Research Paper :

Morphological and aflatoxigenic variation among the isolates of *Aspergillus flavus* isolated from spoiled sweet orange (*Citrus sinensis*)

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SUMMARY

A study was carried out on morphological and aflatoxigenic variability among 19 isolates of *Aspergillus flavus* isolated from spoiled sweet orange (*Citrus sinensis)* fruits. All the 19 isolates varied in colony characters, radial colony growth rate, sporulation, sclerotia formation, size, and number of sclerotia. Colonies of seven isolates were dark parrot green and fast growing with profuse sporulation, whereas eight isolates were dark olive green and slow growing with moderate sporulation in center. Other four isolates showed white fluffy colonies with yellow moderate sporulation and moderately growing. Out of these, six isolates were *non-sclerotia producing* where as 13 isolates were *sclerotia producing*. Among the sclerotia producers, three isolates produced large size (1097 to 1247mm) sclerotia but few in number (30 to 50 sclerotia/plate), seven isolates produced medium size (717 to 912mm) sclerotia and number of sclerotia varied from 53 to 110 /plate. Other three isolates produced sclerotia in very large number (150 to 270 sclerotia/plate) but smaller in size (407 to 709mm). Fast growing isolates showed 90 mm colony diameter, moderately growing isolates showed 42.6 to 77.3 mm colony diameter and slowing growing isolates showed 35.1 to 41.7 mm colony diameter after 72 hours of incubation at 28 °C in BOD. Among 19 isolates, only two isolates were non-aflatoxigenic and other 17 isolates were aflatoxigenic. Among aflatoxigenic isolates, three isolates were weakly aflatoxigenic (207.2 to 321.8 mg/liter), four isolates were moderately aflatoxigenic (334.5 to 531.7 mg/liter) and remaining ten isolates were highly aflatoxigenic (1107.3 to 1787.9 mg/liter). This preliminary information generated on aflatoxigenicity revealed that aflatoxin B_1 produced by these isolated in sweet orange juice ranged from 207.2 to 1787.9 µg/liter of sweet orange juice. Results of this study leads to two important conclusions; firstly sweet orange fruits served as a rich source of ascorbic acid which favours aflatoxin B_1 production by toxigenic *A. flavus* strains. Secondly the quantity of aflatoxin B_1 produced by these isolate is very high than the tolerance limit (5 ppb) fixed by World Health Organization, which is a great concern to the health of consumers especially in a country like India where toxicity by aflatoxin has already been recognized.

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Nutritional value of fruits chiefly depends on their quality and concentration of sugars, vitamins and other essential substances. The fungal pathogens, which are responsible for post-harvest fruits spoilage grow mainly at the expense of various nutrients present in fruits and reduce their nutritional and market values considerably. The fungi responsible for such post-harvest rots may originate within the enclosure of storage houses or they may be carried along with the packing materials like leaves, straw and baskets or may get associated with the surface of the fruits in the field (Meredith, 1961; Sullia and Khan, 1980 and Panduranjan and Suryanarayanan, 1985). According to Indian Horticulture Database, average post-harvest loss of lime and lemon in India ranges from 20–90 per cent (Indian Horticulture Database, 1997). *Aspergillus flavus* not only disfigures or causes rot to fruits but produces aflatoxin which are considered to be potent carcinogens and teratogens to humans and farm animals. Besides causing quantitative loss, *Aspergillus* infections may even increase the health hazards in human beings due to the production of many toxic secondary metabolites known as mycotoxins (Sharma and Sumbali, 1999; Singh and Sinha, 1983; Singh and Sumbali, 2000) whose ingestion by human beings may lead to several disorders (IARC, 1993).

Bamba (2002) isolated five species of *Aspergillus viz., A. niger*, *A. flavus*, *A. nidulans*, *A. terreus* and *A. sulphureous*. Among the various species of *Aspergillus*, those belonging to section Flavi have often

attracted global attention as they are the producers of the important metabolites, aflatoxins and cyclopiazonic acid (CPA) in many of the durables (Rao and Hussain, 1987; Trucksess *et al.*, 1987; Lee and Hagler, 1991; Urano *et al.*, 1992; and Miller, 1995). However, little information is available with respect to contamination of perishables with *A. flavus* and aflatoxins (Singh and Sinha, 1982; Madhukar and Reddy, 1992; Agarwal and Roy 1994; Doster *et al.*, 1996). Aflatoxins, which are a group of highly toxic, mutagenic and carcinogenic polyketide compounds, from rotten fruits could cause a severe health hazard to consumers. Considering the neglected field, greater variability in earlier reports, huge losses, dietary importance and association of *A. flavus* with fruits causing heavy deterioration and aflatoxin B_1 production, the present investigation has been under taken to throw more light on the potential of *A. flavus* to produce aflatoxin B_1 during post harvest pathogenesis in artificially inoculated fruits and in plain juice of sweet orange.

