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Evaluation of strobilurin fungicides Ergon 44.3% (w/w) [Kresoxim methyl 500 g L^{-1} SC] and RIL-070/FI (72WP) against *Phytophthora capsici* infection in black pepper

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Abstract

Two new strobilurin fungicides *viz*., Ergon 44.3% (w/w) [Kresoxim methyl 500 g L^{-1} SC] and RIL-070/FI (72WP) were evaluated *in vitro* and *in planta* against *Phytophthora capsici* causing foot rot disease of black pepper and ED_{50} and ED_{90} values were calculated based on the *in vitro* efficacy. The chemicals were tested *in planta* using the maximum concentration found effective *in vitro*. Ergon when tested from 10-6000 ppm of the product *in vitro,* showed complete inhibition of mycelial growth and sporulation at 6000 ppm. But the zoospore germination was completely inhibited at 2000 ppm. The average ED_{50} and ED_{90} values of Ergon were 845.51 and 1740.71, respectively. Foliar spray of the chemical followed by challenge inoculation showed an overall reduction of lesion development by 44.83% over control and maximum inhibition (57.12%) was observed at five days after spraying at a concentration of 7000 ppm. Soil application of Ergon at different concentrations from 6000-8000 ppm showed no infection or mortality at 7000 ppm. RIL-070/FI at different concentrations from 10-500 ppm of the product when tested *in vitro* against *P. capsici* showed 100% mycelial inhibition at 50 ppm with ED_{50} and ED_{90} values of 22.85 and 45.71, respectively. For inhibition of sporulation, the maximum concentration required was 100 ppm. However, zoospore germination showed 100% reduction at 200 ppm. Foliar spraying of the RIL at concentrations from 100-600 ppm showed lesion development from 0.71 to 100%. When *P. capsici* was challenged five days after spraying of the chemical, 600 ppm showed 100% inhibition of lesion development.

Keywords: black pepper, foot rot, kresoxim methyl, *Phytophthora capsici*, RIL-070/FI (72WP), strobilurin fungicides

Introduction

Phytophthora capsici Leonian causing foot rot disease is a major threat to black pepper cultivation. The pathogen has different growth phases for survival *viz*., hyphae, zoosporangia, zoospores (vegetative phase) and oospores (sexual phase). Fungicide discovery for managing this Oomycete pathogen has undergone a significant change and several novel fungicides have been developed in the past two decades like Oxazolidinediones (famoxadone), Imidazoles (fenamidone),

Benzamides (fluopicolide, zoxamide), Valinamides (iprovalicarb, benthiovalicarb), Mandelamides (mandipropamid), Cyanoimidazoles (cyazofamid), and Thiocarbamates (ethaboxam). Of these the prominent ones being tested against different species in India are mandipropamid, iprovalicarb, benthiovalicarb, fluopicolide, famoxadone, cyazofamid, pyraclostrobin and kresoxim methyl (Qi *et al.* 2009). Since long, *P. capsici* infection in black pepper has been managed through the use of both copper based and systemic fungicide metalaxyl. Since frequent use of single molecule of metalaxyl has conferred resistance to the pathogen, combination products like Metalaxyl-Mancozeb are now being used (Ramachandran *et al.* 1991). However, some other molecules have been reported to have activity on species of *Phytophthora* such as *P. capsici, P. citrophthora* and *P. parasitica*, which include mainly strobilurin fungicides which are fungus-based fungicides having suppressive effect (Matheron & Porchas 2000). The advantages of these new molecules are that they are needed in only very small quantities. They are mostly contact in nature as they are absorbed into the cuticle and not transported any further. However, as per the fact sheet, it is reported that these fungicides are absorbed through the roots and translocated in the xylem to the stems and leaves or through leaf surfaces to the leaf tips and growing edges and are reported to inhibit spore germination, mycelial growth and spore production. Ergon 44.3% (w/w) SC and RIL - 070/FI (72WP), are two new molecules from M/S Rallis India Limited, found effective against many soil borne pathogens including *Phytophthoras* (Matheron & Porchas 2000). The present study is focused on testing the impact of these fungicides against *P. capsici* infection in black pepper. Since the mode of action of the chemicals depends on the different growth stages of the organisms, the major objectives for evaluating the new molecule were (1) evaluation against different growth phases of *P. capsici* (2) estimation of ED_{50}/ED_{90} values and (3) *in planta* evaluation after challenge inoculation at concentrations found effective *in vitro*.

