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In vitro efficacy of promising fungicides and bio control agents against *Fusarium oxysporum* f. sp. *ciceris* causing wilt disease in chickpea (*Cicer arietinum* L.)

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Abstract
The present study was undertaken *in-vitro* to know the efficacy of Fungicides and bio control agents to Manage of *Fusarium oxysporum* f. sp. *ciceris* causing wilt in chickpea. Isolates of *Fusarium* spp. that were isolated from various samples belonged infested with *Fusarium oxysporum* f. sp. *ciceris*. The pathogenicity of fungal isolates was confirmed by Koch's postulates on susceptible chickpea cultivar 'GNG 1958'. The *F. oxysporum* f. sp. *ciceris* isolate from Kherot-KHR Foc-09 was found highly virulent by producing high degree of wilt incidence. Six fungicides were evaluated for their efficacy against *F. oxysporum* f. sp. *ciceris* at 100, 500 and 1000 ppm concentrations, results showed that Carbendazim was the most effective in suppressing the growth of *F. oxysporum* f. sp. *ciceris* at all concentrations which was followed by Saaf. Among bio control agents, *Trichoderma viride-5* was showed severe antagonism and highly effective against *F. oxysporum* f. sp. *ciceris* in dual culture technique.

Keywords: *Fusarium oxysporum* f. sp. *ciceris*, chickpea wilt, fungicides, bio control agents, KHR Foc-09

Introduction
Pulses are cultivated in India since time immemorial. Chickpea (*Cicer arietinum* L.) is commonly known as "Bengal gram" or "Gram". It is also known as Spanish pea, Chestnut bean (English), Poischiche (French), Homos (Arabic), Garbanzo (Spanish), Grao-debico (Portuguese) and Chana (Hindi). Chickpea is a self-pollinated crop belonging to the sub-family: Papilionaceae, family: Leguminaceae. It is believed to be introduced into India from Western Asia.

India is the largest chickpea producer as well as consumer in the world sharing 65.25 percent in area and 65.49 percent in production and is grown on 10.23 million ha area with production 11.35 million tones and productivity 967 kg/ha (Thaware *et al*., 2017) [15]. India is a major international chickpea producer country, producing around 75% of the world's supply (Tomar *et al*., 2010) [16]. In present India grows chickpea on about 105.73 lakh hectares' area, producing 111.58 lakh tonnes which represents 40 percent and 48.1 percent of the national `pulse area and production, respectively (Anonymous, 2020-21) [1].

Chickpea is an important winter legume crop of Rajasthan, grown principally both in irrigated and rain-fed areas. It occupies an area of 21.13 lakh hectares with the production of 23.22 lakh tonnes (Anonymous 2020-21) [1]. The district-wise contribution to overall (chickpea) production in the state, Bikaner leads, with a share of 13%, followed by Churu (9%), Jhunjhunu (9%), Hanumangarh (8%), Shri Ganganagar (8%), Jaipur (8%), Jaisalmer (5%), Sikar (5%), Ajmer (5%), and other remaining districts (30%). (Anonymous 2020-21) [1].

Chickpea cultivation is often subjected to several biotic stresses of which diseases like Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Synd. & Hans, Ascochyta blight [*Ascochyta rabiei* (Pass.) Labr.], Botrytis grey mould (*Botrytis cinerea* Pers.: Fr), Alternaria blight [*Alternaria alternate* (Fr.) Kessel] Powdery mildew [*Oidiopsis Taurica* (Lev.) Salmon] and dry root rot [*Rhizoctonia bataticola* (Taub.) Butler] are important.

Among them, Fusarium wilt in chickpea caused by *Fusarium oxysporum* (*Schlecht and Emnd Synd*) f. sp. *ciceris* (Padwick) Synd. & Hans, has assumed serious proportions in the

recent years (Nene *et al*., 1984a) [11]. It is one of the most important limiting factors in successful cultivation of chickpea and is responsible for a significant reduction in yield. In India, yield losses are estimated to be of the range from 10 – 90 percent every year in different regions and in different cultivars (Singh and Dahiya, 1973; Jalali and Chand**,** 1992a) [14, 6]. In Rajasthan, losses due to this disease have been reported to a range from 10-100percent depending on the prevailing agroclimatic conditions (Grewal and Pal, 1970) [5].