MATERIALS AND METHODS

Collection of samples:

Spoiled sweet orange fruits were collected from various wholesale and retail fruit markets of Udgir city. These samples were brought to the laboratory in pre-sterilized separate polyethylene bags.

Isolation of *A. flavus:*

Nineteen *A. flavus* isolates were isolated from infected fruits within 6 hours of their collection. Small bits of infected fruits were removed carefully and surface sterilized with 0.1% $HgCl_2$ and transferred to Petri dishes containing Potato dextrose agar (PDA) medium supplemented with Streptomycin to prevent bacterial growth. These plates were incubated at 28 °C in BOD for three days. Pure culture of an *A. flavus* was obtained by sub-culture and this pure culture was maintained in culture tube slant at 4^0 C for further studies.

Morphological variation:

The morphological and growth characteristics of all the isolates were studied on Potato dextrose agar medium. After solidification, the agar medium in 90 mm culture plates was seeded with 5 mm culture discs of three days old culture of each isolate in the centre of Petriplates. The plates were incubated at 28^0 C in BOD. After three days of incubation the colony colour, radial diameter and sporulation were recorded. Whereas, the observations on size and number of sclerotia were recoded after ten days of incubation. Five replications were maintained for each isolate.

Aflatoxigenic variation:

Preparation of spore suspension of *A. flavus:*

Spore suspension of 19 isolates of *A. flavus* was prepared by flooding seven days old and fully sporulated cultures grown on PDA slant with 10 ml of sterile distilled water containing 0.1% Tween 20. Spores were rubbed from the surface of PDA slant with a glass rod and filtered through two layers of sterilized muslin cloth. Number of spores were counted with a haemocytometer and adjusted with sterile distilled water to get 10^7 spores per milliliter. All the processes were carried out in aseptic condition.

Inoculation of sweet orange fruits:

Mature and healthy sweet orange fruits were initially sterilized by dipping in 0.1% $HgCl_2$ for 2 minutes, rinsed thrice with sterilized water and then dried under sterilized conditions. Thereafter, a single wound of 4 mm wide and 10 mm deep was made on the fruit with the help of a sterilized cork borer and 100 µl of *A. flavus* spore suspension was injected into the wound through a sterilized micropipette. Three replications were maintained for each isolate of *A. flavus*. Inoculated fruits were incubated in sterilized glass chambers maintained at 28^0 C and 70% relative humidity. Aflatoxin produced by *A. flavus* in these fruits was extracted after 12 days of incubation.

Extraction and estimation of aflatoxin from sweet orange fruits:

Extraction of aflatoxin from *A. flavus* infected sweet orange fruit tissue was done by the indirect competitive ELISA method described by Morgan *et al.* (1986). Extraction of each sample was done by grinding 50 g of *A. flavus* infected fruit pulp with 100 ml of 70 % methanol for five minutes. The homogenate was shaken well for 30 minutes on a mechanical shaker at 300 rpm and then filtered through Whatman filter paper 41. Filtrate was filled in 10 ml sample bottles and kept at 4^0 C for estimation of aflatoxin B_1. 10 µl of sample extract was used for estimation of aflatoxin B_1. Readings of ELISA plate was taken from the ELISA reader at 405 nm. Using the absorbance values for the aflatoxin B_1 and following equation calculated the aflatoxin B_1 content in the sample.

$$\text{Aflatoxin } B_1 \ (\mu g/kg) = \frac{A \times D \times E}{G}$$

where, A= AFB_1 in diluted sample extract, D= Dilution factor, C= Times concentration after cleanup, E= Volume of extraction solvent (ml) and G= sample weight (g).

Estimation of aflatoxin from plain juice of sweet orange:

Potential of *A. flavus* isolates to produce aflatoxin B_1 in plain juice of sweet orange was studied. 100ml plain juice of sweet orange was taken in 250ml capacity conical flasks, autoclaved at 121^0 C for 30 minutes. After cooling, the flasks were inoculated with 1 ml of spores suspension (10^7/ml) of A. *flavus*. For each isolate, three replications were maintained. These flasks were incubated at 28^0C and 70% relative humidity in BOD. After 15 days of incubation, the fresh mycelium of *A. flavus* was removed and cell free filtrate (sweet orange juice) was used for estimation of aflatoxin B_1 by the indirect competitive ELISA method described by Morgan *et al.* (1986).

