Patulin and citrinin production in apple and pear fruits by *Penicillium expansum* strains collected from Jammu markets

YASHPAUL SINGH and GEETA SUMBALI*
Department of Botany, University of Jammu, Jammu 180 006

**ABSTRACT:** An investigation was conducted to elucidate differential toxigenic behaviour of *Penicillium expansum* strains towards various cultivar varieties of pome fruits viz., apple and pear. One hundred and six isolates of *P. expansum* strains (77 from infected apples and 29 from infected pears) were isolated and screened against respective host cultivars. Thin layer chromatographic analysis showed that 58.4 percent of the *P. expansum* strains obtained from diseased apples were positive for both citrinin and patulin production, whereas 15.6 percent produced only citrinin, 22.1 percent produced patulin alone and 3.9 percent were atoxigenic. These toxigenic strains produced variable amount of patulin (14.75-40.08 mg/kg) and citrinin (0.10-8.04 mg/kg) in the infected fruits of various apple cultivars. In contrast, pears were found to be infected with only 24 percent toxigenic *P. expansum* strains that were positive for both citrinin and patulin production, whereas 37.9 percent were positive for only citrinin, 24.1 percent produced only patulin and 13.8 percent were atoxigenic. The level of patulin and citrinin produced by the toxigenic strains in pear cultivars ranged from 11.75-67.50 mg/kg and 0.06-7.02 mg/kg respectively. HPLC analysis of patulin from healthy area surrounding the *P. expansum* rotten area in apples was performed and it was noted that this mycotoxin could migrate to the surrounding non-rotted area in large amount (0.65 to 22.75 mg/kg). These high values show that even the surrounding non-rotted areas of apples heavily infected with blue mold are not fit for consumption.

**Key words:** *Penicillium expansum*, patulin, citrinin, apple, pear

Pome fruits being succulent and rich in nutrients are susceptible to attack by a variety of microorganisms during the various phases of marketing. Both fungi and bacteria are responsible for post-harvest loss of these fruits but losses caused by fungi are more substantial as acidic conditions inhibit bacterial growth (Burchill and Maude, 1986). Besides causing quantitative loss, fungal infections may even increase the health hazard in human beings due to the production of many toxic secondary metabolites known as mycotoxins (Verma *et al*., 1981; Singh and Sinha, 1982).

Among the various post-harvest fungal pathogens of pome fruits, *Penicillium expansum* is the major one that has been reported from almost every Indian fruit market and is known to contribute substantially to the overall losses in stored pomaceous fruits (Kaul and Munjal, 1981; Singh and Sumbali, 2002). This pathogen is reported to produce two mycotoxins of importance, patulin and citrinin (Brian *et al*., 1956; Harwig *et al*., 1973). Among the two, patulin is known to exhibit mutagenic, neurotoxic, gastrointestinal effects and immunosuppressive action in laboratory animals (Stoll and Bullerman, 1975; Mckinley *et al*., 1982) and the most important target organs are liver, spleen, kidney, skin, nervous system and reproductive system. Toxicity of patulin to human beings is unknown; nonetheless several countries have set limits for the patulin content in foods at 50 micrograms/kg or 50 micrograms/L (Van Egmond, 1989; FAO, 1997). According to the report of the Codex Committee on Food Additives and Contaminants, there is an urgent need for a proper sorting and storage of fruits to avoid risk of patulin...
contamination in them as well as in their products (CAC, 1997). Studies conducted in different countries reveal moderate to high patulin incidence in pomaceous and a few other types of fresh fruits (Sylos and Rodriguez-Amaya, 1999; Rupp and Turnipseed, 2000; Martins et al., 2002).

On the other hand, citrinin causes nephrotoxicoses and was first observed in Japan where rice was contaminated with *Penicillium citrinum* (Saito et al., 1971). Toxicity of citrinin is reported to increase in combination with other toxins (Frisvad and Filtenberg, 1983). Many investigators have found cereals and their products contaminated with citrinin (Bilgrami and Jeswal, 1992; Vrabcheva et al., 2000) but few have reported citrinin contamination in fresh fruits (Harwig et al., 1973; Gimeno and Martins, 1983; Martins et al., 2002).