Materials and methods

Fungi and fungicides and test plants

The pathogen *P. capsici* isolate (06-04) was collected from the National repository of *Phytophthora,* ICAR-Indian Institute of Spices Research, Kozhikode. Fungicides Ergon 44.3% (w/w) SC [Kresoxim methyl 500 g L^{-1} SC] and RIL-070/FI (72WP) were supplied by M/S Rallis India Ltd. Plants of variety Sreekara raised under sterile (potting mixture) in green house and grown for three months were taken for the evaluation.

Activity on mycelial growth

Stock solutions of the chemical was prepared and from the stock solution different concentrations such as 10, 20, 30, 40, 50,100, 200, 300, 400 and 500 ppm were prepared in sterile distilled water. This was incorporated into 100 mL of sterilized carrot agar medium so as to get the required concentration and poured into petri dishes. *P. capsici* was grown on Carrot Agar (CA) for 72 h at 24±1°C. Agar plugs of 5 mm were removed from the edges of the actively growing culture of the pathogen and placed in the centre of the sterile CA plates amended with the test fungicide [(10-10000 ppm in case of Ergon and 10-500 ppm in case of RIL-070/FI (72WP)]. The required concentrations of the chemicals were incorporated on to sterilized carrot agar medium and poured into sterile plates and inoculated. Control plates contained only CA inoculated with the pathogen alone. All the treatments were replicated thrice. The plates were incubated for 96 h and radial growth of mycelium was measured and percent inhibition calculated as I= C-T/C × 100 where I= percent inhibition, C= radial growth in control and T= radial growth in treatment. The data was statistically analyzed using MSTAT-C programme.

Activity on sporulation of P. capsici

The stock solution prepared as above was used for studying the sporulation of *P. capsici*. Different concentrations of the chemical were prepared in sterile distilled water. Inoculum plugs of 5 mm size were cut from the advancing margin of 72 h old culture of *P. capsici* and incubated in different concentrations of the chemicals under continuous fluorescent light for 72 h at 24 ± 1°C. There were three replications /treatment with 5 discs/replication. Inoculums plugs in sterile distilled water served as control. Observations for sporulation were taken under the microscope. Three microscopic fields were counted /replication and the average number of sporangia produced was estimated and the reduction in sporulation compared to control was calculated. The ED_{50} and ED_{90} values for sporangial inhibition were also calculated as mentioned above.

Activity on zoospore germination

The stock solution prepared as above was used for studying the zoospore germination of *P. capsici*. Different concentrations of the chemical were prepared in sterile distilled water. Sporulated cultures as above were subjected to cold shock for 10 minutes and kept at room temperature for 20 minutes for zoospore release. The zoospores released were harvested by filtering through three layers of muslin cloth and were taken in an Oakridge tube and centrifuged at 3000 rpm for 7 minutes at 4°C. Supernatant was discarded and pellet containing zoospores were mixed with 1.0 mL sterile water and vortexed. About 20 µL of the zoospore suspension was treated with respective concentrations of the chemical and incubated overnight at 24±1°C. Zoospore suspension without chemical served as control. The treated zoospores were observed microscopically. There were three replications of each treatment and from each replication five microscopic fields were counted. The germinated and non-germinated zoospores were counted and from the means the percent germination was calculated and ED_{50} and ED_{90} values were estimated by probit analysis.

In planta evaluation against P. capsici

Foliar application

Intact plants (rooted cuttings of 3-4 leaf stage) raised in sterile potting mixture in polythene bag of size 21 cm × 15 cm were used for the experiment. The chemical at required concentration was sprayed on the foliage @50 mL plant^{-1} taking care to ensure that the spray fluid reached both sides of the leaf. Here the concentration was fixed based on *in vitro* studies. The maximum concentration of the chemical that showed 100% inhibition *in vitro* was taken as the critical concentration. One concentration below and four concentrations above the critical concentration were also tested. Inoculation was done at 0, 5, 10, 15 days after spraying of the chemical to study the persistence of the fungicide. Inoculum plugs of 5 mm were placed on the lower surface of the leaves and a moist cotton strip was placed over the inoculum plug to keep the inoculums moist. Control plants were maintained with water spray. There were five plants in each treatment. Observations on leaf lesion diameter were recorded at 96 h and the percent inhibition over control was calculated.