Since, *F. oxysporum* f. sp. *ciceris* is a noxious soil and seed borne pathogen that can survive through chlamydospores and mycelia for several years in soil or on plant debris. However, due to its soil borne nature and long survival of pathogen, it is difficult to manage through conventional method. The application of fungicides though effective, but is un-economical. They not only affect associated beneficial microbiota in soil but also are main source contributing towards environmental pollution. As such, use of alternative methods like eco-friendly neem cake and bio-agents integration with fungicides seems to be more appropriate to manage such soil borne diseases.

Materials and Methods

Isolation, purification, identification and pathogenicity of pathogen

Fresh chickpea wilted plants root of showing characteristics wilt symptoms were collected and use for isolation the pathogen. The diseased portion of the plant was cut into small bits in such a way that each bit consists of infected as well as vigorous tissues. The bits were superficial sterilized with 0.1 percent $HgCl_2$ (mercuric chloride) solution for 30 to 45 seconds followed by three washing with sterilized distilled water and then transported aseptically under laminar air flow cabinet on sterilized Petri plates comprising 20 ml PDA (potato dextrose agar) medium and incubated at (27±2 ºC). When the fungal progression growths from the diseased tissues, it was sub-cultured aseptically on potato dextrose slants. The pure culture from altered places sections thus gained was microscopically studied for identification and made confirmation based on the morphological charms *viz.*, mycelia growth and macro & micro-conidia establishment, its size and shape were studied under low (10X) and higher (40X) power amplification from 10 days old culture of *Fusarium* spp. and were acknowledged by using the key of (Burnett and Hunter, 2003; Leslie and Summerell, 2006) [3, 9]. It was promoted refined by using single spore isolation technique. Pathogenicity of the isolated cultures of *Fusarium* spp. remained tested by growing chickpea plants in pots encompassing pathogen-infested soil were mixed in sterilized soil @ 10 g/kg. Then the inoculated soil was filled in sterilized pots. The pots filled with inoculated soil were kept in the cage house for 5 days and were irrigated with sterile water to allow the establishment of pathogen. The seeds of susceptible cultivar of chickpea 'GNG 1958' were sown in the inoculated pots @ 10 seeds/pot. Three replications of each isolate were maintained along with un-inoculated pots as control. The pots were irrigated on alternate days to provide good moisture. The initial symptoms of wilt started as mortality (Damping off) of seedling on 15th day and fully wilting symptoms were manifested within 35 days. The affected plants produced symptoms yellowing and drying of leaves from base to upward, drooping of petioles and rachis, roots showed browning at the soil surface. Eventually the diseased plants wilt and die prematurely. Re-isolation of the pathogen was attempted to prove the Koch's postulates. While in control healthy plants continued to grow without any wilting symptoms.

Evaluation fungicides *in-vitro* against *Fusarium oxysporum* f. sp. *ciceris* (KHR Foc-09)

Efficacy of different fungicides was tested in the laboratory using poisoned food technique (Nene and Thapliyal, 2018) against the most virulent/aggressive isolate of *F. oxysporum* f. sp. *ciceris* (KHR Foc-09) with six fungicides *viz*., Carbendazim 12% WP + Mancozeb 63% WP (Saaf), Copper oxychloride 50 WP (Maincop), Tebuconazole 25 EC (Folicur), Azoxistrobin 23% WP (Amistar), Carbendazim 50 WP (Bavistin), and Hexaconazole 5 SC (Mainex) were tested at three concentrations *i.e.* 100, 500 and 1000 ppm.

The required quantity of each chemical at different concentration was measured and then incorporated aseptically in 100 ml PDA in 250 ml flasks at the time of pouring the Petri plates. Before pouring the medium in plates was shaken well to give uniform distribution of the chemical. After that 20 ml of medium was poured in each Petri plates aseptically and allowed to solidify. The Petri plates were inoculated with 5 mm diameter mycelial disc, cut from the periphery of 10 days old fungus cultures. The mycelial disc was placed in the centre of the plates in an inverted portion to make a direct contact with the poisoned medium and incubated at 27±2 °C for 7-8 days. In each treatment five replications were maintained in Complete Randomize Design (CRD). At the same time a suitable control was also maintained by growing the fungus on chemical free PDA. Observations on linear growth were recorded when full growth of fungus observed in control Petri plate.

The percent inhibition of growth of the fungus in each treatment was calculated by using the following formula given by Vincent (1947) [17].

