Journal of Biosphere, 1: 4-6, 2012 ISSN 2278 – 3342

Penicillium toxins associated with post-harvest fruit rot of crab apples (*Docynia indica* Dcne)

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Abstract: Two *Penicillium* toxins viz., patulin and citrinin were found to be associated with *P. expansum* rotted crab apples cv. Scarlet Siberian. Both these toxins were detected in very high amounts. Patulin was detected up to 36 mg/kg whereas; citrinin level was comparatively lesser (up to 6 mg/kg of the fruit). These amounts are very high in comparison to WHO recommended tolerance level. This research work, therefore, suggested that proper storage and sorting of crab apples before marketing is very necessary, so that *P. expansum* rotted fruits are not consumed.

Key words: Citrinin, crab apple, fruit, mycotoxin, patulin, post-harvest.

1. Introduction

Crab apple cv. Scarlet Siberian (*Docynia indica* Dcne) is an important fresh rosaceous fruit grown in the temperate areas of Jammu and Kashmir. It is juicy, astringent, nutritionally rich in sugars, organic acids, vitamins, Phosphorous, iron and is valued mainly as a dessert fruits. Before harvesting, they are attacked by a few fungi (Chohan and Kaur, 1976) but after harvesting their susceptibility to fungal pathogens increases (Sumbali and Badyal, 1990). During a survey conducted at Jammu fruit market, crab apples were found to suffer extensive post-harvest blue mold rot caused by *Penicillium expansum*. In view of this, an attempt was made to assess the natural occurrence of *Penicillium* toxins associated with this fruit during pathogenesis.

2. Materials and Methods

2.1 Isolation of P. expansum isolates from rotted crab apples

Crab apple suffering from blue mold rot were collected in sterilized begs at regular intervals from wholesale and retailer. Isolation was made within 24 hours of their collection by streaking the spore on sterilized potato dextrose ager medium.

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2.2 Preparation of spore suspension

Spore suspension of *P. expansum* isolates was prepared by flooding 4 days old culture grown on slant with 10 ml of sterile distilled water containing 0.1% Tween 20. Spore were rubbed from surface of the slant with a glass rod, passed through two layers of sterilized cheesecloth, counted with a haemocytometer and adjusted with sterile distilled water to obtain 10^5 spore per milliliter.

2.3 Inoculation of fruits

Mature and healthy crab apple fruits were initially sterilized by dipping in 95 % ethanol for 30 min., rinsed with sterilized water and then dried under sterilized conditions. Therefore, a single wound 4mm wide and 10mm deep was made in the fruit with the help of a sterilized cork borer and 50 ul of spore suspension was inserted into the wound through a sterilized micropipette. Inoculated fruits were incubated at 28.0 ± 2.0 ºC and 100 per cent relative humidity for 15 days.

2.4 Extraction and estimation of patulin and citrinin from diseased fruit

Natural production of patulin and citrinin toxin was estimated at the end of incubation period by taking 30 g of fruit pulps adjacent to the inoculated region and extracting it by the method of Gimeno and Martins (1983). Chloroform extract thus obtained were collected and evaporated to 0.5 ml. This concentrated extract was dissolved to make total volume of 1ml for thin layer chromatography. Quantitative separation of these toxins performed on activated TLC plates using tolune: ethylacetate:chloroform (80:70:50) and 1 ml of 90 % formic acid. For detection of patulin, developed plates were sprayed with freshly prepared phenylhydrazine

hydrochloride (by dissolving 2 gm in 100 ml of H_2O) and the heated for 5 minutes at 110 ºC (Subramanian, 1982). Under visible light, patulin showed yellow spots. For the detection of citrinin, developed plates were directly visualized under 365nm UV light and they showed yellow fluorescent spots. Standard patulin and citrinin samples were also spotted on the TLC plates as reference spots. Further chemical confirmation of citrinin was done by spraying aluminium chloride (dissolved 2gm reagent grade $AlCl_3$.$6H_2O$ in 100ml of ethanol) on the developed plate. Heated it for 5 minutes at 110 ºC, cooled and observed under 365nm UV light. This chemical test changed the yellow fluorescent of citrinin to sky blue fluorescent (Gimeno and Martins, 1983).