In India, production of pome fruits has increased substantially in recent years with the Northern state of Jammu and Kashmir contributing over 62 percent of India’s apple production (Indian Horticulture Database, 1997). Since *Penicillium expansum* was commonly found to cause post-harvest rot of pome fruits (Singh and Sumbali, 2002), investigations were undertaken to elucidate differential toxigenic behaviour of *P. expansum* strains towards various cultivar varieties of apple and pear. This paper reports comprehensive data on the subject.

**MATERIALS AND METHODS**

**Collection of samples**

*Penicillium expansum* infected fruits belonging to five cultivar varieties of apple (Red Delicious, White Dotted Red, American Apirouge, Ambri and Golden Delicious) and two cultivar varieties of pear (William’s and Chinese Sandy) were collected from wholesale and retail outlets of Jammu markets and brought to the laboratory in pre-sterilized polyethylene bags.

**Isolation, purification and maintenance of *P. expansum* isolates**

Isolations from the *P. expansum* infected apples and pears were made within 24 hours of their collection by single spore technique on potato dextrose agar (PDA) medium supplemented with streptomycin sulphate (0.06 g/l) as a bacteriostat. Total of 77 strains of *P. expansum* were isolated from infected apples (20 from cv. Red Delicious, 26 from cv. American Apirouge, 22 from cv. White Dotted Red, 5 from cv. Ambri, and 4 from cv. Golden Delicious). Similarly, 29 strains of *P. expansum* were isolated from rotten pears (15 from cv. William’s and 14 strains from cv. Chinese Sandy). All the *P. expansum* strains were purified by streaking and their cultures were maintained as recommended by International Mycological Institute, UK. (Smith and Onions, 1983).

**Preparation of spore suspension**

Spore suspension of each *P. expansum* strain was prepared from 5 days old culture grown on PDA slant by following Kampp (1994).

**Inoculation of apple and pear fruit cultivars**

Mature and healthy fruits belonging to different cultivar varieties were initially sterilized by dipping in 95% ethanol for 15 minutes rinsed with sterilized water and then dried under sterilized conditions. Thereafter, a single wound (4 mm wide and 10 mm deep) was made in each fruit with the help of a sterilized cork borer (Ippolito et al., 2000). 50 µl of the prepared spore suspension (10⁵ spores per milliliter) of each *P. expansum* strain (isolated from a particular cultivar variety) was inserted by a sterilized micropipette into the wound made in the same variety. Inoculated fruits were incubated at 28°C and 100 per cent relative humidity for 15 days. Three replicates were maintained for each cultivar variety and *P. expansum* strain.

**Extraction of patulin and citrinin**

Extraction of patulin and citrinin from selected varieties of apple and pear inoculated with different strains of *P. expansum* was performed as per the method developed by Gimeno and Martins (1983).

**Qualitative estimation of patulin and citrinin**

For qualitative estimation of patulin and citrinin, 50 µl of the sample extracts were applied on activated silica gel TLC plates using a micropipette and developed in a solvent system consisting of toluene : ethylacetate : chloroform : 90% formic acid (80 : 70 : 50 : 1 v/v). For detection of patulin,
method given by Subramanian (1999) was followed. Under visible light, patulin showed yellow spots. For the detection of citrinin, developed plates were directly visualized under 365 nm UV light and they appeared as yellow fluorescent spots. Standard patulin and citrinin samples were also spotted on the TLC plates as reference spots.

**Quantitative estimation of patulin and citrinin**

Quantitative estimation of patulin and citrinin was done by spectrophotometric method as given by Bacha et al. (1988).