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

Where,

I = Percent inhibition

C = Growth of test fungus in control (mm)

T = Growth of test fungus in respective treatment (mm)

In vitro evaluation of bio-control agents

The dual culture test was carried out to determine the antagonistic action of known four species of fungal *viz*., *Trichoderma viride*-5(IIHR-Tv-5) procured from (Bio pesticide laboratory, RCA, MPUAT, Udaipur), *T. harzianum* (J H. h 89-2), *T. aureoviride* (DG. a 91-5), and *T. viride* (PCI- Bangalore) against wilt pathogen *Fusarium oxysporum* f. sp. *ciceris* in the laboratory.

Twenty (20) ml PDA media was poured aseptically in each petri plates and allowed to solidify. Mycelial disc of five mm diameter of all species *i.e.* each antagonist and test fungus were placed on solid media in the same petri plates

approximately 4 cm away from each other. The mycelia disc at four sides. Simultaneously a suitable control was also maintained by growing the fungus on antagonist free PDA. In each treatment five replications were maintained. All the inoculated and uninoculated plates were incubated at 27±2 °C and observed after 7 days for the growth of antagonist and test pathogen. Antagonistic activity of bio-control agent was calculated by measuring the growth of test pathogen in dual culture and in control plates-2.

Percent growth inhibition zone of pathogen and index of antagonism were determined in each treatment by following standard formula (Vincent, 1947) [17].

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

Where,

I = Percent growth inhibition zone of pathogen

C = Growth of test fungus in control (mm)

T = Growth of test fungus in respective treatment (mm)

Results and Discussion

Evaluation of fungicides against *Fusarium oxysporum* f. sp. *ciceris* (KHR Foc-09) in *In-vitro*. Six fungicides Copper oxychloride (Coc), Carbendazim, Saaf (Carbendazim 12% + (Mancozeb 63% WP), Hexaconazole, Azoxystrobin, and Tebuconazole were evaluated at three concentrations *viz.*, 100, 500 and 1000 ppm by poisoned food technique for their effectiveness against *Fusarium oxysporum* f. sp. *ciceris*. The data depicted in table 7 revealed that all the tested fungicides significantly (*P=0.05*) inhibited the mycelial growth of *F. oxysporum* f. sp. *ciceris* at all concentration.

Carbendazim was found the most effective that completely (94.44 percent) inhibited the mycelial growth of *F. oxysporum* f. sp. *ciceris* at all concentrations (100, 500 and 1000 ppm) followed by Saaf (Carbendazim 12% + (Mancozeb 63% WP) was found effective with inhibition of 70.00, 78.67 and 84.89 percent mycelial growth of *F. oxysporum* f. sp. *ciceris* at 100, 500 and 1000 ppm respectively. It was observed that Saaf (Carbendazim 12% + (Mancozeb 63% WP) found statistically at par with Hexaconazole at 500 ppm. Third most effective fungicide was Tebuconazole which showed 61.78, 72.22 and 77.33 percent inhibition at 100, 500 and 1000 ppm concentration respectively. It was followed by Hexaconazole and Azoxystrobin showed 54.67, 64.00, 72.00 and 46.67, 54.89, 63.56 percent inhibition at 100, 500 and 1000 ppm, respectively. It was found that Coc was more effective at 500 ppm as compare to Azoxystrobin. It also observed that Saaf at 500 ppm found at par with Tebuconazole at 1000 ppm, both had 77.33 & 78.67 percent mycelial growth inhibition. Copper oxychloride was found least effective at all concentrations against *F. oxysporum* f. sp. *ciceris* with with 26.40, 39.11 and 55.33 percent mycelial growth inhibition of *F. oxysporum* f. sp. *ciceris* at 100, 500 and 1000 ppm concentration respectively. (Table-1, Fig.- 1 and Plate-1). Similar results have been reported by several workers. Patra and Biswas (2017) [12] conducted an experiment for selection of superior fungicides for the management of chickpea wilt disease under the red and lateritic zone of West Bengal. Ten fungicides were evaluated against the wilt pathogen *F. oxysporum* f. sp. *ciceris* in *In-vitro* at 500, 1000 and 1500 ppm. Out of them, Carbendazim exhibited 100% fungal growth inhibition at 1000 and 1500 ppm while Copper-oxychloride exhibited least effectiveness. In field condition Carbendazim was found best with the minimum 9.66% wilt incidence of chickpea followed by combination of fungicides (Tebuconazole + Trifloxystrobin) with 10.12% disease incidence. Gadhave *et al.* (2020) [4] conducted *in-vitro* study and concluded that Carbendazim 50% WP, Copper oxychloride 50% WP and Carbendazim 25% + Mancozeb 50% WS were found most effective with maximum growth inhibition (100%), (65.22%) and (100%) respectively.

