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Evaluation of fungicides, botanicals and bio-agents against *Alternaria alternata* incitant of leaf spot of soybean

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Abstract
Alternaria leaf spot of soybean is an important disease of soybean inflicting heavy losses in now days. The present investigation was carried out to test the efficacy of fungicides, botanicals and bio-agents *in vitro.* Among fungicides tested, Hexaconazole, Propiconazole, Propineb and Cymoxonil + Mancozeb showed 100 percent mycelial inhibition of *Alternaria alternata.* Among the botanicals evaluated against *Alternaria alternata in vitro*, Garlic clove extract@ 10% was found most effective giving (87.50%) inhibition against *Alternaria alternata*. Bio-efficacy of bioagent also tested by dual culture technique and results revealed that *Trichoderma harzianum* and *Trichoderma asperellum* gave the best effect against *Alternaria alternata* forming maximum percent mycelial inhibition i.e. 79.65% and 76.55%.

Keywords: Soybean, *Alternaria alternata*, fungicides, botanicals, bio-agent

Introduction
Soybean (*Glycine max* (L.) is one of the most important an Asiatic oil seed crop. It has a prominent place among modern agricultural commodities, as the world's most important seed legume, which contributes 25% to the global edible oil, about two thirds of the world's protein concentrate for livestock feeding and is valuable ingredient in formulated feeds for poultry and fish. Hence, it is spread throughout Asia as well as whole world. The estimates of world soybean area, production and productivity for 2017-18 are 126.64 million ha, 346.31 million tons and 2735 kg/ha and in India soybean grown under area of 10.60 million hectares, production of 8.50 metric tons and average productivity of 802 kg/ha during 2017-18. In Maharashtra total area under soybean is about 34.5 lakh hectares, production of 2.90 million tons and the average productivity of 841 kg/ha during 2017-18 whereas in Vidarbha soybean crop covered area in 2017 was around 16.63 lakh ha and production was 15.80 lakh metric tons with productivity of 950 kg/ha in 2017 (Anonymous, 2017) [2]. Among the causes attributing to low yield, diseases occupy the prime position. Some of the important fungal foliar diseases of soybean were Rust (*Phakopsora pachyrizi*), Cercospora blight (*Cercospora kikuchii*), Alternaria leaf spot (*Alternaria spp*.), Myrothecium leaf spot (*Myrothecium roridum*), Target leaf spot (*Corynospora cassicola*) and Colletotrichum blight (*Colletotrichum dematium*). Among these leaf spot incited by *Alternaria spp*. reported to be the most prevalent and widespread disease. Gupta and Chauhan, (2005) [7] reported 10 to 20% reduction in yield due to Alternaria leaf spot as resulted on account of premature defoliation, pod and seed decay. Conn and Tewari (1990) [6] reported that among the different foliar diseases, leaf blight caused by *Alternaria*, is one of the most dominant one that causes average yield loss in the range of 32-57%. Since last few years Alternaria leaf spot is considered as minor disease in Vidarbha but now days, leaf spot symptoms on soybean are being observed in severe proportion from various places of Vidarbha region on existing cultivated varieties of soybean that becoming potentially threat for soybean cultivation (Anonymous, 2014). Considering the importance of soybean in current legume crops scenario of our country and potential losses caused by Alternaria leaf spot to soybean in all crop growing areas including Vidarbha, the present investigation was carried out under *in-vitro* condition.

Material and Methods
Isolation
Infected soybean leaves used for isolation by tissue method. Infected lesions were selected and centre core of infected tissues separated in pieces of 5 mm, surfaces disinfected with 0.1% $HgCl_2$ for two minute. These pieces were then placed in the sterilized distilled water and bits after dried on sterilized filter paper and around flame of spirit lamp were placed on sterilized

solidified PDA medium in plate. Each plate contained five bits. The plates were incubated at room temperature (28±2 ^{0}C). All these operations were carried out aseptically. The plates were examined regularly. The fungus colonies growing around the each bit were examined and sub cultured. Based on colony character, morphological characters (types of conidia) and published literature the fungi were identified as *Alternaria alternata.* The pure culture was transferred on PDA slants and maintained for further studies.

