

Efficacy of Carbendazim and other Fungicides on the Development of Resistance during Passage in *Alternaria Alternata* Causing Root Rot to Fenugreek

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Abstract: A Survey of Pathogens infecting Fenugreek (*Trigonella foenum graecum*. L), by *Alternaria alternata* (Fr.) Keissler, causing root rot disease, from 12 different localities of Maharashtra was carried out. Fenugreek infected by *Alternaria* was observed as severe, in all pathogens. The MIC of carbendazim among 12 isolates of *Alternaria alternata* on agar plates (*in vitro*) and root rot of fenugreek (*in vivo*) exhibit variation i.e. from 5000 to 2500 µg/ml while 500 to 1000 µg/ml respectively. *In vitro* and *in vivo* culturing of sensitive isolate of *Alternaria alternata* (Aa-2) on carbendazim, continuously for eight successive passages showed significantly increased resistance. Use of carbendazim alternately with Aliette, Roko, Curzate, Dithane M-45 and Ridomil for eight successive passages significantly decreased resistance at 3rd passage. Use of carbendazim in mixture with Aliette, Roko, Dithane M-45, Curzate and Ridomil reduced resistance on agar plates at 2nd passage only while Curzate in mixture with carbendazim, proved to be best fungicide candidate for management of *Alternaria alternata* causing root rot disease to fenugreek.

Keywords: Carbendazim resistance, *Alternaria alternata*, passage.

1. Introduction

Fenugreek (*Trigonella foenum graecum* L.), is native of India and southern Europe. In India it is consumed as vegetable, fodder and source of medicine. The plant is infected by *Alternaria alternata* (Fr.) Keissler and causes root rot disease to fenugreek (Dwivedi *et. al.*, 1982). The valuable biochemical loss was seen after infection, on fenugreek by pathogen like *Alternaria alternata* (Khandare, 2014). Survey of pathogens infecting Fenugreek from 12 different localities of Maharashtra was carried out followed by MIC (Minimum inhibitory concentration) using systemic fungicide carbendazim (Bavistin). *Alternaria alternata*, causing root rot disease to fenugreek is severe in all pathogens infecting Fenugreek. Carbendazim is recommended for the management of Fenugreek diseases hence this fungicide was undertaken for detailed study. Reports on fungicide resistance in plant pathogens of various crops plants are very few. Day by day there is increasing use of fungicides in management of diseases. The aim of the present investigation was therefore to examine the possibility of development of resistance during passage in *Alternaria alternata* against carbendazim.

2. Material and Methods

2.1. Material

Infected samples of fenugreek causing root rot disease to fenugreek by *Alternaria alternata*, fungicides like Bavistin DF (Carbendazim in the form of Benzimidazole), Curzate M8 (Cymoxanil 8%+ Mancozeb 64%), Aliette (Fosetil aluminio), Roko (Thiophanate methyl 70%W/W), Dithane M45 (mancozeb 75% WP), and Ridomil Gold - (R)-2-[(2,6-dimethylphenyl)-methoxyacetyl amino]-propionic acid methyl ester.

2.2. Methods

Infected samples of fenugreek were collected and brought to the laboratory in clean sterilized polythene bags from 12 different localities of Maharashtra state. The pathogen (*Alternaria alternata*) was isolated by direct plating analysis (Pitt and Hocking, 1997) and inoculated on Czapek Dox agar medium. The plates were incubated for 12 days and then screened. The isolated pathogens were identified (Subramanian, 1971; Barnett and Hunter, 1972). The pathogenicity test of which was confirmed by Koch's postulate (Koch 1893). During investigation it was found that *Alternaria alternata* (Fr.) Keissler was found severe in all pathogens infected fenugreek (Khandare and Kamble, 2013). Carbendazim was used to manage the disease. Highly resistant and sensitive isolates were identified and numbered Aa-1 to Aa-12 (Aa-2 - wild sensitive with 500 µg/ml and Aa-8 - highly resistant 5000 µg/ml) using MIC of carbendazim on agar plate (*in vitro*) and (*in vivo*), on fenugreek plant (Khandare and Kamble, 2013). After determining MIC (wild sensitive isolates - Aa-2), the effect was examined during continuous, alternate and mixed passage treatments of carbendazim and other fungicides having different modes of action on the development of resistance in the wild sensitive isolates of *Alternaria alternata* on agar plates (*in vitro*) and on inoculated fenugreek (*in vivo*).