Soil application

Intact plants (rooted cuttings of 5-6 leaf stage) raised in sterile potting mixture in polythene bag of size 21 cm × 15 cm) were drenched with four different concentrations of the chemical *viz*., 200-600 ppm @100 mL plant^{-1}. The treated plants were inoculated with 20 numbers (5 mm size) sporulated inoculum plugs. The inoculum plugs were placed just below the collar portion encircling it and covered with soil. There were 10 plants in each treatment and the experiment was repeated twice. The observations on

mortality of the plants were recorded from the 7th day.

Statistical analysis

The ED_{50} and ED_{90} values are the concentrations of the chemical required for getting 50% and 90% inhibition respectively in mycelial growth, sporangial production and encysted zoospore germination. These values were estimated from the fitted regression line of the log transformed percent inhibition plotted against transformed fungicide concentration using probit analysis.

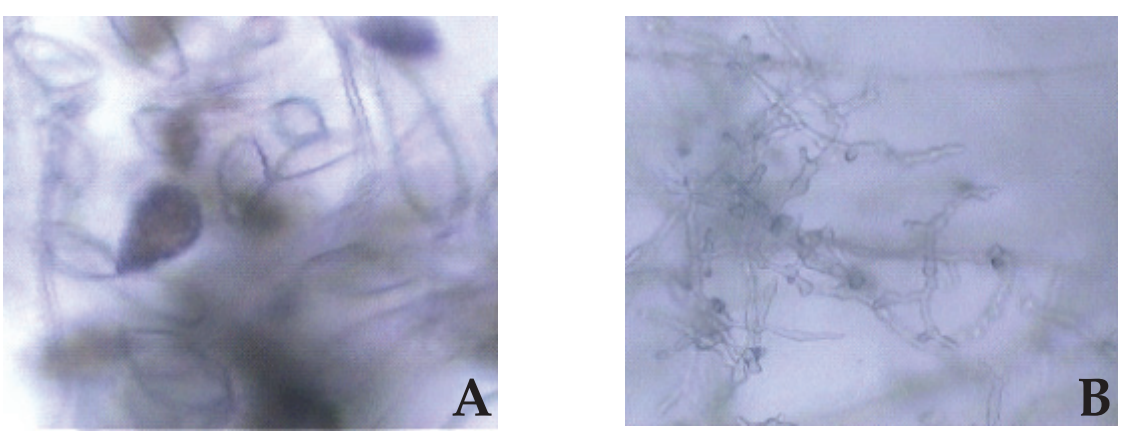
Results and discussion

Evaluation of Ergon 44.3% (w/w) SC [Kresoxim methyl 500 g L^{-1} SC] against P. capsici

Ergon 44.3% (w/w) [Kresoxim methyl 500 g L^{-1} SC] was tested at different concentrations from 10-6000 ppm of the product *in vitro* against mycelial growth *of P. capsici.* Absolutely no inhibition of the pathogen was noticed from 10-500 ppm. But when the concentration was increased from 500 up to 6000 ppm, 100% inhibition of mycelial growth was obtained at 6000 ppm. However, between 1000-3000 ppm,

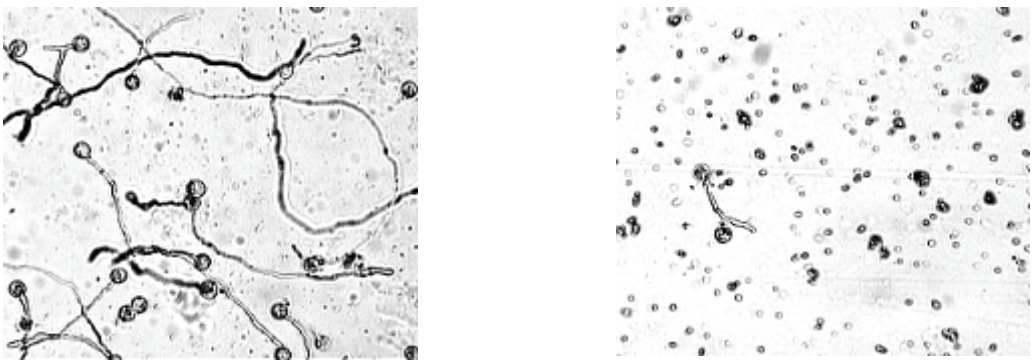
Table 1. Effect of Ergon on mycelial growth, sporulation and zoospore germination of *P. capsici*

