Bioproduction of alkaloids on artificial media and natural substrates
by some strains of Penicillium

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A. G. KOZLOVSKY*1, T. A. RESHETILOVA*1, N. G. VINOKUROVA*1, L. S. L’VOVA*2:
数種 Penicillium 属菌によるアルカロイドの産生

Summary

Some species of the genus Penicillium (P. palitans, P. expansum and P. farinosum) were examined for the ability to produce nitrogen-containing mycotoxins (alkaloids) on synthetic, complex media, summer wheat and apple juice in the submerged and surface culture. Bioproduction of clavine and benzodiazepine alkaloids was observed in all media tested in surface conditions. However, the essential differences were found in the total amounts of alkaloids as well as in the ratio of separate components and their distribution between the mycelium and culture broth. It was suggested that a correlation exists between the ability of fungi to produce N-containing mycotoxins (alkaloids) on artificial media and that on natural substrates.

Key words: Penicillium, Alkaloid, Summer wheat, Apple juice, Artificial media

A number of Penicillium species that contaminate food and feed are able to produce nitrogen-containing mycotoxins. Alkaloids are the most important ones among them. Alkaloid-contaminated feed may be the cause of several cattle diseases1-3. In many cases the ability of alkaloid production depends on the cultivation conditions and the composition of growth medium. The aim of this work was to investigate the toxigenic potential of Penicillium species, P. palitans, P. expansum and P. farinosum, grown on artificial media and that on natural substrates, such as summer wheat and apple juice.

Materials and Methods

Fungal strains of the genus Penicillium P. palitans VKM F-3088, P. expansum VKM F-275 and P. farinosum VKM F-1746, isolated from various food products and feed were obtained from the All-Russian Culture Collection (VKM).

Media and culture conditions Spores from the 4-day-old malt agar slopes were used for inoculation. They were cultivated in submerged (24°C, a rotary shaker, 220–240 rpm) and surface conditions in 750 ml Erlenmeyer flasks containing 150 ml medium of the following composition (g/l distilled water):

1. mannitol—50, succinic acid—5.4 (adjusted to pH 5.4 using concentrated NH₄OH),
MgSO₄·7H₂O—0.3 and KH₂PO₄—1 (Abe’s medium);
2. medium 1 where glucose—50 and malic acid—5.4 were substituted for mannitol and succinic acid, respectively;
3. glucose—50, peptone—10, soy-bean meal—5, KNO₃—2, MgSO₄·7H₂O—0.5 and NaCl (adjusted to pH 6.2 using 1N HCl);
4. Czapek-Dox’ medium with 0.5% yeast extract;
5. medium 1 with 1.5% agar (dense medium);
6. medium 3 with 1.5% agar (dense medium);
7. apple juice (Azovskoe agropromishlennoe ob’edinenie detskogo pitanija, Azov, Russia) diluted twice with distilled water to a final pH of 3.5;
8. summer wheat. Summer wheat (2 kg) was placed into an autoclave and kept for 20 min at 1.2 atm. Then, it was inoculated with the spores from 5 agar slopes to a final humidity of 25%. Fermentation was carried out in glass flasks 280 cm² containing 150 g of summer wheat each at 8° and 25°C. Some of them were closed, others were ventilated periodically. Cultivation on the dense medium (20 ml) was carried out on Petri dishes (diameter 15 cm).

Isolation and purification of alkaloids and assays Clavine and diketopiperazine alkaloids were isolated from the mycelium, culture broth and apple juice as described earlier; quinoline and benzodiazepine alkaloids, α-cyclopiazonic acid (CPA) were purified as described in the literature, respectively. Alkaloids were isolated from summer wheat (20 g) with chloroform (150 ml x 3). Analysis of alkaloids was made by TLC on Silufol plates (Cavalier, Czechoslovakia). The following systems were used:
1. chloroform-methanol—25% ammonia (90: 10: 0.1, v/v) for clavines;
2. ethylacetate-methanol—25% ammonia (85: 15: 10) for CPA;
3. benzene-ethylacetate-methanol-water (70: 30: 5: 0.2) for cyclopenine and cyclophenol;
4. chloroform-methanol-25% ammonia (90: 10: 1) for roquefortine and viridicatine.

Alkaloids were detected as an absorbed zone in UV light or as coloured spots after spraying the plates with appropriate reagents. A quantitative analysis of alkaloids was carried out by UV spectrophotometry (Shimadzu, UV-160A, Japan). The optical density was measured at 328, 282, 317 and 284 nm for roquefortine, clavine alkaloids, viridicatine and CPA, respectively.

Results and Discussion
It was established previously that P. palitans produces clavine alkaloids (fumigaclavine A, fumigaclavine B, pyroclavine, festuclavine, chanoclavine-I), benzodiazepines (cyclopenine, cyclophenol), CPA and prolylvalyl diketopiperazine. P. expansum synthesizes diketopiperazine alkaloids (roquefortine, 3,12-dihydroroquefortine), benzodiazepine (cyclopenine, cyclopenine, cyclopeptin and dihydrocyclopeptin) and quinoline alkaloids (viridication). P. farinosum is able to produce roquefortine, 3,12-dihydroroquefortine and their metabolites PF-1—PF-4. It is known that the production of toxins by fungi depends on many factors, first of all, the composition of culture medium, temperature and pH. Therefore, it is very important to determine the existence or absence of a correlation between the tox-
Table 1. Effect of the medium composition on the contents of alkaloids produced by *P. palitans* (15–20 days storage) and *P. expansum* (10 days storage) in the surface culture conditions