In order to study the effect of passage *in vitro*, wild sensitive isolate (Aa-2) in each passage was cultured on agar plates containing a sub lethal dose of carbendazim (0.5 µg/ml) i.e. 0.1%. The plates without fungicide served as control. Inoculum from the colony of previous passage of the same isolate was placed at the center of each plate. In each passage linear growth was measured after 12 days. The percentage in increase of growth of the isolate from passage to passage was considered as increase in carbendazim resistance. The development of resistance was studied up to 8th passage. Continuous, as well as alternate passage of

carbendazim (Bavistin) with Aliette, Ridomil, Roko, Curzate, Dithane M-45 and mixed passage with the same fungicides were carried out.

In vivo studies were carried out on the roots of fenugreek. Roots were dipped in the solution of carbendazim alone, alternate and in mixture with other fungicides. They were inoculated with the wild sensitive isolate. An infected portion of the root of first passage was used for re-isolation and used as the source of inoculums for second passage. This was continuously carried out up to the last passage. After 12 days of inoculation, the percentage of infection was recorded after incubation periods by 0-4 scale (Kareem, 2007).

2.3. In Vitro studies

2.3.1. Continuous Passage

To study the effect of continuous passage, on carbendazim resistance in *Alternaria alternata* (*in vitro*), wild sensitive isolate (Aa-2) in each passage was cultured on plates containing carbendazim alone (0.5%) in triplicate. 8 mm diameter agar disc from the previous passage of the same isolate was placed at the centre of each plate in triplicate. In each passage, linear mycelial growth was measured after 12 days.

2.3.2. Alternate Passage

To study the effect of alternate passage on carbendazim resistance, *Alternaria alternata* isolate was cultured on plates containing carbendazim, in triplicates. After 12 days, 8 mm diameter agar disc from the previous passage was transferred to the plates containing other fungicides alternately, at the same concentration. The process of such alteration of carbendazim to other fungicides was continued up to 8th passage.

2.3.3. Mixed passage:

To study the effect of mixed passage on carbendazim resistance in *Alternaria alternata* isolate was cultured on plates containing carbendazim with another fungicide, both having equal concentration, in triplicates. After 12 days, 8 mm diameter agar disc from the previous passage was transferred to the plates containing the same mixture of fungicides, in equal concentration. In each type of passage, mentioned above, the increased mycelial growth from passage to passage was considered as criterion for the development of fungicide resistance. The effect of passage on the development of fungicide resistance in the pathogen was studied up to 8th passage, in each case.

2.4. In vivo studies

2.4.1. Continuous Passage

To study the effect of continuous passage, on the development of fungicide resistance in the pathogen (*in*

vivo), mycelial suspension using 1 culture tube of wild sensitive isolate (Aa-2) was prepared. 50 ml mycelial suspension was inoculated on the fenugreek roots, treated with 0.5% carbendazim before 24hrs. After 12 days a mycelial suspension from such infected roots was prepared and applied to healthy plant in the triplicate of *Trigonella foenum graecum* treated with 0.5% carbendazim, 24hrs before inoculation. Same procedure was followed up to 8th passage.

2.4.2. Alternate Passage

To study the effect of alternate passage, on the development of fungicide resistance, in the pathogen, *in vivo*, 50ml mycelial suspension using 1 culture tube of wild sensitive isolate (Aa-2) was inoculated on the healthy plant in the triplicate of *Trigonella foenum graecum*, treated with 0.5% carbendazim 24hrs before it. After 8 days a mycelial suspension from such infected roots was prepared and applied to healthy plant in triplicate of fenugreek treated with another fungicide. Same procedure was followed up to 8th passages alternately.

2.4.3. Mixed Passage

To study the effect of mixed passage, on the development of fungicide resistance, in the pathogen (*in vivo*), mycelial suspension using 1 culture tube of wild sensitive isolate (Aa-2) was inoculated on the healthy plant, treated with mixture of carbendazim and another fungicide in same proportion, 24hrs before it. After 12 days a mycelial suspension from such infected leaves was prepared and applied to healthy plants in the triplicate treated with same mixture of fungicides, in same concentration before 24 hrs. In all above types of passages the fenugreek plants were covered with polythene bags.

2.5. Statistical Analysis

Statistical analysis was calculated using two way analysis of variance by Annova.

