An integrated approach for the reduction of aflatoxin contamination in chilli (*Capsicum annuum* L.)

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Abstract An integrated approach for management of aflatoxin contamination in chilli was undertaken by evaluating the fungicides, bioagents and plant extracts against *Aspergillus flavus* under both in vitro and field condition. Maximum inhibition of radial growth (91.1%) was observed with 0.3% mancozeb followed by captan (85.2%). Carbendazim (73%) was effective and superior over other systemic fungicides. A complete inhibition (100%) of *A. flavus* was observed in neem seed kernel extract (NSKE), nimbecidin and pongamia oil at 5%. An indigenous *Pseudomonas fluorescens* bioagent isolate inhibited (74.9%) the growth of *A. flavus* over *Trichoderma harzianum* (70.4%). The superior performing fungicides, plant extracts and bioagents identified under in vitro were used for challenge inoculation on chilli fruits and so also for field evaluation. The captan treated fruits recorded the least infection of *A. flavus* (1.6%) followed by *P. fluorescens* (2.0%), NSKE (2.2%) and nimbecidin treated fruits (7.8%) as against control (38.3%). As regards to field

evaluation, the least incidence was recorded in NSKE sprayed chilli plot (1.6%) and was on par with captan (2.2%), *P. fluorescens* (2.4%) and *T. harzianum* (2.6%) compared to control (7.4%). Hence, a pre-harvest spray of NSKE (5%) or mancozeb (0.3%) or *P. fluorescens* (1×10^8 cfu/ml) 10 days before harvest of chilli is recommended for field level management of aflatoxin.

Keywords Aflatoxin · *Aspergillus flavus* · Bioagents · Chilli · Fungicides · Plant extracts

Chilli (*Capsicum annuum* L.) is the universal spice and is grown in almost all states of India for the domestic market and export purpose. Chilli crop suffers from many field and post harvest diseases such as damping off, anthracnose or fruit rot, die back, wilt, murda complex, leaf spots and powdery mildew mostly under field conditions but the infection of chilli due to aflatoxin fungi, *Aspergillus flavus* is specific during and after harvest. The infection occurs on stored fruits and the contamination with aflatoxin deteriorates the quality and make the produce unfit for consumption, thereby restricting the export trade. The incidence of *A. flavus* contamination on chilli fruits was recorded up to 29.3% (Naik et al. 2003) in the Northern region of Karnataka. Aflatoxin contamination and pesticide residues are the twin problems faced by Indian chillies in the global market trade. It is essential to formulate proper strategies for management of aflatoxin contamination in chilli. The present paper makes an effort to develop an integrated approach for management of aflatoxin contaminating fungus, *A. flavus* in chilli by evaluating the fungicides, bioagents and plant extracts under in vitro and to evolve a satisfactory management of aflatoxin contamination in chillies using the best performing fungicides, bioagents and plant extracts under field conditions.

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Material and methods

Isolation of the pathogenic *A. flavus* from chilli fruits Infected chilli (*Capsicum annuum* L.) fruits were collected from different fields of North (India) Karnataka regions covering Raichur, Bellary and Gulbarga districts. The fungus was isolated by following standard tissue isolation method (Ajithkumar and Naik 2006) under aseptic conditions. The infected tissues of fruits were cut into small bits of size 1–2 mm and surface sterilized in 1:1000 mercuric chloride solution for 1 min and washed repeatedly thrice in sterile distilled water to remove the traces of mercuric chloride before transferring them to sterile potato dextrose agar (PDA) slants under aseptic condition and incubated at 28 ± 1 °C for growth. The culture, thus obtained was purified by single spore isolation method (Ajith Kumar and Naik 2006). *A. flavus* isolates were identified based on the morphological features such as conidiophores, conidia, metulae, colour of the colony and rate of growth. The conidial heads were bright yellow when young, olive green turning to brown with age, radiate or loosely columnar, conidiophores roughened, vesicles globose to subglobose, flask shaped in smaller heads, sterigmata biseriate or uniseriate, conidia globose to subglobose rarely elliptical, smooth to roughened, Sclerotia red-brown to purple-brown or black, globose to elongate (Raper and Fennell 1965). Further, the identity was confirmed by depositing the cultures at National Centre of Fungal Taxonomy, New Delhi as per No. 1540.07 to 1561.07.