Evaluation of biocontrol agents against *Fusarium oxysporum* f. sp. *ciceris* isolate (KHR Foc-09) *in In-vitro*. Efficacy of four bio control agents *viz*; *Trichoderma viride-5*, *Trichoderma harzianum*, *Trichoderma aureoviride* and *Trichoderma viride* were studied *in-vitro* against *Fusarium oxysporum* f. sp. *ciceris* isolate (KHR Foc-09). All the four bio agents were significantly (*P=0.05*) inhibited the mycelial growth of *F. oxysporum* f. sp. *ciceris* isolate (KHR Foc-09). Results indicated that all the bio control agents were had antagonistic activity against the growth of *F. oxysporum* f. sp. *ciceris in-vitro*. Severe antagonism and significant high percent inhibition of *F. oxysporum* f. sp. *ciceris* growth (73.56 percent) was recorded by *T. viride-5* in dual culture method which was followed by *T. harzianum* and *T. viride* showed moderate antagonism with 62.22 and 58.22 percent growth inhibition respectively. The lowest mycelial growth inhibition and weak antagonism was showed by *T. aureoviride* which was 41.11 percent (Table-2, Fig.-2 and Plate-2). Similar results have been observed by several workers. Rehman *et al.* (2013) [13] evaluated the bioagents *Trichoderma harzianum* and *Trichoderma viride* against the chickpea wilt caused by *F. oxysporum* f. sp. *ciceris* by dual culture method and showed that *T. harzianum* and *T. viride* inhibited the growth upto 81% and 83.33%, respectively. Jambhulkar *et al.* (2011) [7] studied efficacy of bio-control agents against chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* and concluded that *Trichoderma viride* reduced disease incidence between 56 to 61 percent. Keote *et al.* (2019) conducted an experiment by using bio agents against chick pea wilt under *in-vitro* conditions. Under Dual culture, bio agents like *Trichoderma viride*, *Bacillus subtilis* and *Pseudomonas fluorescens* showed significant results.