***In vitro* evaluation of fungicides**

The eleven fungicides (Table 1) were evaluated by employing poison food technique (Nene and Thapliyal, 1993) [12]. Potato Dextrose Agar (PDA) medium was prepared, equally distributed measuring 100 ml in 250 ml conical flask and sterilized in autoclave. Requisite quantity of each of the fungicides (as per concentration) was added in sterilized melted (45 ^{0}C) PDA separately so as to obtain desired concentration. Flask containing poisoned medium was shaken well to have even and uniform distribution of fungicides. About 20 ml of melted poisoned PDA was poured in each sterilized Petri plate and allow solidifying. These Petri plates were inoculated by test fungus separately. Five mm disc of one week old fungus culture was cut with sterilized cork borer, lifted and transferred aseptically in the centre of Petri plate containing the medium poisoned with test fungicide. The control plates were kept the culture disc grown in same condition on PDA without fungicides. Treated plates were incubated at room temperature (25± 2 ^{0}C) for a period of seven days. Colony diameter was recorded in mm and percent mycelial growth inhibition was calculated as per Vincent's formula based on the average colony diameter. The data was subjected to statistical analysis wherever necessary.

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

Where,
PI = Percent Inhibition
C = Growth of fungi in control (mm)
T = Growth of fungi in treatment (mm)

***In-vitro* evaluation of botanicals**

The poisoned food technique (Nene and Thapliyal, 1993) [12] was employed to evaluate the efficacy of various botanicals against *Alternaria alternata*.

Preparation of aqueous leaf extract of Botanicals

The plant leaves extract were prepared by adopting aqueous extracting method. The standard aqueous leaf extract of the selected botanicals (Table 2) was obtained by grinding the washed plant leaves (100 g) in mortal and pestle in presence of equal amount of sterilized distilled water (100 ml). Prepared leaves extract were filtered through folds of muslin cloth, make up the volume and treated as 100% extract. Colony diameter was recorded in mm and percent of mycelial inhibition was calculated as per formula given below based on the average of colony diameter. The data of mycelial growth was also subjected to statistical analysis and conclusion was drawn.

***In-vitro* evaluation of bio-agents**

The lawn culture of test fungi and bio-agents i.e. *Trichoderma asperellum, Trichoderma atroviride, Trichoderma harzianum* were prepared. Autoclaved melted Potato Dextrose Agar was poured in Petri plates and allowed to solidify for obtaining leveled surface. The plates were inoculated with the culture of test fungi and bioagents after solidification of media and then plates were incubated at room temperature for seven days.

Bacterial bio-agents, *Bacillus subtilis* and *Psudomonas fluorescence* were prepared by inoculating a loopful culture in sterilized conical flask containing hundred ml of nutrient broth. Broth culture was incubated at room temperature for three days. Five mm disc of one week old test fungus and bio-agent lawn culture was cut with the help of cork borer lifted and transferred in Petri plates, containing autoclaved solidified PDA medium. In each Petri plates, four discs of bio-agents were inoculated at four peripheral points of the plates and the test fungi was placed in centre of Petri plates. In case of *Pseudomonas fluorescence* and *Bacillus subtilis*, a three days old culture was streaked around the disc of test fungus. The test fungi grown in same condition on Potato Dextrose Agar without bio-agents served as control. All these plates were incubated at room temperature for seven days. After an expiry of seven days incubation period the mycelial inhibition was calculated as per formula mentioned in the poisoned food method.

Results and Discussion

***In vitro* evaluation of fungicides**

Efficacy of 11 fungicides at respective concentration was tested *in-vitro* by following poison food technique for mycelial growth of *Alternaria alternata.*

Table 1: Effect of fungicides on radial mycelial growth of *Alternaria alternate*

S. N	Fungicides	Concentrations	Mean radial mycelial growth (mm) * 7 DAI	Mycelial inhibition (%)
1	Mancozeb 75% WP	0.2	27.67	62.97
2	Carbendazim 50%WP	0.1	62.50	16.36
3	Mancozeb 12%+ Carbendazim 63% WP	0.2	27.00	63.86
4	Hexaconazole 5% EC	0.1	0.00	100.0
5	Propiconazole 25% EC	0.1	0.00	100.0
6	Copper Oxychloride 50% WP	0.3	55.50	25.73
7	Propineb 70% WP	0.2	0.00	100.0
8	Thiophanate Methyl 70% WP	0.2	55.67	25.50
9	Tebuconazole 50% + Trifloxystrobin 2% WG	0.1	15.67	79.03
10	Chlorothalonil 75% WP	0.2	20.00	73.23
11	Cymoxanil 8%+Mancozeb 64% WP	0.1	0.00	100.0
12	Control	-	74.73	
	F ' test	-	Sig.	-
	SE(m)±	-	0.89	-
	CD(P=0.01)		2.59	