Single spore culture of *A. flavus* Ten ml of clean filtered 2% sterile agar solution was poured into sterile Petri plates and allowed to solidify. The dilute spore suspension was prepared in sterile distilled water from 10 days old culture of *A. flavus*. One ml of spore suspension was spread uniformly on solidified 2% of water agar plates. The excess of suspension was aseptically drained off. After 4 h incubation at 28 ± 1 °C, the plates were examined and germinated conidia were located. Single isolated and germinated conidium was marked with ink on the lower surface of the Petri plate. The portion of agar directly below the ink mark was cut and transferred to fresh PDA slants in such a way that the conidium bearing surface is in contact with PDA surface and incubated at 28 ± 1 °C. The pure culture of the fungus was obtained and preserved in refrigerator and sub cultured once in a month.

In vitro evaluation of fungicides against *A. flavus* Four each of systemic and non-systemic fungicides were used for evaluation using poison food technique (Nene 1971) against *A. flavus*. Systemic fungicides were tested at 0.05, 0.1 and 0.15% concentrations, whereas, non-systemic fungicides were evaluated at 0.1, 0.2 and 0.3% concen-

trations. Three replications were maintained for each treatment. The radial growth of the colony was recorded when maximum growth was observed in control and per cent inhibition was calculated by using the formula given by Vincent (1927). Data were analyzed as per the procedures given by Panse and Sukhatme (1985) to determine the level of significance.

In vitro evaluation of plant extracts against *A. flavus* The present investigation was carried out to evaluate the effect of extracts of different plant species viz., neem (*Azadirachta indica*) seed kernel extract (NSKE), pongamia (*Pongamia pinnata*) oil and neem based chemicals like nimbidin (T. Stanes and Company Ltd, Coimbatore, Tamil Nadu, India) (2000 ppm) and rakshak (Pragati Nikem, Mysore, Karnataka, India) (1500 ppm) at 1.0, 2.5 and 5.0% concentrations on *A. flavus* using the poison food technique described earlier.

Preparation of neem seed kernel extract (NSKE) and pongamia oil About 50 g of peeled neem seeds were crushed into small pieces, tied in muslin cloth and soaked in water for 8 h. The squeezed, yellow suspension was taken out and the volume was made up to 1000 ml (Anon 2000). From this, different concentrations of NSKE (1.0, 2.5 and 5.0%) were prepared for evaluation. The extraction of pongamia oil from seeds was done in a sterilized pestle and mortar by adding sterile distilled water. The extracts were filtered through two layers of cheese clothes and were tried at 1.0, 2.5 and 5.0% concentrations for evaluation.

In vitro evaluation of bioagents against *A. flavus* The bioagents obtained indigenously and from the Project Directorate of Biological Control, Bangalore, were evaluated for their efficacy through dual culture technique. The bioagents and test fungus were evaluated side by side on a single Petri dish containing solidified PDA medium. Three replications were maintained for each treatment with one control by maintaining only pathogen and bioagent. They were incubated for 8 days. The diameter of the colony of the pathogen and bioagent was measured in two directions and the average was recorded. The per cent inhibition of the growth was recorded by comparing with the control.

Fruit treatment with fungicides, botanicals and bioagents against *A. flavus* The spray suspension of different treatments of non-systemic fungicides mancozeb and captan (0.3%), botanicals NSKE and nimbidin (5%) and bioagents *P. fluorescens* (Pfl) and *T. harzianum* (ThI) (1×10^8 cfu/ml) were prepared and red ripened chilli fruits were dipped in the suspension comprising of different treatments for 5 min, air dried and then challenge inoculated with *A. flavus* culture using pin prick method of inoculation.