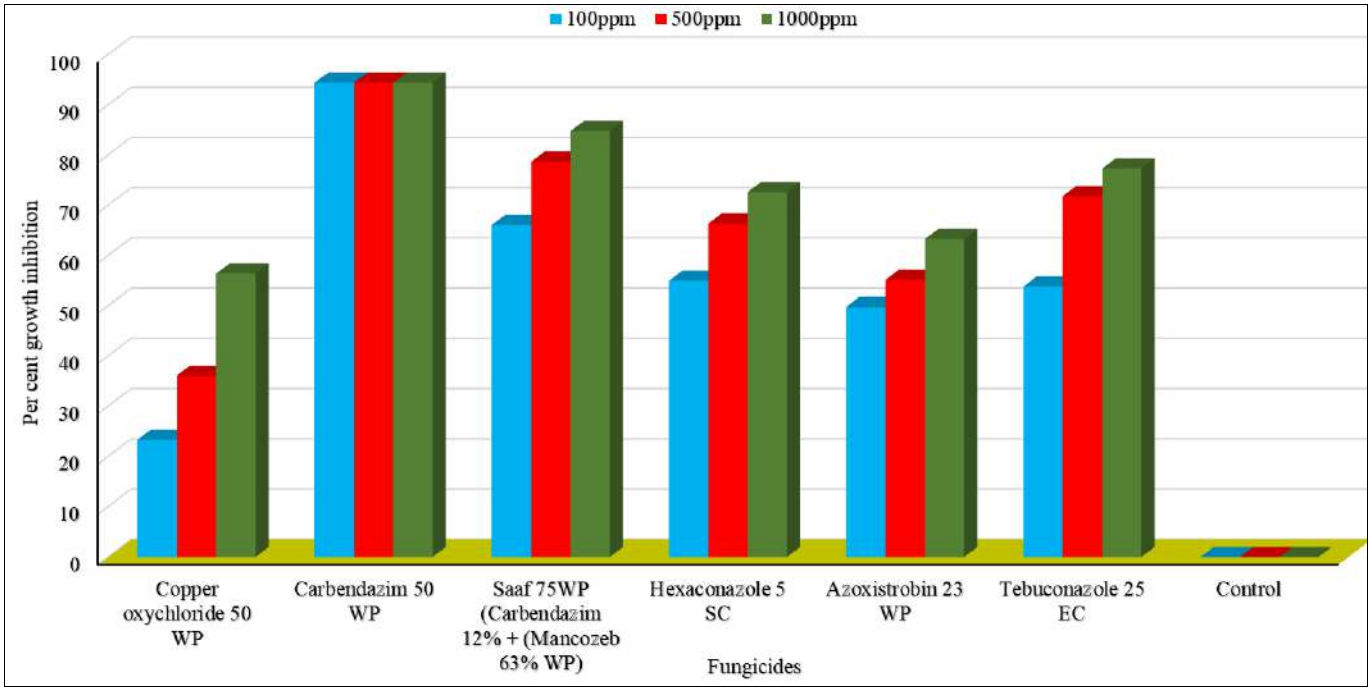

Fig 1: Comparative efficacy of different fungicides on the growth of *Fusarium oxysporum* f. sp. *Ciceris* isolate (KHR Foc-09) *in-vitro*

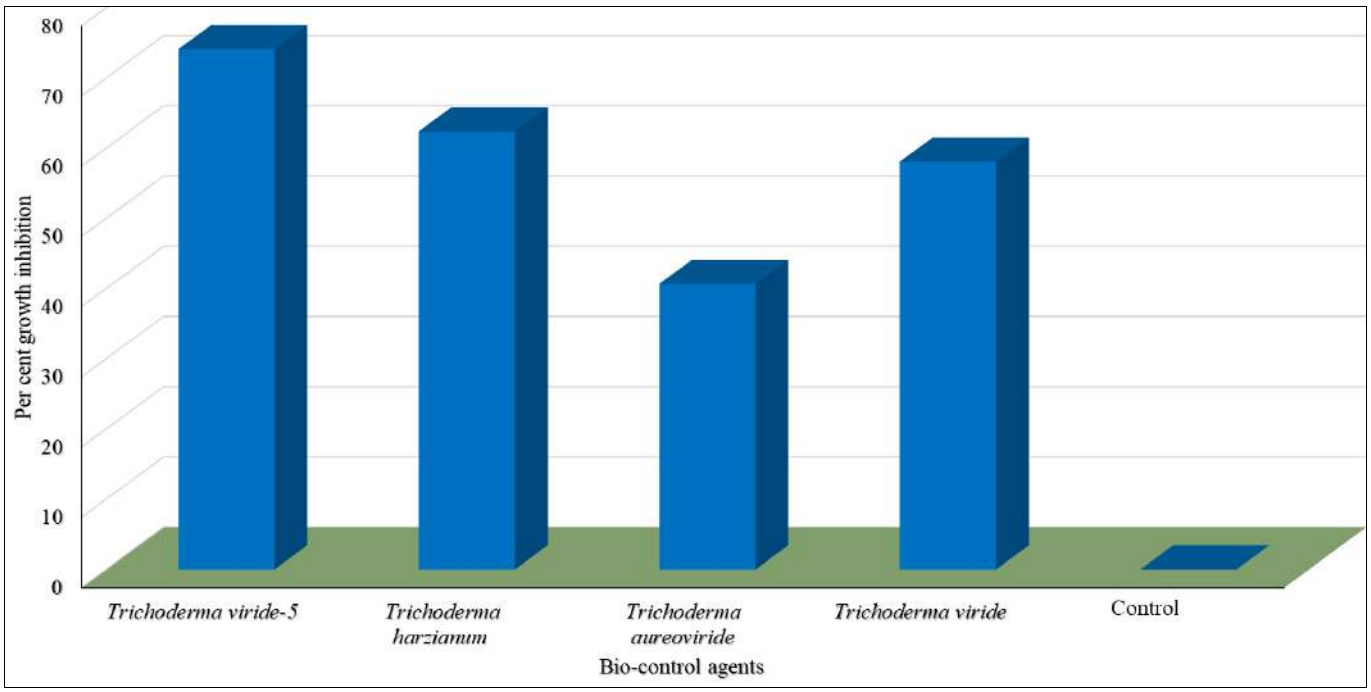

Fig 2: Percent growth inhibition of *Fusarium oxysporum* f. sp. *ciceris* (KHR Foc-09) isolate by different species of *Trichoderma in-vitro*

