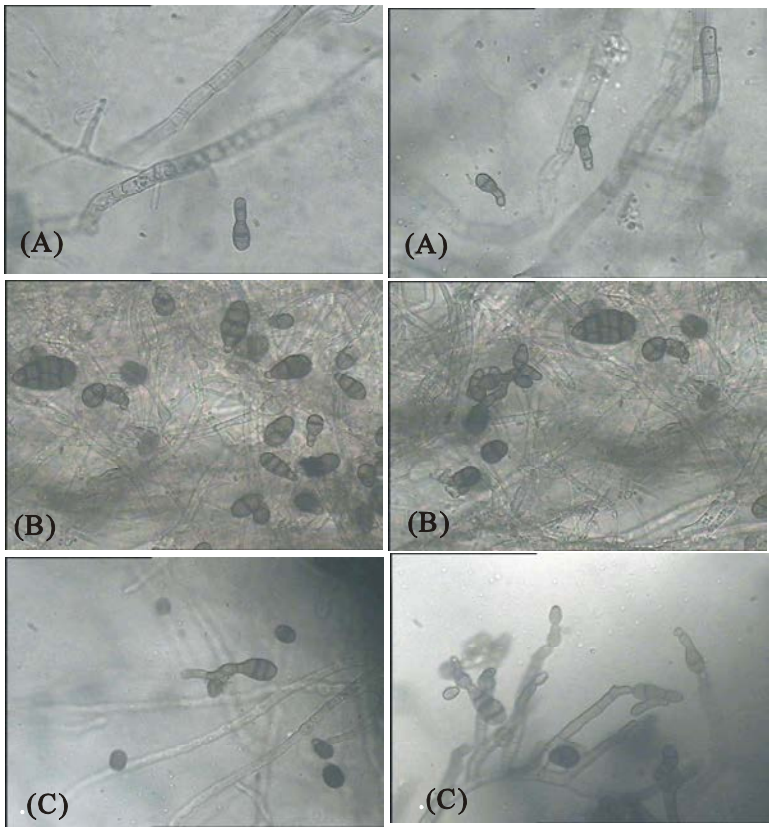

Fig. 1: Image analysis of *A. alternata*. (A): Parent isolate (PI), (B); Clethodim exposed isolate (C.E.I.), and (C); Tetraconazole- tolerant isolate (T.T.I.).

application of metribuzin herbicide may lead to difenoconazole- tolerant isolates of *F. oxysporum* f. sp. *lycopersici* and *A. alternata*.

Morphological Feature: During the continuous exposure of *A. alternata* and *F. solani* to clethodim, regions of slight green color were observed, within the growing colonies. The image analysis (Table 5 and Fig. 1) indicated that *A. alternata* produced thinner conidiophora and mycelia compared to the parent isolate. It was found that the conidiophora and mycelium diameters of *A. alternata* parent isolate (PI) were about 4.89 and 5.17 um, respectively, but they were 3.24 and 3.85 um of clethodim-exposed isolate (C.E.I) and 4.63 and 4.10 um of