Table 1 Effect of fungicides on inhibition of mycelial growth of *Aspergillus flavus* under in vitro conditions

Non-systemic fungicides											
Mancozeb			Chlorothalonil			Copper oxychloride			Captan		
Concentration, C%											
0.10	0.20	0.30	0.10	0.20	0.30	0.10	0.20	0.30	0.10	0.20	0.30
Inhibition,%											
66.5*	78.5	91.1	11.0	16.3	30.0	14.9	21.5	35.9	72.2	80.4	85.2
(54.6)**	(62.4)	(84.6)	(19.1)	(23.8)	(33.2)	(22.7)	(27.6)	(36.8)	(58.2)	(63.7)	(67.4)
F C FXC											
C,% :	0.10	0.20	0.30								
SEm± :	0.22	0.11	0.22								
SEd :	0.31	0.16	0.31								
CD at 1% :	0.51	0.44	0.88								
Systemic fungicides											
Carbendazim			Benlate			Triademefon			Thiophanate methyl		
Concentration, C%											
0.05	0.10	0.15	0.05	0.10	0.15	0.05	0.10	0.15	0.05	0.10	0.15
Inhibition%											
27.4*	35.2	73.0	19.63	30.78	42.55	4.8	16.3	38.9	2.6	8.1	17.4
(31.6)**	(36.4)	(58.7)	(26.3)	(33.7)	(40.7)	(12.7)	(23.8)	(38.6)	(9.3)	(16.5)	(24.7)
F C FXC											
C,% :	0.05	0.10	0.15								
SEm± :	0.21	0.18	0.36								
SEd :	0.30	0.26	0.50								
CD at 1% :	0.82	0.71	1.42								

* Original values ** Figures within the parentheses are Arc sine transformed values

F = Fungicide

Challenge inoculation of chilli fruits with *A. flavus* The pin-prick method of inoculation of chilli fruits was followed (Naik and Rawal 2002). The fruits harvested at red ripened stage were surface sterilized with 0.1% mercuric chloride and then washed in three changes of sterile water. There after, the fruits were pricked with pin bundles specially designed for pricking. The pin pricked fruits were dipped in *A. flavus* spore suspension (1×10^6 spores/ml) for 2–3 min and incubated in humid chamber. The humid chamber was prepared by keeping water in the

tray, which was placed below the perforated tray kept with inoculated fruits. Wet cotton pieces were placed on the tray. The tray was covered with polythene sheet to maintain the RH of over 90% and then incubated at $28^\circ \pm 1^\circ \text{C}$ for a week and the per cent infection of *A. flavus* was recorded (Ajithkumar and Naik 2006). A suitable control was maintained by dipping the chilli fruits in sterile water followed by pricking the fruits without challenge inoculation by pathogen. The experiment was repeated twice.

Table 2 Efficacy of plant extracts on inhibition of mycelial growth (% inhibition) of *Aspergillus flavus* under in vitro conditions

Botanicals (B)	Concentration, C%		
	1.00	2.50	5.00
Neem seed kernel extract	73.3* (58.9)**	93.3* (75.0)**	100.0* (90.0)**
Nimbidin (2000 ppm)	73.3 (58.9)	93.3 (75.0)	100.0 (90.0)
Rakshak (1500 ppm)	53.3 (46.9)	73.3 (58.9)	93.3 (75.0)
Pongamia oil (pure)	53.3 (46.9)	80.0 (63.4)	100.0 (90.0)
SEM	B=±0.487	C=±0.422	BXC=0.844
CD at 1%	B=1.94	C=1.681	BXC=3.362
SED	0.67	0.61	1.19

* Original values ** Figures within the parentheses are Arc sine transformed values

Table 3 Efficacy of bioagents in inhibiting the growth of *Aspergillus flavus*

Bio-agents	Mycelial growth inhibition%
<i>Pseudomonas fluorescens</i> (Indigenous Pf I)	74.9
<i>Trichoderma viride</i> (PDBCH-24)	68.9
<i>Trichoderma harzianum</i> (PDBCH-10)	70.4
SEm±	0.552
SEd	0.78
CD at 1%	2.621