Chemicals and reagents for indirect competitive ELISA:

Aflatioxin B_1 standard, bovine serum albumin (BSA), alkaline phosphates, p-nitrophenyl phosphate disodium, Tween –20 and glutaraldehyde were procured from Sigma Chemical Co., St. Louis, MO, USA. Polystyrene microtitre plates were purchased from Dynatech Lab, Virginia, USA. Other solvents and chemicals used in were of the highest analytical grade.

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been presented under following heads:

Morphological variation:

The isolates varied for their colony colour, growth rate, sporulation, number and size of sclerotia. Colonies of seven isolates were dark parrot green and fast growing with profuse sporulation, whereas eight isolates were dark olive green and slow growing with moderate sporulation in centre. Other four isolates showed white fluffy colonies with yellow moderate sporulation and moderately growing. Out of these, six isolates were non-sclerotia producing where as 13 isolates were sclerotia producing. Among the sclerotia producers, three isolates produced large size (1097 to 1247 mm) sclerotia but few in number (30 to 50 sclerotia/plate), seven isolates produced medium size (717 to 912 mm) sclerotia and number of sclerotia varied from 53 to 110 /plate. Other three isolates produced sclerotia in very large number (150 to 270 sclerotia/ plate) but smaller in size (407 to 709 mm). Fast growing isolates showed 90 mm colony diameter, moderately growing isolates showed 42.6 to 77.3 mm colony diameter and slowing growing isolates showed 35.1 to 41.7 mm colony diameter (Table 1).

Table 1: Morphological and aflatoxigenic variation in different isolates of *Aspergillus flavus*

Isolates	Colony diameter (mm)*	Colony colour	Sporulation	No. of sclerotia*	Size of sclerotia (µm)*
AF-1	90.0	Dark parrot green and fast growing	Profuse	163	535
AF-2	40.3	White fluffy mycelium and moderately growing	Moderate	194	721
AF-3	35.1	Dark olive green and slow growing	Scanty	53	912
AF-4	90.0	Dark parrot green and fast growing	Profuse	47	1097
AF-5	72.7	White fluffy mycelium and moderately growing	Moderate	270	407
AF-6	90.0	Dark parrot green and fast growing	Profuse	210	695
AF-7	38.7	Dark olive green and slow growing	Scanty	92	752
AF-8	77.3	White fluffy mycelium and moderately growing	Moderate	201	521
AF-9	36.9	Dark olive green and slow growing	Scanty	61	901
AF-10	90.0	Dark parrot green and fast growing	Profuse	31	1247
AF-11	38.1	Dark olive green and slow growing	Scanty	59	874
AF-12	42.6	White fluffy mycelium and moderately growing	Moderate	150	709
AF-13	90.0	Dark parrot green and fast growing	Profuse	187	514
AF-14	41.7	Dark olive green and slow growing	Scanty	110	796
AF-15	90.0	Dark parrot green and fast growing	Profuse	44	1142
AF-16	59.3	White fluffy mycelium and moderately growing	Moderate	54	814
AF-17	39.1	Dark olive green and slow growing	Scanty	71	717
AF-18	73.2	White fluffy mycelium and moderately growing	Moderate	63	789
AF-19	90.0	Dark parrot green and fast growing	Profuse	193	469

*Mean of five replications

Estimation of aflatoxin from sweet orange fruits:

In the present study, 19 isolates of *A. flavus* recovered from spoiled sweet orange fruit, were tested for aflatoxin B_1 production potential during post-harvest pathogenesis. It was found that out of 19 isolates of *A. flavus* tested, 17 isolates were found to be aflatoxigenic whereas, others two isolates (AF-4 and AF-16) were non-toxigenic. Lowest amount of aflatoxin B_1 (207.2 µg/kg) was produced by isolate No. AF-2 whereas, highest amount of aflatoxin B_1 (1787.9 µg/kg) was produced by isolate No. AF-19 (Fig.1).

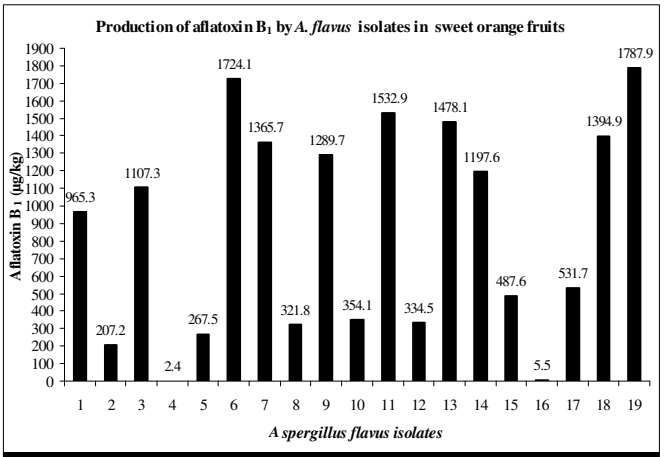

Fig. 1 : Production of aflatoxin B_1 in artificially inoculated sweet orange fruits by 19 different isolates of *A. flavus*

