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Efficacy of fungicides and *Trichoderma viride* mutants against *Alternaria helianthi* causing leaf blight of sunflower

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Abstract
In vitro efficacy of fungicides *viz*., carbendazim 75 WP, propiconazole 25 EC, metalaxyl 35 SD and azoxystrobin 23 SC and bio-agents *viz*., *Trichoderma viride* and its mutants (*TV*M1 and *TV*M2) and *Pseudomonas fluorescens* were tested against *Alternaria helianthi* causing leaf blight of sunflower. Among the fungicides propiconazole @ 0.1% was found most effective followed by carbendazim @ 0.2% and azoxystrobin (0.05%). Among the bio-agents *Trichoderma viride* Mutant 1 (*TV*M 1) was proved as best bio-agent against test pathogen followed by *Trichoderma viride* mutant 2 *(TV*M 2) and mother culture of *Trichoderma viride*.

Keywords: Sunflower, *Alternaria helianthi*, symptoms, pathogenicity, bio-agent

Introduction
Sunflower (*Helianthus annus* L.) is major edible oil seed crop next to soyabean and groundnut at the global level (Shilpa *et al*, 2015) [14]. India is the largest grower of sunflower with an area of 0.90 million hectares, production of 0.62 million tones and the average productivity of 696 kg/ha. Sunflower suffers from many diseases caused by fungi, bacteria, and viruses. Sunflower is the known host of more than 30 pathogens mostly fungi which under certain climatic condition may impair the normal physiology of the plant, so that yield and oil quality are reduced significantly (Gulya *et al*., 1994) [9]. Among them Alternaria leaf blight caused by *Alternaria helianthi* (Hansf.) Tubaki and Nishahara has been considered as a potential and destructive disease in India and other countries like Yugoslovia, Australia, Tanzania, Uganda and North Africa (Balasubrahmanyam and Kolte, 1980; Zimmer and Hose, 1978) [3, 18] and is devastating under humid tropical conditions (Hiremath *et al*., 1990) [10]. Alternaria leaf blights are considered as a major disease and can cause yield losses from 15 to 90% (Berglund, 2007) [4]. The disease can cause severe leaf spots, stem spots and blight resulting in premature defoliation and stem breakage. It can also infect other parts such as capitulum, disc and ray florets. It is more serious in India and yield losses go up to 80% (Agrawal *et al.*, 1979) [1]. Considering the importance of sunflower in current oil seed crops scenario of our country and potential losses caused by Alternaria leaf blight to sunflower in all crop growing areas including vidarbha so the present investigation was carried out under *in-vitro* condition.

Material and Methods
The experiment was carried out in department of Plant Pathology Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola to evaluate different fungicides and bio-agents against *Alternaria helianthi* causing leaf blight of sunflower. The mother culture of *Trichoderma viride* and pure culture of *Pseudomonas fluorescens* were procured from department of plant pathology Dr. P.D.K.V., Akola. The mutants of *Trichoderma viride* (*Tv* M1 and *Tv* M2) were obtained through mutation induced by Gamma Radiation**.**
Induction of mutation by gamma radiation was carried according to the procedure of Gadgil *et al*. (1995), Migheli *et al*. (1998) and Rey *et al*. (2000) at Bhabha Atomic Research Centre, Mumbai. The 10 days Sporulated culture of *Trichoderma viride* was irradiated with cobalt – 60 gamma radiation @41.6 gray/min. The applied doses level were 25 k-rad, 50 k-rad and 75 k-rad with time interval of 15,30,45 min. After irradiated culture were transfers on fresh PDA medium and grown up to six generations to check the stability of *Trichoderma* mutants.

Isolation
The fresh naturally infected diseased leaves were collected from field of oilseeds Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

The isolation of *Alternaria helianthi* was done by standard tissue isolation method on SLEM (sunflower leaf extract medium). The infected leaves were washed with water then cut into small bits and surface sterilized with 1% sodium hypochlorite solution for two minute, subsequently washed with three changes of sterilized distilled water. These small bits of infected sample were dried on sterilized blotter paper and aseptically transferred to solidified sterilized media in Petri plates and were incubated at 28±2 °C in BOD incubator for 7 days. After seven days, brown scanty colonies were observed with a brown diffusible pigmentation into the medium. Colonies on SLEM were light brown to olive brown, velvety, slowly growing and sporulation was abundant. Microscopic examination of the culture revealed branched, septate, brown coloured mycelium. Conidia were cylindrical, long ellipsoid, straight or lightly curved, light yellowish brown with round ends. They were without beaks, mostly transverse septate (2-7) or rarely having longitudinal septa (0-2). The growth was examined and identified on the basis of morphological character. After identification under stereoscopic binocular morphologically unique colonies of *Alternaria* spp. (Ellis, 1971) [7] were transferred to potato dextrose agar medium (PDA) slants and after 5 days all slants were preserved at 4 °C for further use.

