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# Effective inoculation technique for identification of resistance source in chilli (*Capsicum annuum* L.) against aflatoxin and management through *Trichoderma* species

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**Abstract**

The present study was carried out to develop an effective inoculation technique for screening chilli fruits against *Aspergillus flavus* and to identify the most effective bioagents against *A. flavus* to integrate them into management strategies. The study revealed that dipped method is better than pin- pricked method for creating maximum infection pressure on fruits and can be successfully utilized for screening against the pathogen. Artificial screening revealed that chilli varieties Tabasco, PBC-535, IIHR-10 and LCA-429 were resistant to *A. flavus*. Among three tested species of *Trichoderma*, *T. viride* was comparatively more effective against 21 isolates of *A. flavus*. All the *Trichoderma* species showed fungistatic behaviour against *A. flavus* even after 14 days of incubation. Differential response of *Trichoderma* species against *A. flavus* indicated distinct variability among the isolates of *A. flavus* collected from different regions of the country.

**Keywords:** aflatoxin, *Aspergillus flavus*, bioagents, chilli, management, resistance

## Introduction

Chilli (*Capsicum annuum* L.) is an important vegetable and spice crop of India. Red dried fruits of chilli are usually stored in warehouse after packing in moist condition. Water is sprinkled over the dried fruits just before packing in gunny bags to avoid breakage of dry red fruits. The gunny bags are thin and netted due to which external moisture penetrates into the fruits during rainy season. Most of the time, chilli powder and paste available in the market are prepared using substandard, infected chilli fruits. All these factors lead to contamination by mycotoxin producing fungi like *Aspergillus flavus*, *A. parasiticus*, *A. ochraceous*, *A. niger* and *Fusarium* spp. Several types of mycotoxins particularly aflatoxin, ochratoxin and fumonisins are commonly reported in dry red chilli, powder and paste in subtropical and tropical countries of the world. Aflatoxin is a carcinogenic mycotoxin harmful to human beings at very low concentration. The aflatoxin level in chilli powder in Addis Ababa market of Ethiopia was 100-525 ppb and 13.3% samples were positive to aflatoxin (Fuba & Urga 1996). Aflatoxin contamination in pepper ranged from 32.2-48.1 μg $kg^{-1}$ in Punjab and Sindh region of Pakistan (Jaffar *et al*. 1994). Aflatoxin B1 was detected (upto 234 μg $kg^{-1}$) in samples of Cayenne pepper powder (Finnli & Ferari 1994). The acceptable

aflatoxin content in food products should be less than 30 μg $kg^{-1}$ (Halt 1994). The maximum residue level of aflatoxin in food permitted in Japan is 10 ppb. However, more than 75% samples of paprika were found to be contaminated with aflatoxin (Tabata *et al*. 1998). The maximum permissible limit (MRL) of aflatoxin is 20 ppb in different parts of world. Putzka (1994) reported that 55% samples of chilli and paprika were carrying above the permissible limits of aflatoxin B1. In United Kingdom, highest concentration of aflatoxin (48 μg $kg^{-1}$) in retail samples of chilli powder was recorded (Macdonald & Castle 1996). Sun drying of red chilli fruits reduced the level of aflatoxin to < 2.0 μg $kg^{-1}$ (Adegoke *et al*. 1996). Though, aflatoxin in chilli is a worldwide problem, no effective management strategies have been developed as yet. Besides, resistance, source against *A. flavus* is neither available nor screening techniques have been developed. However, the possibility of the use of bioagents can be explored for the management of *A. flavus* contamination. *Trichoderma* species inhibited the growth of *A. flavus* (Calistru *et al*. 1997). *Trichoderma viride* and *A. niger* were strongly antagonistic to the growth of *A. flavus* by 87% and 66%, respectively (Aziz & Shahin 1997). The objective of the present study was to find out the best screening technique, effective resistance source and potential bioagent for management of *A. flavus* in chilli.