Estimation of aflatoxin from plain juice of sweet orange:

Similar observations were obtained for aflatoxigenic potential of 19 isolates of *A. flavus* in plain juice of sweet orange. The highest yield of aflatoxin B_1 (1577.3µg/liter) in sweet orange juice was produced by isolate No. AF-19, while the lowest amount of aflatoxin B_1 (301.5 µg/liter) was yielded by isolate No. AF-2. The two isolates (AF-4 and AF-16) were found to be non-toxigenic even in plain sweet orange juice (Fig.2).

High level of aflatoxin in this citrus fruit may be attributed to the rich ascorbic acid (vitamin C) content, which is known to be good source for aflatoxin production by the toxigenic *A. flavus* strains. Similar results have been reported by (Singh and Sinha 1982a; Singh and Sinha, 1982b) in orange (mandarin) and sweet orange (musambi) infected with *A. flavus* and *A. parasiticus*. Pandey *et al.* (1988), reported aflatoxin B_1 production by *A. flavus* isolates obtained from dry fruits and vegetables. Buchanan *et al.* (1975) and Barkai Golan (1980) have also reported some *A. flavus* isolates to be non-toxigenic and the percentage of such isolates varies depending upon the

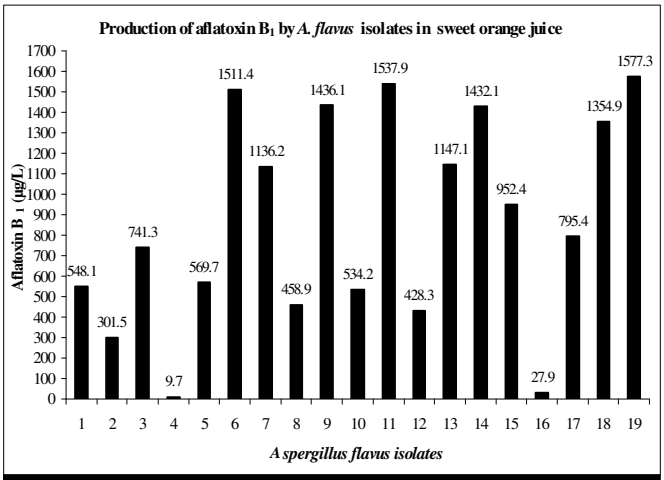

Fig. 2 : Production of aflatoxin B_1 by *A. flavus* isolates in artificially inoculated sweet orange juice

substrate. Since aflatoxin B_1 is the most toxic member of the group, its tolerance limit fixed by World Health Organization (1979) is 20 ppb. Besides, 66 other countries have also introduced legislation on the maximum permissible levels of aflatoxin ranging from zero to 50 ppb in different food commodities for human consumption Sinha (1995).

Aflatoxins produce four distinct effects: acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects. Ingestion of aflatoxins has been suggested as one of the causes for the Indian childhood cirrhosis (Bhat, 1989). Amla *et al.* (1971) detected aflatoxin B_1 in the urine samples of cirrhotic children and the affected children showed liver lesions and skeletal muscle changes who had accidentally consumed aflatoxin-contaminated groundnut flour. Gopal *et al.* (1969); Kishan Rao (1980) and Choudary and Rao (1982) have reported outbreaks of aflatoxicosis in India. Aflatoxins are both acutely and chronically toxic to animals, including man.

In view of hepatotoxic, carcinogenic and other toxic properties of aflatoxin B_1, its detection in *A. flavus* infected sweet orange may create a health risk to such domestic consumers who may consume such infected fruits. There is also another risk that such contaminated sweet orange may be included into processed products through faulty sorting procedures and thus may constitute another potential hazard to human health. Therefore, there is a need for a concerted effort to ensure better post-harvest technology, especially in a developing country like India where acute toxicity by aflatoxin has already been documented (Singh and Sumbali, 2000).

Conclusion:

Results of this study leads to the following

conclusions :

– *A. flavus* was found to be associated with post-harvest spoilage of sweet orange fruits.

– Considerable aflatoxin B_1 was produced in *A. flavus* infected sweet orange fruits during pathogenesis and in plain sweet orange juice.

– Awareness should be created among the consumers about fruit spoilage by toxigenic fungi and aflatoxins.

– More research and training is needed to improve fruit quality and minimize health risk of consumers.

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