3. Results

3.1. In vitro studies:-

Continuous and alternate treatment with carbendazim:-

It was seen that growing of *Alternaria alternata* on the medium containing carbendazim for eight successive passages continuously, increased the resistance constantly till 4th passage and after that remain constant, till 8th passage. When it was cultured alternately with other fungicides there was decrease in the development of carbendazim resistance as compare to continuous use. Carbendazim (Bavistin) when used alternately with Aliette, Roko, Curzate, Dithane M-45 and Ridomil, there was inhibition of *Alternaria alternata* after 2nd passage. (Table.1)

Table 1: Effect of exposure of *Alternaria alternata* to carbendazim continuously and alternating with other fungicides on the development of carbendazim resistance during eight successive passages (*In Vitro*)

Fungicide	Passage Numbers							
	1	2	3	4	5	6	7	8
Carbendazim(Bavistin) individual	11.00	14.33	16.00	17.33	18.66	18.66	18.66	18.66
Carbendazim Alternate Aliette	10.00	13.66	00.00	00.00	00.00	00.00	00.00	00.00
Carbendazim Alternate Roko	10.33	12.66	00.00	00.00	00.00	00.00	00.00	00.00
Carbendazim Alternate Dithane M-45	10.00	11.66	00.00	00.00	00.00	00.00	00.00	00.00
Carbendazim Alternate Ridomil	10.66	14.66	00.00	00.00	00.00	00.00	00.00	00.00
Carbendazim Alternate Curzate	9.66	10.33	00.00	00.00	00.00	00.00	00.00	00.00

P<0.0001.

Treatment of carbendazim in mixture of selected fungicides (*In vitro*):-

It was interesting to note that carbendazim when used along with select fungicides like Aliette, Curzate, Roko, Dithane M-45, and Ridomil; there was complete inhibition of the

pathogen after 2nd passage but interesting notice is that, use of curzate in mixture with carbendazim show inhibition of pathogen after first passage (**Table.2**).

Table 2: Effect of exposure of *Alternaria alternata* to the mixture of Carbendazim with other fungicides on the development of resistance during eight successive passages (*In Vitro*)

Fungicide	Passage Numbers							
	1	2	3	4	5	6	7	8
Carbendazim (Bavistin) individual	11.00	13.33	15.66	17.00	18.66	19.33	18.33	18.33
Carbendazim + Aliette	10.33	12.66	00.00	00.00	00.00	00.00	00.00	00.00
Carbendazim + Roko	10.66	11.33	00.00	00.00	00.00	00.00	00.00	00.00
Carbendazim + Dithane M-45	10.00	11.33	00.00	00.00	00.00	00.00	00.00	00.00
Carbendazim + Ridomil	9.66	11.66	00.00	00.00	00.00	00.00	00.00	00.00
Carbendazim + Curzate	11.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00

P<0.01

3.2. In vivo studies:-

Continuous and alternate treatment with carbendazim:-

The wild sensitive isolate (Aa-2) was inoculated on the carbendazim treated Fenugreek roots continuously, shows that there was increase in resistance constantly till 4th

passage and after that remain constant, till 8th passage. It shows that the increased resistant remains constant. The alternate use of selected fungicides with carbendazim when used showed inhibition of growth at second passage except Aliette (Table. 3).

Table 3: Effect of exposure of *Alternaria alternata* to Carbendazim continuously and alternating with other fungicides on the development of resistance during eight successive passages (*In Vivo*)

Fungicide	% of infection and Grade	Passage Numbers							
		1	2	3	4	5	6	7	8
Carbendazim (Bavistin) individual	% of infection	25.00	33.33	33.33	41.66	41.66	41.66	41.66	41.66
	Grade	1	2	2	2	2	2	2	2
Carbendazim Alt. Aliette	% of infection	41.66	25.00	8.33	00.00	00.00	00.00	00.00	00.00
	Grade	2	1	1	0	0	0	0	0
Carbendazim Alt. Roko	% of infection	25.00	8.33	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	1	1	0	0	0	0	0	0
Carbendazim Alt. Dithane M-45	% of infection	25.00	8.33	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	1	1	0	0	0	0	0	0
Carbendazim Alt. Ridomil	% of infection	25.00	8.33	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	1	1	0	0	0	0	0	0
Carbendazim Alt. Curzate	% of infection	25.00	16.66	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	1	1	0	0	0	0	0	0

P<0.0001

Treatment of carbendazim in mixture of selected fungicides:

The wild sensitive isolate (Aa-2) was inoculated on the carbendazim treated Fenugreek roots continuously, shows that there was increase in resistance constantly till 4th passage and after that remain constant till 8th passage. The use of carbendazim in mixture with other fungicides noticed that percentage infection over untreated control, calculated

was showing decrease as compare to continuous and alternate use where at 2nd passage, inhibition of pathogen was seen. It was also observed that carbendazim in mixture with Roko show inhibition of *Alternaria alternata* and growth of pathogen was observed at first passage. The important out come of the experiment was that, the use of carbendazim in mixture of Curzate showed total inhibition of pathogen (Table.4).