## Materials and methods

Chilli growing areas as well as markets were surveyed and samples of dry red chilli fruits were collected from Uttar Pradesh, Punjab, Haryana, Karnataka, Andhra Pradesh, Delhi, Bihar, Himachal Pradesh, Jammu and Kashmir states in the months of September and October during 2007-08. A total of 21 cultures of *A. flavus* was isolated and purified from these samples by hyphal-tip method. Mature red fruits of chilli from different varieties and advanced germplasm lines (39) were harvested from the field and sun dried completely at the stage of storage in the first set of experiment. In another set, fresh red fruits were harvested and used without drying. The moisture level in dried chillies of different tested cultivars or germplasm ranged from 10.0-10.30%, however, the moisture level in fresh chilli fruits ranged from 84.50-85.10% at the time of inoculation. These red fruits were inoculated by two methods *viz*., pin-pricked and dipped method. The intact chilli fruits were dipped in the inoculum. The spore concentration of *A. flavus* was prepared using seven days old pathogenic culture. The inoculum concentration was maintained at $1 \times 10^8$ conidia $mL^{-1}$. Inoculation was done by placing 5 μL spore suspension on pin-pricked surface of fruits while in another set, the red fruits were dipped for one minute in the spore suspension. All the inoculated fruits were kept in indigenously designed moisture box prepared with the help of plastic trays and blotting papers. The relative humidity was maintained at > 95% inside the moisture box. The relative humidity in the box was measured with the help of Clock & Hygro Termo (Max. & Min.) digital meter (Eurolab-412CTH). The inoculated chilli fruits were incubated for 14 days in BOD incubator at 28±2°C. The observations were recorded after 7 and 14 days of the inoculation. The rating was given on the basis of percent infected area of chilli fruits by *A. flavus* and accordingly categorized for the host reactions. The chilli fruit surface colonized in the range of 0-10% area was categorized as resistant, 11-25% as moderately resistant, 26-50% as moderately susceptible, 50-75% as susceptible and 75-100% as highly susceptible. A total of 39 chilli varieties/germplasm lines were screened against *A. flavus and T. harzianum, T. viride* and *T. koningii* were tested against different location specific pathogenic isolates of *A. flavus* by dual culture method. Mean radial growth of *A. flavus* was recorded after five and 10 days of inoculation. It was compared with the radial growth of *A. flavus* from control and the differential antagonistic potential of *Trichoderma* species was recorded.

## Results and discussion

### *Effect of inoculation techniques on* A. flavus *severity in chilli*

Two different inoculation techniques were used against *A. flavus* at two different maturity

stages of chilli fruits. Maximum infection was recorded by dipped method in comparison to pin-pricked method. The colonization capacity of *A. flavus* and its severity on most of the varieties were greater in dipped method after 7 and 14 days of inoculation. The average severity was 42.71% and 85.71% in dipped method after 7 and 14 days of inoculation, respectively. However, the corresponding value was comparatively less (12.2% and 24.3%, respectively) in pin-pricked method (Table 1). Therefore, dipped method was selected as best inoculation technique for *A. flavus* on chilli which can be used for disease resistance screening and host pathogen interaction study. Chilli varieties *viz*., Tabasco, PBC-535, LCA-429 and IIHR-10 were found to be resistant under the test screening by both the methods. Tabasco and PBC-535 were free from infection even after 14 days of incubation. It was also observed that the infection significantly increased from 7 to 14 days of incubation in all the varieties and in both the methods of inoculation. The maximum infection (95-100%) was recorded only after 14 days of incubation in dipped method. KCS-2013 and Ajeet-3 were identified as highly susceptible varieties of chilli and can be used as susceptible check in any pathological and biochemical study. It clearly indicated that optimum time for incubation should be 14 days to record any pathological observation of *A. flavus* in red chilli fruits.

*Colonization of* A. flavus *on fresh red and dry red chilli fruits*

Red fruits freshly harvested from the field having more than 80% moisture and completely dried red chilli fruits having less than 10% moisture were used in the present study to find the colonization potential of *A. flavus*. The results revealed that majority of the chilli varieties were severely infected by *A. flavus* when moisture was very low in fruits. The corresponding severity percentage was less in fresh red chilli fruits at higher moisture level (Table 2). It is clear that moisture of fresh fruit and the inherent defense mechanism of its living cells play important role for infection of *A. flavus*. Dry red chilli fruits were more prone to infection rather than fresh red fruits. It is well known that some of the *Aspergillus* species are prevalent in warm and dry environment. The study indicated that red chillies are susceptible to aflatoxin production compared to green chillies. Reasons for less production of aflatoxin in green chillies have not yet been studied. The fresh red chilli fruits were more susceptible to *Colletotrichum* and *Fusarium* spp. rather than *A. flavus*. Also, less infection by *A. flavus* on fresh fruits is due to the biologically active defense mechanism and higher concentration of compounds like phenolics, phytoalexins, systemic acquired resistance etc. Resistance in Tabasco, PBC-535, IIHR-10 and LCA-429 was further confirmed and consistently present in fresh and dry red chilli fruits which showed its inherent genetic character.