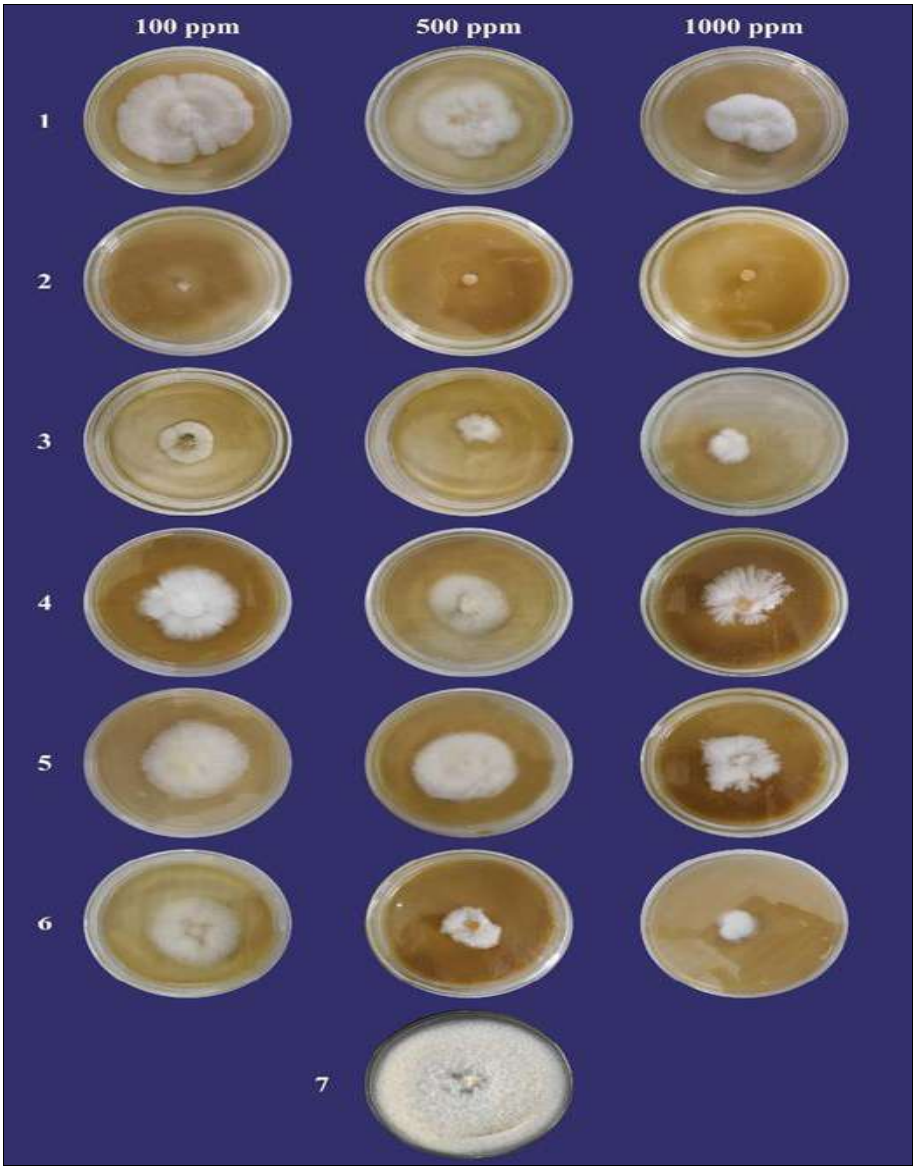

Plate 1: (Fig 1-7) Inhibition of mycelia growth of *Fusarium oxysporum* f. sp. Ciceris (KHR foc-09) at different concentration of various fungicides *in vitro*

1. Copper oxychloride	2. Carbendazim 50 WP
3. Saaf 75 WP	4. Hexaconazole 25 EC
5. Azoxistrobin 23 WP	6. Tebuconazole 25 EC
7. Control	

Fig. 1: *Trichoderma viride*-5

Fig. 2: *Trichoderma harzianum*

Fig. 3: *Trichoderma aureoviride*

Fig. 4: *Trichoderma viride*

Fig. 5: Control

Plat 2: (Fig 1-5) Efficacy of different bio-agents against *Fusarium oxysporum* f. sp. ciceris (KHR foc-09) isolate *in vitro*

Table 1: Comparative efficacy of different fungicides on the growth of *Fusarium oxysporum* f. sp. *ciceris by* isolate (KHR Foc-09) *in-vitro*