Field evaluation of fungicides, botanicals and bioagents Field experiments were conducted twice during *khari* 2007 and 2008 at Horticulture Garden, College of Agriculture, Raichur. The superior performing treatments identified under laboratory condition were imposed under field condition. The experiments were conducted in a plot measuring 42.5 m×12.5 m with spacing of 75×45 cm in randomized block design with seven treatments such as mancozeb, captan, NSKE, Nimbicidin, *T. harzianum*, *P. fluorescens* and control which were replicated thrice and randomized. The cultivar ‘Byadagi Kaddi’, a regional variety of chilli, was used in this experiment. All other cultural and pest management practices were imposed as recommended in package of practices. The spray suspension of different treatments were prepared and sprayed at 10 days before harvesting. The fruits were harvested and stored, 8 days after storage the percent infection of aflatoxin contaminated chilli fruits was recorded in different treatments. The chilli yield in each treatment was noted; the 2 years data were pooled and statistically analyzed. Statistical analysis was carried out as per the procedures given by Panse and Sukhatme (1985). Actual data in percentage were converted to angular values, before analysis according to the table given by Snedecor and Cochran (1967).

Results and discussion

In vitro evaluation of fungicides against *A. flavus* The inhibition of radial mycelial growth of the fungus varied proportionately according to concentration of the fungicides used. Among non-systemic fungicides (Table 1) the maximum inhibition of radial mycelial growth (91.1%) was observed with 0.3% mancozeb, followed by captan (85.2%). Mancozeb was superior to all other fungicides followed by captan, whereas copper oxy chloride and chlorothalonil were least effective against *A. flavus*. There was a significant difference between systemic fungicides, concentration and also its interaction (Table 1). Carbendazim (73%) was effective and superior to rest of the fungicides at 0.15% concentration, whereas benomyl, triademefon and thiophanate methyl were not effective in inhibiting the mycelial growth of *A. flavus*. In fact, they are known to be ineffective against coloured spore producing fungi such as *A. flavus*. The results are also in conformity with Tandan et al. (1975) and Baqar Imam Zaidi et al. (1991) wherein aptan (0.2%) was found to be most effective in inhibiting the mycelial growth. However, Savitri et al. (1998) and Suryavanshi and Deokar (2001) reported the effectiveness of mancozeb and carbendazim against *Aspergillus* spp.

Table 4 Effect of different fungicides, plant extracts and bioagents on aflatoxin fungal infection on chilli fruits under laboratory and field conditions

Mancozeb	Captan	NSKE	Nimbicidin	<i>Trichoderma harzianum</i>	<i>Pseudomonas fluorescens</i>	Control
Concentration, C%						
0.3	0.3	5	5	1 × 10 ⁸ cfu/ml	1 × 10 ⁸ cfu/ml	—
Chilli fruits infected under laboratory condition,%						
1.90*	1.57	2.20	7.75	5.17	2.00	38.33
(7.91)**	(7.18)	(8.52)	(15.96)	(13.13)	(8.12)	(38.25)
SEm ± : 0.25	SEd: 0.35	CD at 1% : 1.10				
Chili fruits infected under field condition,%						
3.45*	2.16	1.65	2.80	2.60	2.42	7.41
(10.69)**	(8.35)	(7.33)	(9.59)	(9.25)	(8.84)	(15.79)
SEm ± : 0.36	SEd: 0.50	CD at 1% : 1.09				

* Original values ** Figures within the parentheses are Arc sine transformed values

NSKE: Neem Seed Kernel Extract

In vitro evaluation of plant extracts against *A. flavus* A significantly higher inhibition (100%) of *A. flavus* was observed with NSKE, nimbicidin and pongamia oil, which were on par with each other and were significantly superior over all other treatments (Table 2). Least inhibition of growth (93.3%) was recorded by Rakshak. But all the four plant extracts showed maximum inhibition (99.6%) of *A. flavus* growth at 5.0% concentration. However, the percent inhibition decreased with decrease in concentration of plant extracts. Antifungal property of *Azadirachta indica* in inhibiting the AFB1 production by *Aspergillus flavus* was also established by Reddy (1987).

In vitro evaluation of bioagents against *A. flavus* *P. fluorescens* inhibited the mycelial growth of *A. flavus* significantly (74.9%) over the rest of the bio-agents. *T. harzianum* inhibited the growth of *A. flavus* to the extent of 70.4% followed by *T. viride* with least inhibition of mycelial growth (68.9%), as presented in Table 3. The antagonistic nature of *P. fluorescens* in inhibition of mycelial growth of *A. flavus* was shown by Ray et al. (1990) and Manwar et al. (2000). Bhuvaneshwari and Subbarao (2001) reported that *T. viride* was very effective in reducing the infection of *A. flavus*.