*Antagonistic effect of* Trichoderma *species on* A. flavus

Three important species of *Trichoderma* were tested *in vitro* for their inhibitory potential against 21 pathogenic isolates of *A. flavus*. Observations after 5 and 10 days of incubation in dual culture revealed that inhibition of *A. flavus* was almost constant after 5 days of inoculation. The radial growth of *A. flavus* varied from 12.9-14.2 mm after 5 days and was almost constant (11.9-14.0 mm) even after 10 days of incubation. However, the growth of *A. flavus* in control increased from 45.76 to 58.62 mm during the test period (Table 3). It clearly indicated the fungistatic behavior of *Trichoderma* species rather than fungicidal activity. The inhibitory effect of *Trichoderma* spp. on all the isolates of *A. flavus* was prominent but mycoparasitic antagonism was not as prominent as observed with *Pythium, Sclerotium* and *Fusarium* spp. *A. flavus* isolates Lu-1, So-1, Si-1, Gu-59 and Gr-1were more susceptible to all the three test species of *Trichoderma* and the maximum radial growth inhibition was observed after 10 days of antagonistic interaction. Isolate Sr-2, Ch-2 and Dh-2 were more tolerant to *Trichoderma* spp. The radial growth inhibition potential of *T. viride* was comparatively better than *T. harzianum* and *T. koningii*. The radial growth of all the isolates of *A. flavus* varied from 30-88 mm in control which indicated significant variability among

**Table 1.** Progressive *A. flavus* severity by two inoculation methods on different chilli cultivars

| Cultivars/ germplasm | Pin-pricked method (%) | | Dipped method (%) | | Average | Host |
|---|---|---|---|---|---|---|
| | 7 DAI[a] | 14 DAI | 7 DAI | 14 DAI | (14 DAI) | Reaction |
| BS-20 | 10 | 20 | 15 | 18 | 19.0 | MR |
| BS-35 | 10 | 40 | 40 | 50 | 45.0 | MS |
| P-1649 | 0 | 0 | 70 | 85 | 42.5 | MS |
| A-7 | 0 | 0 | 30 | 80 | 40.0 | MS |
| *Pusa Jwala* | 10 | 15 | 25 | 50 | 32.5 | MS |
| PBC-574 | 0 | 8 | 70 | 90 | 49.0 | MS |
| *Jawahar Chilli*-218 | 50 | 70 | 90 | 95 | 82.5 | HS |
| LCA-429 | 0 | 0 | 0 | 10 | 5.0 | R |
| *Indira Chilli* | 10 | 22 | 55 | 60 | 41.0 | MS |
| PBC-367 | 10 | 20 | 50 | 85 | 52.5 | S |
| A-3 | 0 | 8 | 10 | 55 | 31.5 | MS |
| R-Line | 0 | 0 | 85 | 90 | 45.0 | MS |
| CCH-3 | 0 | 18 | 0 | 10 | 14.0 | MR |
| PBC-379 | 10 | 20 | 20 | 60 | 40.0 | MS |
| KA-2 | 0 | 10 | 70 | 80 | 45.0 | MS |
| NCH-338 | 20 | 30 | 25 | 35 | 32.5 | MS |
| CCH-1 | 25 | 35 | 40 | 90 | 62.5 | S |
| IIHR-10 | 0 | 8 | 0 | 0 | 4.0 | R |
| *Ac-Assam*-10 | 23 | 25 | 40 | 95 | 60.0 | S |
| 9950-5197 | 40 | 60 | 85 | 90 | 75.0 | S |
| KCS-2013 | 33 | 90 | 75 | 100 | 95.0 | HS |
| PDG-1 | 15 | 18 | 60 | 90 | 54.0 | S |
| SHSC-1111 | 0 | 12 | 15 | 25 | 18.5 | MR |
| *Tabascco* | 0 | 0 | 0 | 0 | 0.0 | R |
| BC-28 | 0 | 30 | 0 | 40 | 35.0 | MS |
| LCA-301 | 15 | 18 | 15 | 45 | 31.5 | MS |
| Ajeet-3 | 20 | 65 | 90 | 95 | 80.0 | HS |
| PBC-535 | 0 | 0 | 0 | 0 | 0.0 | R |
| CCH-2 | 15 | 35 | 50 | 75 | 55.0 | S |
| LCA-206 | 23 | 60 | 45 | 80 | 70.0 | S |
| MS-12 | 0 | 15 | 12 | 30 | 22.5 | MR |
| F-S-112 | 10 | 25 | 25 | 85 | 55.0 | S |
| *Japani Longi* | 10 | 25 | 55 | 70 | 47.5 | MS |
| A-4 | 0 | 0 | 50 | 90 | 45.0 | MS |
| *Capsicum Chilli* | 0 | 0 | 0 | 10 | 5.0 | R |
| CCA-426 | 0 | 0 | 70 | 80 | 40.0 | MS |
| AKC-89/38 | 0 | 15 | 0 | 40 | 27.5 | S |
| PKM-1 | 65 | 70 | 85 | 90 | 80.0 | HS |
| 97-7125-2 | 50 | 60 | 65 | 80 | 70.0 | S |
| Mean Value | 12.15 | 24.28 | 42.74 | 85.71 | - | - |