High performance liquid chromatography (HPLC) (Torres et al., 1987) was used to assess the migration of patulin in apples (cv. Red Delicious) from rotted areas into surrounding non-rotted areas. The analytical equipment for HPLC consisted of a liquid chromatography pump 305 model, Gilson (France), an injection system Rheodyne 7125 (USA) with a 50 µl sample loop and a variable wavelength absorbance UV detector 115 Gilson (France) set at 254 nm. The analytical column was a lichrocrat 250 (30 cm by 3.9 mm), filled with Lichrosphere 100 RP-18 material, 5 µm particle size (Merck). The mobile phase consisted of methanol : water (30 : 70 v/v) and was used at a flow rate of 1 ml/min. Injection volume for extract solution was 2 µl. Analysis was performed at room temperature (20°C) and data was recorded in Winchrom chromatographic software (Nucon Engineers, India). Quantification of patulin was done by comparison of the retention time and peak areas observed in the samples with those observed for patulin standard.

All reagents (analytical grade) were obtained from Merck. Standards of patulin and citrinin were purchased from Sigma Aldrich company.

**RESULTS AND DISCUSSION**

Results summarized in table 1 show that among the various pome cultivars investigated, majority of the patulin producing *P. expansum* strains (more than 90 percent) were detected from rotten fruits belonging to cv. American Apirouge and cv. White Dotted Red. Some of these toxigenic strains produced very high amount of patulin (upto 40.08 mg/kg). Next in decreasing order was cv. Golden Delicious whose fruits were infected with 75 percent patulin positive *P. expansum* strains, whereas, cvs. Red Delicious and Ambri were observed to be infected with 60 percent patulin positive strains.

In case of *P. expansum* infected pear fruits, it was observed that cv. William's elaborated more toxigenic strains (66.8%) than the cultivar Chinese

<table>
<thead>
<tr>
<th>Fruit/Cultivar</th>
<th>Strains positive for patulin only/n*</th>
<th>%</th>
<th>Strains positive for citrinin only/n*</th>
<th>%</th>
<th>Strains positive for both patulin + citrinin/n*</th>
<th>%</th>
<th>Strains negative for both toxins/n*</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apple</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. Red Delicious</td>
<td>3/20</td>
<td>15.0</td>
<td>7/20</td>
<td>35.0</td>
<td>9/20</td>
<td>45.0</td>
<td>1/20</td>
<td>5.0</td>
</tr>
<tr>
<td>cv. American Apirouge</td>
<td>6/26</td>
<td>23.0</td>
<td>2/26</td>
<td>7.7</td>
<td>18/26</td>
<td>69.2</td>
<td>0/26</td>
<td>0.0</td>
</tr>
<tr>
<td>cv. White Dotted Red</td>
<td>5/22</td>
<td>22.7</td>
<td>1/22</td>
<td>4.5</td>
<td>15/22</td>
<td>68.2</td>
<td>1/22</td>
<td>4.5</td>
</tr>
<tr>
<td>cv. Ambri</td>
<td>1/5</td>
<td>20.0</td>
<td>1/5</td>
<td>20.0</td>
<td>2/5</td>
<td>40.0</td>
<td>1/5</td>
<td>20.0</td>
</tr>
<tr>
<td>cv. Golden Delicious</td>
<td>2/4</td>
<td>50.0</td>
<td>1/4</td>
<td>25.0</td>
<td>1/4</td>
<td>25.0</td>
<td>0/5</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17/77</td>
<td>22.1</td>
<td>12/77</td>
<td>15.6</td>
<td>45/77</td>
<td>58.4</td>
<td>3/7</td>
<td>3.9</td>
</tr>
<tr>
<td><strong>Pear</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. William's</td>
<td>5/15</td>
<td>33.3</td>
<td>3/15</td>
<td>20.0</td>
<td>5/15</td>
<td>33.3</td>
<td>2/15</td>
<td>13.3</td>
</tr>
<tr>
<td>cv. Chinese Sandy</td>
<td>2/14</td>
<td>14.2</td>
<td>8/14</td>
<td>57.1</td>
<td>2/14</td>
<td>14.2</td>
<td>2/14</td>
<td>14.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>7/29</td>
<td>24.1</td>
<td>11/29</td>
<td>37.9</td>
<td>7/29</td>
<td>24.1</td>
<td>4/29</td>
<td>13.8</td>
</tr>
</tbody>
</table>

*n = number of strains screened*
Sandy pear (28.5%). Patulin amount in these cultivars varied in the range of 11.75 to 67.50 mg/kg (Table 2). These results are comparable with the data reported by other workers from contaminated apples (Mortimer et al., 1985; Martins et al., 2002), pears (Gimeno and Martins, 1983) and other fruits (Mortimer et al., 1985; Larsen et al., 1998).