*Average of 3 replication. * DAI- Day after inoculation

Results presented in Table 1 showed that the effect of fungicides on radial mycelial growth of *Alternaria alternata.* After 7 days of inoculation hexaconazole, propiconazole, propineb and cymoxonil + mancozeb was found most effective in arresting growth of *Alternaria alternata* as complete (100%) inhibition obsereved in respective tested fungicides. Tebuconazole + Trifloxystrobin, Chlorothalonil and mancozeb next in order to restrict the growth by 79.03, 73.23 and 62.97% respectively. Carbendazim, Thiophanate Methyl and Copper-oxychloride were found least effective as recorded the 16.36, 25.50, and 25.73% inhibition. Maximum radial growth was recorded in control plate (74.73 mm).

The present findings are in agreement with Thaware *et al.* (2010) [17], Maheswari and Krishna (2013) [11] and Nidhika Rani (2018) [13] who reported that propiconazole and hexaconazole was most effective fungicides against *Alternaria alternata*. Singh and Majumdar (2002) [15] also reported that propiconazole was the most effective fungicide in controlling *A. alternata* by 100% in 8 day after inoculation. No related information could be traced during literature hunt in respect to propineb and cymoxonil + mancozeb efficacy against *A. alternata.* Thus, it provides additional information as effective fungicides for management of *A. alternata.*

Under laboratory studies, relatively higher 79.03, 73.23 and 62.97% percent inhibition recorded in Tebuconazole + Trifloxystrobin, Chlorothalonil and mancozeb respectively. This finding are almost similar to those of Hiremath and Sundaresh (1985) [8]; Amaresh and Nargumd (2002) [1] and Jakatimath *et al.* (2017) [9] who reported the judicious inhibition of *Alternaria alternata* by tebuconazole, chlorothalonil and mancozeb. In present investigation carbendazim, and copper-oxychloride were found least effective as recorded the minimum percent inhibition against *A. alternata*. These findings seem to be in contrast with those of Kantwa *et al.* (2014) [10] who recorded copper oxychloride and carbendazim exhibit maximum mycelial inhibition. Thiophanate Methyl also found less effective in *in vitro* studies in present investigation with 16.36% inhibition of *A. alternata.* Pareek *et al.* (2012) [14] also observed that thiophanate methyl was less effective against *A. alternata.*

Efficacy of botanicals

Eleven plant extract were evaluated against *Alternaria alternata* isolated from soybean leaves by poisoned food technique and data presented in Table 2.

Table 2: Efficacy of botanicals against *Alternaria alternata* by poisoned food technique

S. N	Botanical	Botanical name	Plant parts used	Conc. used	Mean radial mycelial growth (mm)	Percent mycelial inhibition
1	Neem	*Azadirachta indica*	Leaves	10%	44.53	47.34
2	Cogress grass	*Parthenium hysterophorus*	Leaves	10%	75.07	11.23
3	Ghaneri	*Lantana camara*	Leaves	10%	54.43	35.63
4	Mehandi	*Lawsonia innermis*	Leaves	10%	50.83	39.89
5	Datura	*Datura metal*	Leaves	10%	63.00	25.50
6	Garlic	*Allium* sativum	Clove	10%	10.57	87.50
7	Nilgiri	*Eucalyptus spp.*	Leaves	10%	64.67	23.53
8	Beshram	*Ipomea carnea*	Leaves	10%	68.20	19.35
9	Ginger	*Zingiber officinale*	Rhizome	10%	56.67	32.99
10	Turmeric	*Curcuma longa*	Rhizomes	10%	64.67	23.53
11	Onion	*Alium cepa*	Bulb	10%	47.80	43.47
12	Control	--	--	--	84.57	
	F' test				Sig.	
	SE(m)±				0.83	
	CD(P=0.01)				2.41	