Concentration (ppm)	Mycelial growth (mm)	Mycelial growth inhibition over control (%)	Sporulation (%)	Reduction in sporulation over control (%)	Germination (%)	Reduction over control (%)
1000	41.33	45.38	29.75	50.47	0.39	99.50
2000	40.33	46.69	23.70	60.54	0.00	100.00
3000	34.33	54.62	22.00	63.38	0.00	-
4000	20.00	73.57	13.20	78.03	0.00	-
5000	13.33	82.38	17.43	70.98	0.00	-
6000	0.00	100.00	0.00	100.00	0.00	-
Control	75.67	-	60.08	0	87.07	-
CD (P <0.05)	0.78		2.65			
CV %	2.07		10.11			



A. Sporulation in control **B.** Sporulation in 6000 ppm of Ergon

Fig. 1. Effect of Ergon on sporulation of *P. capsici*



A. Zoospore germination in control **B.** Zoospore germination at 1000 ppm of Ergon

Fig. 2. Effect of Ergon on *P. capsici* zoospore germination

the difference in growth was only marginal. The ED_{50} and ED_{90} values were 210.61 μg mL^{-1} and 311.06 μg mL^{-1}, respectively (Table 1). When tested *in vitro* against sporulation, complete inhibition of sporulation occurred at 6000 ppm and no significant difference was noticed between 1000-3000 ppm. However >50% inhibition was obtained even at 1000 ppm (Table 1 & Fig. 1). Similarly, almost complete inhibition of zoospore germination was obtained at 1000 ppm. The chemical was found to be highly sensitive to encysted zoospores germination when compared to untreated zoospores where >87% germination was obtained (Table 1 & Fig. 2).

The ED_{50} and ED_{90} values for mycelial growth inhibition were 210.64 μg mL^{-1} and 311.06 μg mL^{-1}, respectively, whereas it was 1480.41 μg mL^{-1} and 3170.35 μg mL^{-1} for inhibition of sporangial production. The average ED_{50} and ED_{90} values were 860.69 and 1487.17 μg mL^{-1}, respectively. The result clearly showed that sporangial formation is the hardiest stage where the fungicide shows comparatively low sensitivity when compared to mycelial growth and zoospore germination (Table 2).

Foliar application of Ergon followed by challenge inoculation on leaves with *P. capsici* showed an average of 44.83% reduction in lesion development over control and maximum reduction (57.12%) was observed at five days after spraying at a concentration of 7000 ppm and was reduced to 46.38% after 10 days and 43.52% after 15 days. Absolutely no reduction in lesion development could be noticed after 20 days showing that the residual period of the chemical is only up to 15-20 days (Table 3). However, no significant difference in lesion development was observed among the different concentrations of the chemical. Soil application of the chemical at concentrations ranging from 6000-8000 ppm showed only 28.5% morality at 6000 ppm as compared to control (57.14%), while no infection or mortality was observed

Table 2. The ED_{50} and ED_{90} values of Ergon 44.3% (w/w) Kresoxim methyl 500 g/l^{-1} SC and RIL-070/FI for mycelial growth, sporangium production and encysted zoospore germination of *P. capsici*

Growth phases of *P. capsici*	Kresoxim methyl 500 g L^{-1} SC (μg mL^{-1})		RIL (μg mL^{-1})	
	ED_{50}	ED_{90}	ED_{50}	ED_{90}
Mycelial growth	210.64	311.06	22.85	45.71
Sporangial production	1480.41	3170.35	34.47	47.47
Zoospore germination	891.03	945.45	30.38	70.11
Average	860.69	1487.17	29.23	54.43