Treatments	Name of the Fungicide	Different concentrations(ppm)					
		100		500		1000	
		Colony Diameter (mm)*	Percent Growth inhibition*	Colony Diameter (mm)*	Percent Growth inhibition*	Colony Diameter (mm)*	Percent Growth inhibition*
T_1	Copper oxychloride 50 WP	66.20 (54.44)	26.44 (30.90)	54.80 (47.74)	39.11 (38.69)	40.20 (39.33)	55.33 (48.04)
T_2	Carbendazim 50 WP	5.0 (12.92)	94.44 (76.33)	5.0 (12.92)	94.44 (76.33)	5.0 (12.92)	94.44 (76.33)
T_3	Saaf 75WP (Carbendazim 12% + (Mancozeb 63% WP)	27.00 (31.28)	70.00 (56.78)	19.20 (25.97)	78.67 (62.47)	13.60 (21.62)	84.89 (67.11)
T_4	Hexaconazole 5 SC	40.80 (39.68)	54.67 (47.66)	32.40 (34.67)	64.00 (53.12)	25.20 (30.11)	72.00 (58.04)
T_5	Azoxistrobin 23 WP	48.00 (43.83)	46.67 (43.07)	40.60 (39.56)	54.89 (47.79)	32.80 (34.91)	63.56 (52.86)
T_6	Tebuconazole 25 EC	34.40 (35.89)	61.78 (51.79)	25.00 (29.98)	72.22 (58.18)	20.40 (26.83)	77.33 (61.55)
T_7	Control	90.00 (71.54)	0.00	90.00 (71.54)	0.00	90.00 (71.54)	0.00
SEm±		0.452	0.509	0.423	0.463	0.445	0.480
CD (P=0.05)		1.317	1.484	1.232	1.349	1.294	1.399

*Mean of five replications; Figures in parentheses are arcsine √ percent angular transformed values.

Table 2: Percent growth inhibition of *Fusarium oxysporum* f. sp. *ciceris* (KHR Foc-09) isolate by different species of *Trichoderma in vitro*

Treatments	Name of the Bio-control agent and procured places	Mycelial growth (mm) *	Percent growth Inhibition *	Antagonism Index**
T_1	*Trichoderma viride*-5 (IIHR-TV-5)	23.8 (29.14)	73.56 (59.07)	+ + + +
T_2	*Trichoderma harzianum* (Jh.h.89-2)	34.0 (35.65)	62.22 (52.05)	+ + +
T_3	*Trichoderma aureoviride* (DG. a 91-5)	53.0 (46.70)	41.11 (39.86)	+ +
T_4	*Trichoderma viride* (PCI- Bangalore)	37.6 (37.80)	58.22 (49.71)	+ + +
T_5	Control	90.0 (71.5)	0.00	--
SEm±		0.561	0.607	
CD (p=0.05)		1.667	1.803	

*Average of five replications. Values in the parentheses are arcsine √ percent angular transformed values.
** Antagonism index (Bell *et al.*, 1982) [18]
+ + + + = Severe antagonism
+++ = Moderate antagonism
++ = Weak antagonism
-- = No antagonism

Summary and Conclusion

In view of rising wilt incidence in chickpea growing areas, efforts were made to evaluate six (systemic and non-systemic) fungicides *viz.*, Copper oxychloride (Coc), Carbendazim, Saaf, Hexaconazole, Azoxystrobin and Tebuconazole at 100, 500 and 1000 ppm concentrations against *F. oxysporum* f. sp. *ciceris* under *in-vitro*. Current experiment was conducted to unveil the efficacy of fungicides and bio control agents (fungal) against growth inhibition of pathogen. The findings and conclusions resulted from the study are here as follows. Efficiency of Carbendazim, Saaf and Tebuconazole was found more pronounced against *F. oxysporum* f. sp. *ciceris*, whereas Carbendazim was highly effective in controlling the mycelial growth of *F. oxysporum* f. sp. *ciceris* at all the concentrations tested.

In-vitro studies on comparative efficacy of four bio control agents (fungal) were tested against *F. oxysporum* f. sp. *ciceris.* The antagonism activity of *Trichoderma viride* found to be highly effective against *F. oxysporum* f. sp. *ciceris* under *in-vitro*. However, *T. aureoviride* was found to be least effective with weak antagonism in nature comparing to other bio-agents.

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