Pathogenicity test

Fifteen days old culture of the organism was used for proving the pathogencity by applying Koch's postulates. For this purpose, seeds of morden variety which is susceptible to Alternaria blight (*Alternaria helianthi* (Hans f.) were surface sterilized with 0.1% sodium hypochlorite solution and sown in the earthen pots filled with steam sterilized potting mixture of soil: sand: FYM (2:1:1). Healthy growing sunflower seedlings were maintained, watered regularly and kept in the shade net green house for further development. Three weeks old healthy seedlings were selected for inoculation. The spore suspension was prepared and filtered through two layers of sterile muslin cloth to remove residual mycelia. Filtrate obtained was suitably diluted with sterile distilled water to get inoculum concentration of 1 x 10^6 spores per ml. The seedlings were inoculated with 15 days old test fungus. Uninoculated seedlings of the same age sprayed with sterilized water served as control. After inoculation, the seedlings pots (both inoculated and uninoculated) were incubated in the shade net green house, where relative humidity is > 80-90% and optimum temperature (27±1 °C) were maintained for further development of Alternaria blight symptoms. Re-isolation was made from inoculated leaves by the isolated fungus which resembled in all respect with the culture used for inoculation.

Several workers found that foliar spray of spore suspension of the pathogen, *Alternaria helianthi* would be the best method for conducting the pathogenicity test. The above results were in agreement with Prathibha (2005) [13] proved the pathogenicity of *A. helianthi* on sunflower by inoculating spore suspension (10^6spores/ml) grown on PDA. The symptoms appeared 8-9 days after inoculation.

***In vitro* evaluation of fungicides**

Efficacy of four fungicides *viz*., Carbendazim 75 WP, Propiconazole 25 EC, Metalaxyl 35 SD and Azoxystrobin 23 SC were evaluated *in vitro* against *Alternaria helianthi* by applying poison food technique (Nene and Thapliyal, 1993) [12]. Prepared potato dextrose agar medium was added @ 100 ml per 250 ml conical flask and autoclaved at 15 psi for 15 min. the chemical fungicides from stock solution were added so as to get desired concentration. About 20 ml melted poisoned potato dextrose agar medium was poured in each sterilized Petri plates were inoculated by 15 days old test fungus culture, cut with sterilized 5 mm diameter cork borer and placed aseptically with the help of forceps in the centre of petriplates containing poisoned medium. The surface of inoculums disc was kept in inverted position on medium in petriplates. Five plates were inoculated by fungal disc for each kind of chemical fungicides. Control plates were run parallel and the plates were incubated at room temperature 28±2° C.

The observations of colony diameter were recorded at the three days interval after inoculation. Maximum mycelial diameter of 7 days growth was recorded concerning to each treatment. Mycelial diameter of each treatment was compared with control plate and data was analyzed statistically and per cent inhibition of growth of the test fungus was calculated by applying following formula (Vincent, 1927) [17].

$$\text{Per cent Growth inhibition} = \frac{C - T}{C} \times 100$$

Where,

C = Growth of fungus in control.

T = Growth of fungus in treatment.

***In vitro* efficacy of bio-agents**

Three fungal antagonist *viz.*, *Trichoderma viride*, *Trichoderma viride* mutant 1 and *Trichoderma viride* mutant 2 and one bacterial antagonist *viz.*, *Pseudomonas fluorescens* were evaluated *in vitro* against *Alternaria helianthi* by applying by dual culture technique (Dennis and Webster 1971) [6]. In dual culture technique, 20 ml of sterilized and cooled potato dextrose agar was poured into sterilized petri plates. Fungal antagonists were evaluated by inoculating the pathogen at one side of the petri plate and the antagonist was inoculated exactly on opposite side of the same plate by leaving 3-4 cm gap. For this, actively growing cultures were used. In case of bacterial antagonist mycelial disc (5 mm) of test pathogen were placed at the centre of petriplate containing solidified PDA medium and a loopful 24 hour old culture of bacterial antagonist were streaked at both side by leaving 2 cm gap. Control plate containing only pathogen was maintained and all the treatments were replicated five time. The radial mycelial growth was measured in three directions and average was recorded, per cent inhibition of growth of the test fungus was calculated (Dennis and Webster, 1971) [6].

$$\text{Per cent inhibition} = \frac{C - T}{C} \times 100$$

Where,

C = Mycelial growth in control (mm).