Table 3. Effect of Ergon on foliar infection by *P. capsici*

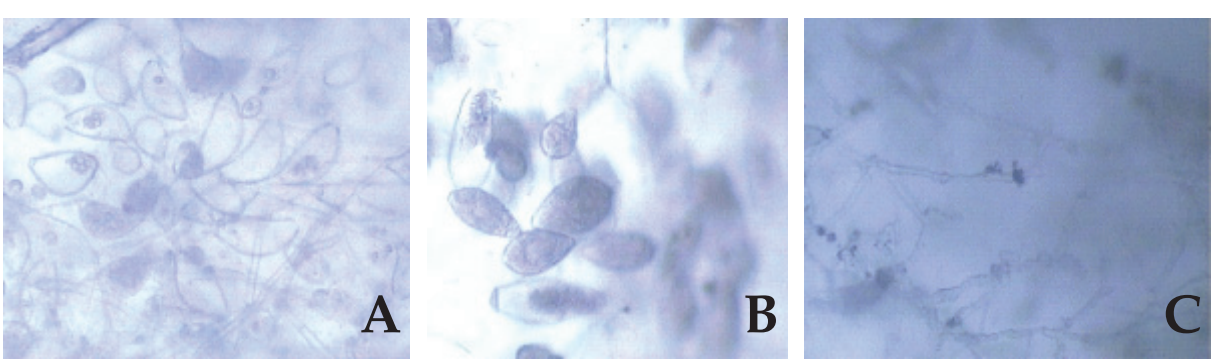
Concentration (ppm)	Lesion size (mm)					Reduction over control (%)				
	0	5	10	15	20	0	5	10	15	20
5000	13.60	63.04	32.00	6.98	33.00	5.17	30.60	16.39	37.00	0.00
6000	17.66	52.01	27.60	19.77	22.15	36.35	24.00	34.42	33.80	8.65
7000	18.00	51.09	14.75	57.12	18.66	46.38	20.67	43.52	36.80	0.54
Control	36.80	-	34.40	-	34.80	-	36.60	-	37.00	-
CD (P <0.05)	21.52	-	27.19	-	27.15	-	27.97	-	33.70	-

DAS=days after spraying

Table 4. Effect of RIL-070/FI on mycelial growth, sporulation and zoospore germination of *P. capsici*

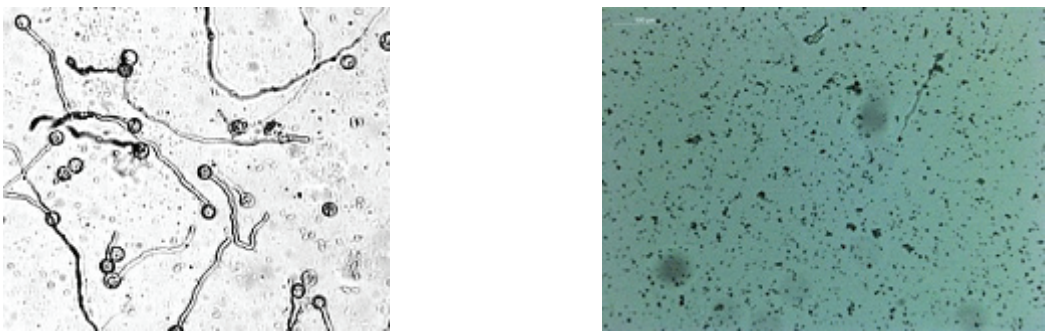
Concentration (ppm)	Mycelilal growth (mm)	Reduction of mycelial growth over control (%)	Sporulation (%)	Reduction in sporulation over control (%)	Zoospore germination (%)	Reduction over control (%)
10	76.00	14.3	36.7	11.0	68.7 (56.0)	17.0 (14.6)
20	60.33	32.0	29.4	28.9	70.5 (57.2)	14.8 (12.8)
30	34.00	61.7	24.4	40.9	41.7 (40.2)	49.7 (38.7)
40	12.67	85.8	14.2	65.5	46.6 (43.0)	43.8 (34.4)
50	0.00	100.0	8.6	79.3	37.4 (37.7)	54.8 (42.8)
100	0.00	100.0	0.0	100.0	41.6 (40.2)	49.6 (38.6)
200	0.00	100.0	0.0	100.0	0.0 (0.0)	100.0 (100)
Control	88.67		41.3		82.8 (65.6)	-
CD (P <0.05)	1.40		4.5		3.2 (1.99)	
CV (%)	3.10		18.31		6.09	
ED_{50}		22.85 ppm		34.47 ppm		30.38 ppm
ED_{90}		45.71 ppm		47.47 ppm		70.11 ppm

The figures in parenthesis are arc-sine transformed values.



A. Sporulation in control **B.** Sporulation in 10 ppm **C.** Sporulation in 100 ppm

Fig. 3. Effect of RIL-070/FI on sporulation of *P. capsici*



A. Germination in control **B.** Germination at 200 ppm

Fig. 4. Effect of RIL-070/FI on zoospore germination of *P. capsici*

at 7000 ppm. However, all the plants treated with 8000 ppm showed complete death of the plants.