It is of concern that most of the P. expansum strains positive for patulin were in the production range of 21-30 mg/kg, whereas, at least 20 other positive strains produced patulin in amounts that even exceeded 31 mg/kg of infected pome fruit (Table 2). Previously, levels of patulin as high as 1 g/kg rotten fruit have been observed elsewhere (Gimeno, 1979). There is currently no evidence to prove that patulin has the potential to produce adverse human health effects (Hopkins, 1993; Machinsky and Midio, 1995). But World Health Organization (1979) and even Food and Agriculture Organisation (1997) have recommended a maximum patulin level of 50 µg/kg or 50 µg/l. Various European and other countries have also recommended maximum patulin levels of 30-50 µg/kg (Van Egmond, 1995; Italian Ministry of Health, 1999). Since patulin is a heat stable mycotoxin (Pohland and Allen, 1970; Harrison, 1989), the products made from apples rotted by P. expansum may also contain patulin and may present a health hazard (Watkins et al., 1990). This awareness had repercussions on the limits for patulin in apple products in Italy where the usual limit of 50 µg/kg or 50 µg/l was reduced to 25 µg/kg or 25 µg/l for baby foods (AIIPA, 1996). Later, it was shown that patulin was significantly reduced in apple juice when clarification procedures were employed but nevertheless, clarification resulted in high patulin levels in the pressed pulp and this could be harmful if they were used as animal feeds (Bissessur et al., 2001).

In the present investigation, patulin was not always detected in P. expansum rotted apples and pears, thereby showing that patulin is not involved in the pathogenesis of pome fruits. Our results agree with those obtained by Brian et al. (1956) who worked on P. expansum infected apple fruits.

Comparative analysis of patulin from the rotted and surrounding non-rotted area was performed by HPLC to evaluate if this mycotoxin could migrate to the non-rotted area. As depicted in table 3, all the P. expansum infected apples showed the presence of patulin in the surrounding non-rotted area. Patulin value in the surrounding non-rotted area ranged between 0.65 to 22.75 mg/kg. These high values show that even if rotten areas of P. expansum infected apples are removed, the seemingly unaffected areas are also not fit for consumption or for preparation of products as the levels of patulin detected, far exceed the safe limits established by the international committees. Similar observations have been recorded earlier by Beretta et al. (2000) who found nearly 81% of the samples taken from unaffected areas to be patulin positive.

While investigating the toxins produced by P. expansum isolates during pathogenesis of pome fruits, citrinin was detected to co-occur frequently with patulin. It was further observed that 58.4 percent of the P. expansum strains pathogenic on apples were positive for both citrinin and patulin production, whereas 15.6 percent were positive for only citrinin, 22.1 percent were positive for only patulin and 3.9 percent were atoxigenic (Table 1). Among the various apples cultivars investigated (Table 2), maximum citrinin producing P. expansum strains (80%) were isolated from diseased cv. Red Delicious fruits followed by cv. American Apirouge (76.9%), cv. White Dotted Red (72.7%), cv. Ambri (60.0%) and cv. Golden Delicious (50.0%). As depicted in table 2, these toxigenic strains produced variable amount of citrinin ranging from 0.10 to 8.04 mg/kg of apple.

In comparison to apple fruits, pears were found to be infected with only 24.1 percent of P. expansum strains that were positive for both citrinin and patulin production, whereas 37.9 percent were positive for only citrinin, 24.1 percent were positive for only patulin and 13.8 percent were atoxigenic (Table 1). Among the pear cultivars investigated, cv. Chinese Sandy was noted to be infected with more toxigenic strains (71.4%) than cv. William’s (53.3%) and the amount of citrinin produced by these toxigenic strains ranged from 0.06 to 7.02 mg/kg of the fruit (Table 2).