Table 4: Effect of exposure of *Alternaria alternata* to the mixture of Carbendazim with other fungicides on the development of resistance during eight successive passages (*In Vivo*)

Fungicide	% of infection and Grade	Passage Numbers							
		1	2	3	4	5	6	7	8
Carbendazim (Bavistin) individual	% of infection	25.00	25.00	33.33	33.33	33.33	41.66	41.66	41.66
	Grade	1	1	2	2	2	2	2	2
Carbendazim + Aliette	% of infection	25.00	8.33	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	1	1	0	0	0	0	0	0
Carbendazim + Roko	% of infection	8.33	00.00	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	1	0	0	0	0	0	0	0
Carbendazim + Dithane M-45	% of infection	25.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	1	0	0	0	0	0	0	0
Carbendazim + Ridomil	% of infection	25.00	8.33	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	1	1	0	0	0	0	0	0
Carbendazim + Curzate	% of infection	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00
	Grade	0	0	0	0	0	0	0	0

P<0.001

4. Discussion

Carbendazim is as a systemic fungicide used for management of many fungal diseases. Treatment of carbendazim continuously, alternately and in mixture show variable results during eight passages *in vitro* and *in vivo*. These results are agreeing with other workers, regarding resistance of pathogen and application of fungicide and its mode of action on pathogen. Horsten (1979), observed that alternate use of ediphenphos with carbendazim reduced carbendazim resistance in *Septoria nodorum* and *Cercospora herpotrichoides*. Kable and Jaffery (1980), gave a mathematical model to test different chemicals for their alternate use. Anitha *et al.*, (1989) also supported this work in *Macrophomina phaseolina* for acquired and cross fungicide tolerance to carbendazim. Gangawane *et al.*, (1993) studied the effect of passage on the development of metalaxyl resistance in *Phytophthora infestans*. Khilare and Gangawane (1998) reported the effect of passage on the development of thiophonate methyl resistance in *Penicillium digitatum*. Hartill (1983), advised alternate use of mancozeb with metalaxyl to control late blight of potatoes. Multisided action of carbendazim with mancozeb, benomyl, captafol and thiram might be responsible for the complete inhibition or the development of resistance in the *Macrophomina phaseolina* causing charcoal rot of potato was recorded by (Kamble, 1999). Again he observed development of carbendazim resistance in *Macrophomina phaseolina* mutants having resistance factor from 5-15, mutants having high resistant factor exhibition higher growth and all mutants were stable for carbendazim resistance. This might happen due to change in target sites. According to Griffin (1981), alternately used fungicide must have different mode of action. This opinion was supposed by many pathologists (Kumar *et al.*, 2007; Dekker and Gielink, 1979; Gangawane and Reddy, 1987; Dahiwale *et al.*, 2009; Magie, and Wilfret, 1974; Gullino and Garibaldi, 1981; Sanders and Gilbride, 1981; Jarvis and Slingsby, 1975; Penrose and Koffman, 1977; Sable and Gangawane, 2012) in various pathogens.

5. Conclusions

The outcome of the experiment during 8 passages showed following conclusions

1. Use of carbendazim alone continuously, showed increase of resistance both *in vitro* and *in vivo*.
2. Alternate use of carbendazim with select fungicides show better results in inhibition of pathogen both *in vivo* and *in vitro* as compare to continuous application of carbendazim.
3. Use of Carbendazim in mixture with select fungicides show best results during *in vivo* and *in vitro* application.
4. Use of Curzate in mixture with carbendazim was proved to be best fungicide candidate for management of *Alternaria alternata* causing root rot disease to fenugreek.

6. Abbreviations

MIC- Minimum inhibitory concentration

Aa- *Alternaria alternata*

Ridomil- Ridomil Gold

Alt- Alternate

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