<table>
<thead>
<tr>
<th>Medium</th>
<th><em>P. palitans</em></th>
<th><em>P. expansum</em></th>
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<tbody>
<tr>
<td></td>
<td>biomass (g d.w.<em>1</em>/l)</td>
<td>clavine alkaloids</td>
</tr>
<tr>
<td>Abe's medium (1)</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Abe's modified medium (2)</td>
<td>10.4</td>
<td>2.8</td>
</tr>
<tr>
<td>glucose-peptone (3)</td>
<td>20.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Czapek-Dox' medium (4)</td>
<td>12.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Abe's medium with agar (5)</td>
<td>20.8</td>
<td>0.5</td>
</tr>
<tr>
<td>glucose-peptone with agar (6)</td>
<td>31.9</td>
<td>0.6</td>
</tr>
<tr>
<td>apple juice (7)</td>
<td>9.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*1 dry weight.*  
*2 no data.*  
*3 30 days culture.*

genic potential of these fungi on artificial media and that on natural substrates.

*P. palitans* was able to synthesize benzodiazepine alkaloids only on the synthetic medium in surface conditions (on Abe's medium cyclopenol—0.31 mg/g dry weight; cyclopenine—traces). Ergot alkaloids were produced in all media tested (Table 1). In submerged culture these metabolites were detected in a negligible amount (about 0.1 mg/l) only on Abe's medium. CPA was synthesized in all the cases in surface culture (Table 1), in submerged conditions on Abe's and Czapek-Dox’ media—2 and 3 mg/g dry weight, respectively.

Ergot alkaloids and CPA were also produced on the dense media (media 5 and 6, Table 1). The spectra of alkaloids were the same as in the submerged culture on Abe's medium.

Alkaloids were also found in the apple juice used as a natural substrate (5.5 mg/l). The culture productivity was the same on glucose-peptone and apple juice media, but its excretic activity differed essentially. The contents of extracellular alkaloids were two times higher than those of intracellular ones in the apple juice: in the case of glucose-peptone medium, the contents of alkaloids were higher in the mycelium than those in the medium (Fig. 1).

Summer wheat is another natural substrate of *P. palitans*. We analyzed summer wheat infected with the spores of this fungus to evaluate the possibility of this strain to synthesize N-containing mycotoxins (alkaloids). One can see (Fig. 2) that the composition of alkaloids in extracts from the infected wheat was same to that from the mycelium of the culture grown on glucose-peptone. In the course of fermentation the content of alkaloids in wheat increased (Table 2).

*P. farinosum* was not known as a typical contaminant of wheat. We detected small quantities of roquefortine, 0.2, 0.6 and 2.0 mg/kg wheat at 12, 16 and 22 days, respectively, in the infected wheat (ventilation, 25°C). Roquefortine was found earlier in barley silage...
Fig. 1. Effect of the medium composition on the contents intra- (A) and extracellular (B) clavine alkaloids in the surface cultures of P. palitans. The fungi were cultured on apple juice (1) and glucose-peptone (2) media for 30 days.

Fig. 2. Profiles of the alkaloids production by P. palitans on the surface cultures of summer wheat (a—12, b—16, c—22 days) and glucose-peptone medium (d—20 days). 1—fumigaclavine A; 2—pyroclavine; 3—festuclavine; 4—fumigaclavine B; 5—CPA; 6—chanoclavine.

which was contaminated by P. roqueforti).

P. expansum is a typical contaminant of apple, grape and canned fruits. We investigated the ability of this strain to produce N-containing mycotoxins on various media: Abe’s
Table 2. Effect of the culture conditions on the contents of clavine alkaloids produced by *P. palitans* in summer wheat (mg/kg).

<table>
<thead>
<tr>
<th>Days of storage</th>
<th>ventilated</th>
<th>hermetically sealed</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>8°C</td>
<td>25°C</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>4.2</td>
</tr>
<tr>
<td>16</td>
<td>—</td>
<td>9.5</td>
</tr>
<tr>
<td>22</td>
<td>—</td>
<td>39.0</td>
</tr>
</tbody>
</table>

* alkaloids were not detected.

medium which is favourable for alkaloid bisynthesis; complex media (media 3 and 4) and a natural substrate, such as, apple juice (Table 1). When *P. expansum* was grown on glucose-peptone medium, the largest amounts of roquefortine were found in the mycelium. Thirty six mg and 19 mg were present in the mycelium and filtrate, respectively, from 1L of the fermentation broth. Similar results were obtained on Czapek-Dox medium (30.4 mg, mycelium; 3.3 mg, medium). The contents of alkaloids in mycelium and filtrate in Abe’s medium were 3.2 and 5.0 mg, respectively, and those in apple juice were 0.4 and 0.6 mg, respectively. It may be concluded that the ability of fungi to excrete alkaloids depends on the composition of media.

The biosynthesis of ergot alkaloid and benzodiazepine mycotoxins occurred in all media tested in the surface culture. However, essential differences were observed in the total amount of alkaloids produced as well as in the ratio of separate components and their distribution between the mycelium and culture broth.

Using the results obtained, we may suggest that the ability of some fungal species to synthesize N-containing mycotoxins (alkaloids) in laboratory conditions correlates with such in nature.

References