Evaluation of RIL-070/FI (72WP) against P. capsici

RIL-070/FI when tested at different concentrations from 10 - 500 ppm *in vitro* against mycelial growth of *P. capsici* showed 100% inhibition at 50 ppm and the ED_{50} and ED_{90} values were 22.85 and 45.71, respectively (Table 4). However, sporulation of *P. capsici* was completely inhibited at 100 ppm. The ED 50 and ED 90 values were 34.47 and 47.47, respectively (Table 4 & Fig. 3). Likewise RIL-070/FI showed complete inhibition of zoospore germination at 200 ppm and the ED_{50} and ED_{90} values were 30.38 ppm and 70.11 ppm, respectively (Table 2, Table 4 & Fig. 4). The average ED_{50} and ED_{90} values were 29.23 and 54.43 µg mL^{-1}, respectively (Table 2).

Foliar spraying of the chemical at concentrations from 100-600 ppm showed reduction in lesion development from 0.71-100% and 100-400 ppm showed only less than 40% reduction whereas 500-600 ppm showed more than 60% reduction. Immediately after spraying of the chemical, 500 and 600 ppm showed 92% and 96% reduction in lesion development, respectively. When *P. capsici* was challenged five days after spraying, 79.86% reduction in lesion development was noticed at 500 ppm whereas 600 ppm showed 100% reduction and the effect was reduced when challenged 10 days after spraying (Table 5). A significant reduction in mortality due to *P. capsici* infection was observed in treated plants when compared to control by soil application and 400 and 600 ppm were found effective and showed 100% reduction in mortality. The *P. capsici* population was also reduced to 77.58%.

Table 5. Effect of RIL-070/FI on foliar infection by *P. capsici*

Concentration (ppm)	Lesion size (mm)			Reduction over control (%)		
	0	5	10	0	5	10
100	27.80	0.71	24.00	16.67	24.20	10.37
200	23.40	16.42	25.20	12.5	18.40	31.85
300	26.75	4.46	17.80	38.19	15.60	42.22
400	21.60	22.85	20.40	29.16	16.40	39.25
500	2.20	92.14	5.80	79.86	9.60	64.44
600	1.00	96.42	0.00	100.00	5.60	79.25
Control	28.00	-	28.8	-	27.0	-
CD (P<0.05)	6.246		9.722		9.229	
CV (%)	30.97		50.56		49.88	

Table 6. Effect of soil application of RIL-070/FI on *P. capsici* infection

Concentration (ppm)	Mortality (%)	Reduction in mortality over control (%)	*P. capsici* population	Reduction of *P. capsici* population (%)
200	14.29	74.99	36.06 (38.57)	46.01
300	14.29	74.99	19.29 (21.43)	71.11
400	0.00	100.00	14.97 (11.42)	77.58
600	0.00	100.00	14.97 (11.42)	77.58
Control	57.14	-	66.79 (74.43)	-
CD (P<0.05)			48.88 (54.54)	

But 800 ppm and above was found to be phytotoxic (Table 6).

Similar study was conducted by Matheron & Porchas (2000). They evaluated the effect of azoxystrobin (Strobilurin analogue) on developmental stages of *P. capsici* along with other species like *P. citrophthora,* and *P. parasitica*. The ED_{50} and ED_{90} values for *P. capsici* was found to be >3000 μg mL^{-1} for mycelial growth, whereas it was 3.3 μg mL^{-1} and 57.8 μg mL^{-1}, respectively for sporangial formation and 700 and >1000 μg mL^{-1} for zoospore germination, respectively and is in agreement with the result obtained in our present study. Similarly, the average ED_{50} and ED_{90} values for Ergon were 860.69 μg mL^{-1} and 1487.17 μg mL^{-1}. However ED_{50} and ED_{90} values for zoospore germination were 891.04 μg mL^{-1} and 945.45 μg mL^{-1} respectively. The average ED_{50} and ED_{90} values for *in vitro* inhibition was 29.23 and 54.43 ppm, respectively.