T = Mycelial growth in treatment (mm).

Results and Discussion

***In vitro* evaluation of fungicides**

Four fungicides were evaluated for their efficacy against *Alternaria helianthi* by poisoned food technique as described in “Material and Methods”. All the treatments were replicated five times and a suitable untreated control (without fungicide) was also maintained. The results (table 1) revealed that the fungicides tested significantly inhibited growth of the test fungus. At seventh day the maximum inhibition of mycelial

growth over control was recorded in Propiconazole 25EC @ 0.1% (91.66%) which was significantly superior over other treatments. Carbendazim 75WP @ 0.2% (88.26%) was second best followed by Azoxystrobin 25SC (70.77%). Least inhibition was observed in Metalaxyl 35SD @ 0.6% (65.18%).

Previously Thaware *et al*. (2010) [10] has reported the *in vitro* effectiveness of propiconazole 0.05% in controlling *Alternaria alternata* causing leaf blight of cowpea which is completely inhibit the mycelial growth. And also correlate with Thejakumar *et al*. (2016) [16] evaluated ten fungicides *in vitro* against *Alternaria alternata* causing leaf spot of chilli in different concentration among them propiconazole at all concentration *viz*. 500, 1000, and 2000 ppm was showed complete inhibition of mycelial growths followed by mancozeb.

Table 1: *In vitro* evaluation of different fungicides against *Alternaria helianthin*

Tr. No.	Treatments	Conc. (%)	Radial mycelial growth (mm)	Per cent growth inhibition
T_1	Carbendazim	0.2	10.56	88.26 (69.96)*
T_2	Propiconazole	0.1	7.50	91.66 (73.21)
T_3	Metalaxyl	0.6	31.33	65.18 (53.84)
T_4	Azoxystrobin	0.05	26.30	70.77 (57.27)
T_5	Control		90.00	00.00
	'F' test		Sig.	
	S.E.(m) ±		0.12	
	CD at (p =0.01)		0.55	

***** Figure in the parentheses are arc sin transformed values

In vitro efficacy of bio-agents

Three fungal antagonist i.e. *Trichoderma viride* and its mutants *T. viride* M_1, *T. viride* M_2 and one bacterial bio-agent i.e. *Pseudomonas fluorescens* were evaluated *in vitro* against *A. helianthi* by using dual culture technique (Dennis and Webster, 1971) [6]. The result (Table 2) revealed that all the bioagents were significantly inhibited the mycelial growth of *A. helianthi* over control. Of the four bio-agents tested *T. viride* M 1 was found most effective which recorded least mycelial growth (16.52 mm) and corresponding highest mycelial growth inhibition (81.64%) of the test pathogen over untreated control (90.00 mm and 00.00%, respectively), followed by *T. viride* M 2 (growth: 21.43mm and inhibition: 76.18%) and *T. viride* (growth: 29.52mm and inhibition: 67.2%). Bacterial antagonist *P. fluorescens* were found significantly least effective with 36.50mm linear mycelial growth and 59.44% inhibition of the test pathogen.

Table 2: *In vitro* evaluation of different bio-agents against *Alternaria helianthin*

Tr. No.	Treatments	Radial mycelial growth (mm)	Per cent growth inhibition
T_1	*T. viride*	29.52	67.2 (55.05)*
T_2	*T. viride* M_1	16.52	81.64 (64.63)
T_3	*T. viride* M_2	21.43	76.18 (60.78)
T_4	*Pseudomonas fluorescens*	36.50	59.44 (50.44)
T_5	Control	90.00	00.00
	'F' test	Sig.	
	S.E.(m) ±	0.19	
	CD at (p =0.01)	0.87	

***** Figure in the parentheses is arc sin transformed values

Thus the fungal bio-agents was found effective than bacterial bio-agent for inhibition of test pathogen are in conformity to those reported earlier by several workers Deokar *et al*. (2010) [5] tested three botanicals and two fungal bio-agents against *A. helianthi* among them *T. viride* (29.26 mm) was found most effective and recorded least mean colony diameter and also Shilpa *et al*. (2015) [14] evaluated four bio-agents against *Alternaria helianthi*, maximum inhibition of mycelial growth was noticed in *T. viride* (83.30%) followed by *Streptomyces griseus* and *P. fluorescens*.

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