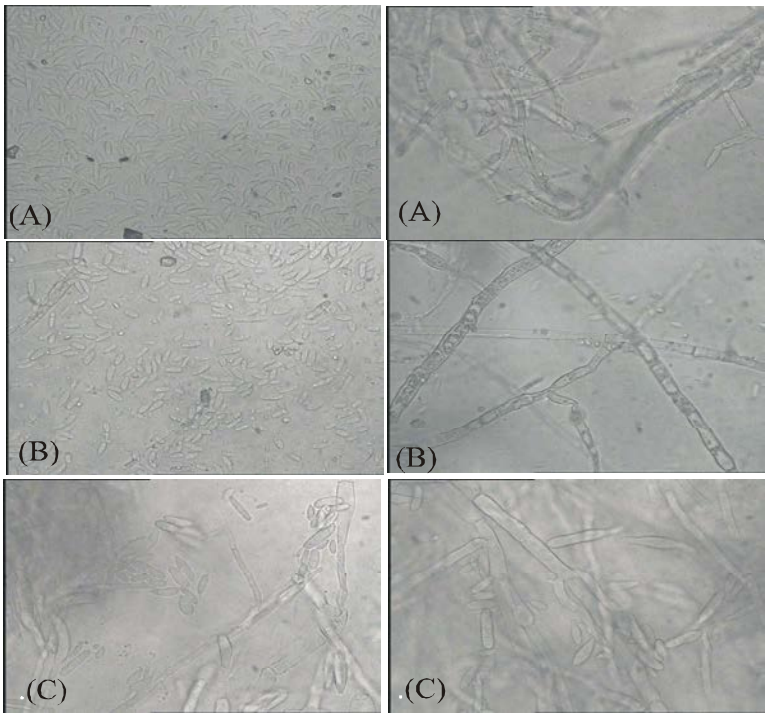

Fig. 2: Image analysis of *F. solani.* (A): Parent isolate (PI), (B): Clethodim exposed isolate (C.E.I.) and (C); Tetraconazole- tolerant isolate (T.T.I.).

tetraconazole- tolerant isolate (T.T.I), respectively. On contrary, this fungus produced more size of spores compared to (PI), whereas, the width and length of conidia of (PI) were about 7.68 and 14.84 um, respectively, but they became 13.92 and 22.39 um of C.E.I. and 10.02 and 15.68 um of T.T.I., respectively. Regarding *F. solani*, the image analysis (Table 5 and Fig. 2) showed that the fungus produced enlarged conidiophora, mycelium and conidia which were markedly different from the parent isolate, which were more obvious in tetraconazole-tolerant isolate. It was found that the conidiophora and mycelium diameter and width and length of conidia of *F. solani* parent isolate (PI) were 3.37, 4.38, 3.12 and 8.43 um, respectively, but they became 4.41, 5.76, 3.32 and 8.52 um of C.E.I. and 4.61, 7.09, 4.32 and 13.15 um, respectively, when the fungus tolerated tetraconazole (T.T.I.). These morphological differences of these isolates may play a part for the tolerance mechanism. Mahmoud and Khalifa [26] reported the same observations. They found that *F. graminarum* exposed to the herbicide tralkoxydim produce enlarged terminal chlamydospores and intercalary chlamydospores which differed markedly from the parent isolate, however, this isolate tolerated the fungicides toloclofos-methyl and thiabendazole. Mahmoud and Khalifa [27] indicated that *F. oxysporum* f. sp. *Lycopersici* and *A. alternata* exposed to the herbicide metribuzin produced thinner mycelia compared to the parent isolates, however, these isolates tolerated the fungicide difenoconazole.

Persistence of Tolerance: Serial sub culturing of C.E.I. or T.T.I. of both fungi on herbicide or fungicide- free medium and assessment the EC_{50} value of each step were illustrated in table (6). According the detected EC_{50} values, tolerance in *A. alternata* and *F. solani* to tetraconazole decreased after the subsequent culturing of the tolerant isolates on fungicide- free PDA medium. It was found that the EC_{50} values were 28.82 and 10.46 µg / ml of tetraconazole after the 1st sub culturing of *A. alternata* tolerant isolate on tetraconazole or clethodim-free medium, respectively. The same result was detected for the tolerant isolates of *F. solani*, where the EC_{50} values of tetraconazole were 72.29 and 13.56 µg / ml after the same sub culturing step. Subsequent cultivation up to 5th step on the same media resulted in great reduction, whereas the EC_{50} values of tetraconazole were 8.24 and 1.16 µg / ml against growth of T.T.I. and C.E.I. isolates of *A. alternata*, respectively and they were 9.42 and 1.86 µg / ml of T.T.I. and C.E.I. of *F. solani*, respectively. Indeed, the sub culturing of C.E.I. on clethodim- free medium

resulted in reduction of tolerance to the fungicide tetraconazole, as indicating by EC_{50} values. The 10th sub culturing step of T.T.I. of both fungi led to the disappearance of tolerance, whereas the EC_{50} of tetraconazole became o.83 and 1.61 µg / ml against growth of *A. alternata* and *F. solani*, respectively, which were somewhat equally to the EC_{50} of the fungicide tetraconazole against growth of the parent isolates. The disappearance of fungicide tolerance was recorded after the 5th step of sub culturing of C.E.Is on clethodim-free medium. However, this observation was achieved by 10 sub culturing steps of T.T.I. on tetraconazole- free medium. Generally, it can be concluded that excessive application of clethodim might lead to tetraconazole-tolerant isolates of *A. alternata* and *F. solani*, forthrightly; this type of tolerance was lost when the fungi were grown on pesticide- free medium indicating that the tolerance is due to physiological adaptation rather than mutation and selection.

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