*Average of four replication

The results presented in the Table 2 indicated that all the tested botanicals showed significantly differences compared with control. Among the plant extracts garlic clove extracts @ 10% recorded maximum inhibition (87.50%) of mycelial growth of test fungus and was significantly superior to rest of the treatments. This was followed by Neem leaf extract and Onion bulb extract recorded 47.34 and 43.47 percent mycelial inhibition of *A. alternata*. Rest of the plant extract *viz.* Parthenium Leaves extract, Ghaneri leaves extract, Mehandi leaves extract, Datura leaves extract, Nilgiri leaves extract, Beshram leaves extract, Ginger rhizome extract and Turmeric rhizome extract recorded percent inhibition in the range of 11.23 to 39.89% which least effective against test pathogen. These results are almost similar to those of Bhosale *et al.* (2014) [4] who observed the mycelial inhibition of *Alternaria alternata* due to Garlic clove extract by 80.30% in soybean. Singh *et al.* (2014) [16] also observed the effectiveness of garlic and neem extract against *Alternaria alternata*. Likewise Thaware (2010) [17] moreover reported that the garlic clove extract showed maximum mycelial inhibition (63%) followed by neem (33%), karanj (26.66%) and tulsi (27.77%) against *A. alternata.*

The findings of Nidhika Rani *et al*. (2018) [13] are on the similar line of the present results; while testing the different efficacy of botanicals they reported that garlic clove extract @ 10% concentrations exhibited the maximum (84.31%) inhibition against *Alternaria alternata* whereas 82.18% inhibition of mycelial growth was observed in case of *Alternaria tenuissima* followed by onion and neem. The fungi toxicity of plant extracts in the present study might be due to antifungal metabolites present in different plant species.

Efficacy of bio-agents

Antagonistic activity of bio-control agents namely *Trichoderma asperellum, Trichoderma atroviride, Trichoderma harzianum, Pseudomonas fluorescence* and *Bacillus subtilis* were investigated by using dual culture technique.

Table 3: Efficacy of bio-agents against *Alternaria alternata* by dual culture technique

S. N	Bioagents	Mean radial mycelial growth (mm)*	Percent mycelial inhibition (%)
1	*Trichoderma asperellum*	19.97	76.55
2	*Trichoderma atroviride*	30.10	64.65
3	*Trichoderma harzianum*	17.33	79.65
4	*Pseudomonas fluorescence*	26.67	68.68
5	*Bacillus subtilis*	44.03	48.30
6	Control	85.17	-
	F' test	Sig.	-
	SE(m)±	0.48	-
	CD (P=0.01)	2.06	-

*Average of four replication

All the bio-agents tested showed clear significant effect compared with were control (Table 3). Antagonist *Trichoderma harzianum* and *Trichoderma asperellum* gave the best effect against *Alternaria alternata* forming maximum percent mycelial inhibition i.e. 79.65% and 76.55% and decreased the mycelial growth from 85.17 to 17.33 and 19.97 mm respectively. *Pseudomonas fluorescence* was next best recorded (68.68%) inhibition while the least mycelial inhibition was observed in case of *Bacillus subtilis* (48.30%).

In current experiment fungal antagonist found effective than bacterial known antagonist. The present findings are conformity with the results of Jakatimath *et al.* (2017) [9] who reported that *Trichoderma spp*. was found most effective against *Alternaria alternata* as compared *Pseudomonas fluorescens* and *Bacillus subtilis*. These results are also in accords with Thaware *et al.* (2010) [17] who reported effectiveness of *Trichoderma harzianum* and *Trichoderma viride* against mycelial growth of *Alternaria alternata* by 85.88% and 81.88% respectively. The similar observation was also reported by Chaitali (2014) [5] who recorded that *Trichoderma harzianum* was most effective against *Alternaria* fungus with significant mycelial inhibition (83.3%) followed by *Trichoderma viride* (81.4%), *Pseudomonas fluorescence* (66%) and *Bacillus subtilis* (67.8%).

The inhibition of fungal growth due to *Trichoderma spp*. may have been due to secretion of extracellular cell degrading enzymes such as chitinase ɐ-1, 3-glucanase, cellulose and lectin, which may have helped myco-parasites in the colonization of their host. The inhibition of pathogen may also be attributed to the production of secondary metabolites by antagonists such as glioviridin, viridian and gliotoxin (Behairy *et al.*, 2014) [3].

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