The ultimate effect of any chemical compound on disease management depends on its mode of action at the physiological level on one or more phases of the life cycle of a pathogen (Matheron & Porchas 2000). Considering all the stages in the life cycle of *P. capsici*, sporangial formation and zoospore release provide the greatest opportunity for a rapid buildup in the infective propagules and subsequent higher potential for host infection and disease development (Duniway 1983; Gregory 1983; van der Plank 1963). Therefore, any chemical that significantly suppresses formation of sporangia and release/germination of zoospores should reduce the ability of the pathogen to cause disease. The present study clearly indicated that strobilurin fungicide Kresoxim methyl 500 g L^{-1} SC at 1000 ppm completely inhibited the germination of encysted zoospores. However, the same at 6000 ppm was required for inhibiting the sporulation when compared with untreated control, thus restricting the pathogenicity of *P. capsici*. The average ED_{50} values and ED_{90} values of Kresoxim methyl 500 g L^{-1} SC were 860.69 μg mL^{-1} and 1487.17 μg mL^{-1}, respectively. For inhibition of mycelial growth of *P. capsici* the ED_{50} values and ED_{90} values were-210.64 μg mL^{-1} and 311. 06 μg mL^{-1}, respectively, which indicated that the chemical is highly sensitive to mycelial phase.

Godwin *et al.* (1992) reported that azoxystrobin a Strobilurin analogue is more active in suppressing the sporangium formation (mean EC_{90} value of 29.2 μg mL^{-1}) and zoospore motility (mean EC_{90} value of 14.5 μg mL^{-1}) than zoospore cyst germination (EC_{90} value >1000 μg mL^{-1}). The data suggested that the fungicide is more sensitive to zoospore germination than mycelial growth and sporulation. According to Brandt *et al.* (1988), the likely reason for low mycelial sensitivity to Strobilurin displayed by several pathogens is due to its specificity to inhibit mitochondrial respiration by blocking electron transport at the cytochrome bc1 complex and circumvention of the cytochrome bc1 target site by induction of the alternative

oxidase respiratory pathway. EC 90 value is often calculated in studies assessing the potential value of a molecule to control growth or sporulation of the pathogen. Qi *et al.* (2009) evaluated four commercial formulations of strobilurin fungicides, kresoxim-methyl, azoxystrobin, enestroburin and pyraclostrobin *in vitro* and *in vivo* for their efficacy, curative and protective action against pepper blight caused by *P. capsici*. Their *in vitro* experiments showed that kresoxim-methyl could inhibit the mycelial growth of *P. capsici* with EC_{50} at 8.88 and sporangium formation at 1.31 μg mL^{-1}. The fungicide showed stronger inhibition against sporulation germination with EC_{50} values at 0.94 μg mL^{-1}. Similarly *in vivo* experiments, the fungicide showed protective and curative activities and protection action was better than curation.

Foliar spray of Ergon 44.3% (w/w) showed maximum reduction in lesion development (57.12%) at fifth day of spraying at a concentration of 7000 ppm and was reduced to 46.38% after 10 days and 43.52% after 15 days of application of the chemical. Whereas foliar spraying of RIL at 100-600 ppm showed lesion development from 0.71 to 100%. When *P. capsici* was challenged five days after spraying of the chemical, 600 ppm showed 100% reduction of lesion development and the effect was reduced further 10 days after spraying. So the residual effect of the chemical remained only up to 10-15 days. This may be because of their sensitivity to light, and high vapor pressures that causes them to rapidly disappear when applied to the surface of a leaf as in the case of other strobilurins.

However, soil application of Ergon showed 50.12% reduction in mortality at 6000 ppm while no mortality could be observed at 7000 ppm while soil application of RIL at 400 ppm showed 100% reduction in plant mortality/ infection as well as 77.58% reduction in *P. capsici* population. The data revealed that the fungicide is more effective as soil application than foliar spray. Foliar application of the chemical did not show any significant difference in lesion development when compared to untreated control. This is supported by the work of Gupta & Jarial (2010) where they evaluated two strobilurins along with different systemic and non – systemic fungicides for their efficacy against *Phytophthora* leaf blight and fruit rot of bell pepper. They observed that kresoxim methyl was least effective. In our study, soil application at the same concentration showed complete inhibition of the pathogen and disease development. The plant retained the effect even after six months of application. This is supported by the fact that these chemicals are absorbed through the roots and translocated in the xylem to the stems and leaves or through leaf surfaces to the leaf tips and growing edges. However, it was observed that soil application at concentration higher than what is recommended is detrimental to the plant system. Phytotoxicity of the chemical was already reported by Gullino *et al.* (2002). So it was found that Kresoxim methyl 500 gL^{-1} SC is effective as a soil drench @7000 ppm against foot rot disease caused by *P. capsici* whereas RIL @ of 400 ppm is effective as soil drench. But more studies are required to confirm the efficacy of the chemical's under field condition.

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