[a]DAI=Days after inoculation
MR=Moderately resistant; MS=Moderately susceptible; HS=Highly susceptible; S=Susceptible; R=Resistant

**Table 2.** Comparative colonization capacity of *A. flavus* on two types of chilli fruits and its host resistance reaction

| Cultivars/ germplasm | Fresh red fruits | | Dried red fruits | | Average Severity (%) | Host reaction |
|---|---|---|---|---|---|---|
| | Severity (%) | Host reaction | Severity (%) | Host reaction | | |
| BS-35 | 40.0 | MS | 96.7 | HS | 68.4 | S |
| A-7 | 40.0 | MS | 76.7 | HS | 58.4 | S |
| *Pusa Jwala* | 25.0 | MR | 83.3 | HS | 54.2 | S |
| *Jawahar Chilli*-218 | 72.0 | S | 76.2 | HS | 74.1 | S |
| LCA-429 | 5.0 | R | 13.0 | MR | 9.0 | R |
| *Indira Chilli* | 40.0 | MS | 31.7 | MS | 35.9 | MS |
| A-3 | 30.0 | MS | 28.3 | MS | 29.2 | MS |
| IIHR-10 | 5.0 | R | 9.0 | R | 7.0 | R |
| R-Line | 45.0 | MS | 25.0 | MR | 35.0 | MS |
| CCH-3 | 14.0 | MR | 80.0 | HS | 47.0 | MS |
| PBC-379 | 40.0 | MS | 82.5 | HS | 61.3 | S |
| NCH-338 | 32.5 | MS | 76.6 | HS | 54.6 | S |
| CCH-1 | 62.5 | S | 78.3 | HS | 70.4 | S |
| KCS-2013 | 35.0 | MS | 95.0 | HS | 65.0 | S |
| PDG-1 | 54.0 | S | 50.0 | MS | 52.0 | S |
| SHSC-1111 | 18.5 | MR | 93.3 | HS | 55.9 | S |
| BC-28 | 35.0 | MS | 80.0 | HS | 55.0 | S |
| LCA-206 | 70.0 | S | 40.0 | MS | 65.0 | S |
| MS-12 | 22.5 | MR | 60.0 | S | 41.3 | MS |
| Tabasco | 0.0 | R | 2.0 | R | 1.0 | R |
| F-S-112 | 55.0 | S | 75.0 | S | 65.0 | S |
| *Japani Longi* | 47.0 | MS | 28.3 | MS | 37.7 | MS |
| LCA-301 | 31.5 | MS | 76.7 | S | 54.1 | S |
| Ajeet-3 | 56.7 | S | 80.0 | HS | 68.4 | S |
| PBC-535 | 0.0 | R | 9.0 | R | 4.5 | R |

R=Resistant; S=Susceptible; MS=Moderately susceptible; MR=Moderately resistant; HS=Highly susceptible

the chilli isolates collected from different regions of the country. Guntur district of Andhra Pradesh is a major chilli growing area of country and famous for its dry red chilli. The test isolates Gu-59 and Gu-98 showed significant variability in radial growth as well differential antagonism by *Trichoderma* sp. which clearly indicated the existence of variability in the isolates of *A. flavus* collected from the same region and climatic conditions.