Investigations also revealed that in all the tested pome fruit cultivars, pathogenic P. expansum strains were able to produce quantitatively more patulin (ranging from 11.75 to 67.50 mg/kg) than citrinin (ranging from 0.06 to 8.04 mg/kg). Similar observations have been recorded earlier by Gimeno
Table 2. Patulin and citrinin concentration in the rotted areas of *Penicillium expansum* inoculated apples and pears

<table>
<thead>
<tr>
<th>Fruit/Cultivar</th>
<th>Concentration of patulin detected (mg/kg)</th>
<th>Number (%) of <em>P. expansum</em> strains showing patulin production (mg/kg) range of</th>
<th>Concentration of citrinin detected (mg/kg)</th>
<th>Number (%) of <em>P. expansum</em> strains showing citrinin production (mg/kg) range of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt; 11</td>
<td>11-20</td>
<td>21-30</td>
</tr>
<tr>
<td>Apple cv. Red Delicious</td>
<td>14.66-29.72</td>
<td>-</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(21.93±4.17)**</td>
<td>(4.17)</td>
<td>(58.3)</td>
<td>(1.48±2.05)**</td>
</tr>
<tr>
<td></td>
<td>(30.50±4.92)</td>
<td>(54.2)</td>
<td>(45.8)</td>
<td>(2.07±1.47)</td>
</tr>
<tr>
<td>Apple cv. White Dotted Red</td>
<td>15.66-40.08</td>
<td>-</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>(28.41±3.27)</td>
<td>(10.0)</td>
<td>(55.0)</td>
<td>(1.57±2.19)</td>
</tr>
<tr>
<td>Apple cv. Ambri</td>
<td>25.42-31.16</td>
<td>-</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(27.38±3.27)</td>
<td>(66.7)</td>
<td>(33.3)</td>
<td>(3.82±3.22)</td>
</tr>
<tr>
<td>Apple cv. Golden Delicious</td>
<td>20.66-27.33</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(23.52±3.43)</td>
<td>(100.0)</td>
<td>(100.0)</td>
<td>(1.52±0.29)</td>
</tr>
<tr>
<td>Pear cv. William's</td>
<td>12.00-33.50</td>
<td>-</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(19.92±6.55)</td>
<td>(60.0)</td>
<td>(30.0)</td>
<td>(1.0±1.05)</td>
</tr>
<tr>
<td>Pear cv. Chinese Sandy</td>
<td>11.75-67.50</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(32.18±24.34)</td>
<td>(25.0)</td>
<td>(50.0)</td>
<td>(1.39±2.12)</td>
</tr>
</tbody>
</table>

** = value within parenthesis are mean ± SD,
- = not detected.
and Martins (1983). This evaluation, therefore, shows that proper storage and sorting of apples is very necessary before marketing so that intake of patulin and citrinin above the tolerance limit is avoided. If apple juice and apple-based foods are prepared with these low quality fruits, the presence of patulin and citrinin can be much higher than the safe limits established by international committees.

ACKNOWLEDGEMENT

The first author wishes to thank Council of Scientific and Industrial Research, New Delhi, India for granting financial assistance in the form of Senior Research Fellowship.

REFERENCES


Table 3. Patulin levels in P. expansum rotted tissue and surrounding non--rotted tissue (cv. Red Delicious)

<table>
<thead>
<tr>
<th>P. expansum strains tested</th>
<th>Patulin production (mg/kg) in</th>
<th>Rotted tissue</th>
<th>Surrounding non-rottled tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE-145</td>
<td>49.72</td>
<td>6.50</td>
<td></td>
</tr>
<tr>
<td>PE-210</td>
<td>31.20</td>
<td>3.25</td>
<td></td>
</tr>
<tr>
<td>PE-254</td>
<td>19.82</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>PE-257</td>
<td>221.0</td>
<td>22.75</td>
<td></td>
</tr>
<tr>
<td>PE-365</td>
<td>111.15</td>
<td>0.65</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Patulin levels in P. expansum rotted tissue and surrounding non--rotted tissue (cv. Red Delicious)


Received for publication July 2, 2007