Fruit treatment with fungicides, plant extracts and bioagents Among non-systemic fungicides, captan treated fruits recorded least infection by *A. flavus* (1.6%) and was on par with mancozeb treated fruits (1.9%). Among botanicals, NSKE showed least infection (2.2%), which significantly differed from nimbicidin treatment (7.6%). Whereas, bioagent *P. fluorescens* (Pfl) reduced the colonization of *A. flavus* (2.0%) compared to *T. harzianum* (ThI) (5.2%). Contrary to this, in control the infection rate was to the extent of 38.3%. The results are also in conformity with Baquar Imam Zaidi et al. (1991), who have observed effective inhibition the mycelial growth of *A. flavus* with captan at 0.2%. The high efficacy of captan in inhibiting mycelial growth of *A. flavus* was reported by Meena and Mariappan (1993). The efficacy of neem extract in reducing the *A. flavus* incidence in various crops was reported by several workers (Bansal and Sobti 1988; Meena and Mariappan 1993; Srivatsava et al. 1997; Sinha et al. 1999). The effectiveness of neem as pesticide is due to the presence of azadirachtin chemical, which has fungicidal effect. Antifungal property of *A. indica* was confirmed by Reddy (1987) and Srivastava et al. (1997). Manwar et al. (2000) identified the antagonistic nature of two strains of *P. fluorescens*, against mycelial growth of *A. flavus* in plates under in vitro. Praveen Kumar et al. (2000) reported the efficacy of talc powder based formulation of *P. fluorescens* against *A. flavus* and *A. niger*, which inhibited the growth of *Aspergillus* on seeds. The treated seeds exhibited increased germination, seedling vigour and emergence compared to control.

Field evaluation of fungicides, plant extracts and bioagents All treatments showed reduced incidence of *A. flavus* compared to control (7.4%) as presented in Table 4. Among different treatments, least incidence was recorded in NSKE treated plot (1.6%) and was on par with captan (2.2%), *P. fluorescens* (Pfl) (2.4%) and *T. harzianum* (ThI) (2.60%). Antifungal property of *A. indica* in inhibiting aflatoxin B1 was established by Reddy (1987). Even mancozeb was fairly good in reducing the incidence (3.4%) as against control with an incidence of 7.4%. Among the fungicides, the efficacy of thiram and dithane M-45 in reducing the incidence of *A. flavus* on groundnut was reported by Bansal and Sobti (1988). Similarly, the least incidence of *A. flavus* on groundnut seeds (1 and 3%) was obtained using thiram (0.3%) and dithane M-45 (0.2%), as compared to control (9%) (Tripathi and Singh 1991). Mancozeb and captan have been found to be quite safe with no residual effect. Maximum residual effect of mancozeb and captan is 3 mg/kg and is quite safe (Debi Sharma et al. 2002). Hence, their use in management of aflatoxin fungus is quite acceptable and safe not only for domestic consumption even for export purpose.

In the present experiment, bioagents *P. fluorescens* (Pfl) and *T. harzianum* (ThI) are equally efficient in reducing the *A. flavus* infection on chilli. The overall seed infection of *A. flavus* in groundnut was reduced by application of the antagonists in pot cultures (Vanamala et al. 2001). Anjaiah et al. (2006) obtained more than 50% reduction of the *A. flavus* population in groundnut by inoculation of fluorescent *Pseudomonas*, *Bacillus* and *Trichoderma* spp. Of the *Trichoderma* isolates evaluated against *A. flavus*, culture filtrate of *T. virens* showed complete inhibition (100%) of growth of *A. flavus* at 15% concentration (Reddy et al. 2004). The use of bioagents has additional advantage as these are eco-friendly without any pesticide residues problem on chilli fruits and this aspect is essentially important for export of chilli (Dorner et al. 1992). Hence, a pre-harvest spray of NSKE (5%) or mancozeb (0.3%) or *P. fluorescens* (1×10^8 cfu/ml) 10 days before harvest of chilli is recommended for field level management of aflatoxin producing fungus.

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