For quantitative estimation of patulin and citrinin, spectophotometric method of Bacha et al. (1988) was followed. For patulin estimation, silica gel of each yellow spot was scraped in a clean dry centrifuge tube, dissolved in 5 ml of n-butanol and centrifuged at 3000 rpm for 3 min. Citrinin estimation was done by marking yellow fluorescent spots under long wave UV light (365 nm) scraped them individually in clean, dry centrifuge tubes, dissolved them in 5ml of cold methnol and centrifuged at 3000rpm for 3 minutes. Colour intensity of supernant was immediately determined in a spectrophotometer at 540nm for patulin (Subramanian, 1982) and at 332 nm for citrinin (Bacha et al., 1988).

Table 1. Production of patulin and citrinin in *P. expansum* infected crab apples

Isolates screened	Total no. of isolates screened	= 15.0
	% of toxigenic isolates	= 86.7
	% of patulin producer	= 33.3
	% of citrinin producer	= 80.0
	% of both patulin and citrinin producer	= 26.7
	Patulin toxin	Citrinin toxin
PE-C1	-	6.04 ± 7.1
PE-C2	29.50±6.5*	0.94 ± 2.0
PE-C3	-	0.26 ± 1.7
PE-C4	-	0.09 ± 2.4
PE-C5	-	0.72 ± 1.8
PE.C6	-	0.15 ± 3.4
PE-C7	-	4.21 ± 2.4
PE-C8	-	0.39 ± 1.8
PE-C9	-	-
PE-C10	28.00±3.5	1.15 ± 6.4
PE-C11	33.50±7.5	-
PE-C12	-	-
PE-C13	36.00±6.2	0.14 ± 2.4
PE-C14	-	0.18 ± 1.4
PE-C15	33.50±8.5	2.00 ± 1.6

*Each value represents the mean ± SD, n = 3, and -, not detected.

3. Results and Discussion

Results depicted in table 1 show that 86.7 % of the recovered isolates were found to be toxigenic producing either patulin or citrinin or both. Among these, positive 33.3 % were found to be patulin producers (28 to 36 mg/kg) whereas, 80.0 % of the tested *P. expansum* isolates were found to be positive for citrinin production (0.09 to 6.04 mg/kg). Similar results were obtained by Brian et al. (1956). The production of patulin in fruits may be attributed to the sugar, organic acid, fruit species and variety (Menniti et al., 2010).

From the result it was also observed that 26.7 per cent isolates of *P. expansum* could produce both patulin and citrinin and their levels of production were very high. Similarly, level of patulin and citrinin contamination from *P. expansum* infected apples (*Malus pumila* Mill.) has been reported to be as high as 1 and 3 mg/kg, respectively, from elsewhere (Gimeno, 1979; Gimeno and Martins, 1993; Ritieni, 2003). Although there is currently no evidence to prove that patulin and citrinin have the potential to produce adverse human health effects (Hopkins, 1993; Machinosky and Midio, 1995), yet the finding in animals that these mycotoxins are carcinogenic, teratogenic and nephrotoxic (Krough et al., 1970; Dickens and Jones, 1961; Mayer and Legator, 1969), emphasise the need for concern. World health Organisation(WHO) has recommended a maximum patulin level of 50 ug/kg. Various European and other countries have also recommended maximum patulin levels of 30-50 ug/kg (Van Egmond, 1995 and Italian Ministry of Health, 1999). But so far no recommendation for the tolerance level of citrinin has been made. Since patulin is a stable mycotoxin (Poland and Allen, 1970; Harrison, 1989), and its co-occurrence with citrinin in the infected crab apples and the products made from crabapples rotted by *P. expansum*, which may present a health hazard.

This evaluation, therefore, shows that proper storage and sorting of crab apples is very necessary before marketing so that intake of these mycotoxins above the tolerance limit is avoided. This will essentially make a difference for children and vegetarians who consume a higher quantity of fruits.

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