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**Table 3.** Efficacy of *Trichoderma* species on different isolates of *A. flavus* collected from different regions of the country

| *A. flavus* Isolate | Location | Mean radial growth (mm) of *A. flavus* in dual culture | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 5 DAI[a] | | | | 10 DAI | | | |
| | | *T. viride* | *T. harzianum* | *T. koningii* | Control | *T. viride* | *T. harzianum* | *T. koningii* | Control |
| Me-1 | Meerut | 16.0 | 11.0 | 13.5 | 42.5 | 13.0 | 13.5 | 11.0 | 58.0 |
| Mu-1 | Muzaffar nagar | 12.5 | 16.5 | 17.5 | 50.0 | 12.0 | 15.5 | 16.5 | 58.0 |
| Ka-1 | Kanpur | 13.5 | 13.0 | 18.0 | 58.0 | 12.5 | 12.5 | 17.0 | 65.0 |
| Lu-1 | Lucknow | 15.0 | 13.0 | 13.0 | 62.0 | 12.5 | 12.5 | 19.0 | 88.0 |
| Si-1 | Sitapur | 12.0 | 12.5 | 11.5 | 66.0 | 11.5 | 11.0 | 15.0 | 73.0 |
| Va-1 | Varanasi | 16.5 | 12.0 | 12.0 | 45.0 | 15.0 | 12.0 | 12.0 | 55.0 |
| Gh-1 | Gaziabad | 14.5 | 12.5 | 12.5 | 30.0 | 12.0 | 12.0 | 11.5 | 52.0 |
| De-1 | Delhi | 13.0 | 12.5 | 11.5 | 35.0 | 12.0 | 14.0 | 11.0 | 55.0 |
| Fa-1 | Faridabad | 14.5 | 12.5 | 10.5 | 40.0 | 15.0 | 11.5 | 11.5 | 58.0 |
| Ro-1 | Rohtak | 12.0 | 10.5 | 11.5 | 40.0 | 12.5 | 11.0 | 10.5 | 51.0 |
| Gr-1 | Gurgaon | 14.5 | 16.5 | 18.0 | 60.0 | 14.0 | 15.0 | 17.5 | 70.0 |
| Ra-1 | Rajapura | 14.5 | 10.0 | 12.5 | 40.0 | 14.0 | 12.5 | 11.5 | 57.0 |
| Pa-1 | Patiala | 13.0 | 12.0 | 18.0 | 37.5 | 11.0 | 13.5 | 12.5 | 48.0 |
| Ch-2 | Chandigadh | 11.0 | 11.5 | 11.5 | 22.0 | 9.5 | 11.5 | 17.0 | 30.0 |
| Sa-1 | Sangroor | 11.5 | 13.0 | 13.0 | 54.0 | 11.0 | 12.5 | 14.0 | 70.0 |
| So-1 | Solan | 12.0 | 17.5 | 17.5 | 62.0 | 11.0 | 17.0 | 19,0 | 75.0 |
| Sr-2 | Srinagar | 11.0 | 17.5 | 15.0 | 30.0 | 12.0 | 17.0 | 12.5 | 35.0 |
| Bh-1 | Bhagalpur | 12.0 | 17.0 | 17.0 | 55.0 | 9.0 | 15.5 | 17.5 | 65.0 |
| Dh-2 | Dharwad | 8.5 | 11.5 | 16.5 | 36.0 | 8.5 | 11.0 | 11.5 | 42.0 |
| Gu-59 | Guntur | 10.5 | 13.0 | 13.0 | 54.0 | 9.5 | 12.5 | 12.0 | 72.0 |
| Gu-98 | Guntur | 12.5 | 16.5 | 15.0 | 42.0 | 13.0 | 16.0 | 14.0 | 54.0 |
| | Mean | 12.9 | 13.4 | 14.2 | 45.4 | 11.9 | 13.3 | 14.0 | 58.6 |
| | CD (P<0.05) | 2.43 | 1.96 | 3.21 | 4.63 | 1.88 | 2.74 | 2.36 | 4.79 |

[a]DAI=Days after inoculation

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