Antibacterial effects and toxigenesis of *Penicillium aurantiogriseum* and *P. viridicatum*

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The toxigenesis of one *Penicillium aurantiogriseum* and one *Penicillium viridicatum* isolates was investigated. Sterile culture filtrates of both fungi had a clear antibacterial effect only against *Bacillus subtilis*. The effect on *B. subtilis* varied with amount of filtrate used and temperature. The antibacterial activity of chloroform extracts varied with the nature of media used to grow the fungi. Different mycotoxins were identified in the fungal cultures using thin-layer chromatography. *P. aurantiogriseum* was found to produce penicillic acid, terrestric acid and aurantiamine, while penicillic acid, terrestric acid, brevianamide A and xanthomegnin were produced by *P. viridicatum*.

**Key words:** *P. aurantiogriseum*, *P. viridicatum*, mycotoxins, antibacterial activity.

**INTRODUCTION**

Mycotoxins are secondary toxic metabolites elaborated by toxigenic molds in foods. Mycotoxin ingestion represents a real threat to human and animal health. Some of them are very strong carcinogens (Applebaum et al., 1982; Mills et al., 1995). Numerous investigations have reported that various mycotoxins can be produced by *Penicillium* (Faid and Tantaoui-Elaraki, 1989) including industrially used species when under favourable physicochemical and trophic conditions. Previous works have pointed out that *Penicillium aurantiogriseum* and *Penicillium viridicatum* are among major *Penicillium* species represented in foods such as olives, cereal grains and derivatives (Maouni, 1997; Mills et al., 1995; Sebti and Tantaoui-Elaraki, 1994; Tantaoui-Elaraki et al., 1990). Furthermore, both species have shown more resistance to antifungal agents (sorbic and benzoic acids, calcium propionate, cinnamon extract) than other fungi (Sebti and Tantaoui-Elaraki, 1994).

The aims of this work were to determine whether culture sterile filtrates and extracts of the two species are toxic to selected bacteria and to identify the toxic metabolites produced, if any.

**MATERIALS AND METHODS**

**Microorganisms**

*P. aurantiogriseum* was isolated from “Pastilla papers” in a previous work (Sebti and Tantaoui-Elaraki, 1994), and its identification was confirmed by the “Institut Scientifique de la Santé Publique Louis Pasteur”, Brussels, Belgium.
**P. viridicatum** was isolated from wheat at the “École Normale Supérieure” (ENS) Food Microbiology Laboratory, Tétouan, Morocco. Four of the bacteria used were furnished by the ENS Food Microbiology Laboratory collection: *Bacillus subtilis, Bacillus megaterium, Escherichia coli* and *Lactococcus (L.) lactis* subsp. *diacetylactis* (hereafter called *L. diacetylactis*). The fifth one, a non pathogenic mutant of *Salmonella typhimurium* TA 100, was given by the Toxigenetics Laboratory, Biology Department, Faculty of Sciences, Tétouan, Morocco.

**Fungal cultures**

For preparation of fungal inocula, *Penicillium* strains were cultivated in petri dishes on malt extract agar (MEA) and incubated 5 days at 25°C. Then the spores were collected in sterilized 0.1% Tween 80 water solution. After enumeration of the spores in the suspension by hematometry, their numbers were adjusted to 10⁷ spores/ml by dilution in 0.1% Tween solution. The fungi were cultivated in flasks containing 25 ml MEA, Malt Broth (MB), Czepeck Yeast extract Agar (CYA), Yeast Extract Sucrose (YES) broth or YES agar (Table 1). Each flask was inoculated with 2 ml fungal inoculum and incubated 10 d at 25°C.

**Preparation of culture filtrates and extracts**

In order to underline fungal toxigenesis, sterile culture filtrates and extracts were prepared. At the end of incubation period on YES broth, 5 ml of liquid culture were taken and filtered first through Whatman paper to remove the mycelium, and then through 0.45 µm millipore membrane for sterilization. The sterile culture filtrates were used for antibacterial activity tests.

It is well established that mold toxigenesis is widely influenced by numerous environmental conditions including nutritional parameters (Håggblom and Ghosh, 1985). Same fungus may produce a given mycotoxin in a given medium while it may synthesize another toxic metabolite under different nutritional conditions. Therefore, known mycotoxins of the two *Penicillium* were searched in various media (MEA, MB, CYA, YES broth and YES agar). At the end of incubation period, 25 ml chloroform was added to each fungal culture flask. After shaking 10 min, the chloriformic phase was recovered, filtered through anhydrous sodium sulfate to remove aqueous phase droplets, and concentrated with a rotavapor. The concentrate was dried under distilled water. This aqueous solution was sterilized by millipore filtration to be tested for antibacterial activity. The other half was used to search for mycotoxins by thin layer chromatography.

**Identification and separation of the mycotoxins by thin layer chromatography (TLC)**

Kieselguhr 60 F₂₅₄ TLC plates were used, according to the technique described by Mills et al. (1995). Standard mycotoxins used for reference of migration forehead (RI) were patulin, citrinin and ochratoxine A. 10 µL of each ethanol extract, and 10 µL of standard solutions (1 mg/ml) were spotted on TLC plates. The elution systems used were as follows: toluene - ethyl acetate - formic acid (5/4/1, v/v/v) and chloroform - acetone - 2-propanol (85/15/20, v/v/v). The plates were developed under darkness and examined at daylight, 365 and 254 nm. Spots with RI different from standard on either elution systems were not considered. Confirmation of toxins was performed as follows: spraying of the spots by ANIS: 0.5% (v/v) solution of p-anisaldehyde in absolute ethanol - glacial acetic acid -concentrated sulfuric acid (17/2/1, v/v/v), heating 8 min at 120°C. Some toxins specifically react with ANIS at this temperature, leading to characteristic colours under daylight, i.e. lilac blue for penicillic acid, yellow for terrestric acid and grey for aurantiamine. On TLC plates none treated with ANIS, areas corresponding to identify toxins were scratched and recovered with chloroform. The chloroform was completely evaporated and the residue redissolved in ethanol. Half the ethanol volume was used to confirm mycotoxin purity on a new TLC plate with ANIS. The other half was evaporated again, redissolved in water and millipore filtered in order to be used for antibacterial activity.

**Evaluation of antibacterial activity**

*B. subtilis* was first reactivated by cultivation on TYG and incubation at 37°C for 24 h. Suspensions containing 10⁵ cells/ml in sterile distilled water was used for inocula. Petri dishes (Ø: 90 mm) containing Tryptone Yeast extract Glucose (TYG) were surface inoculated with 0.2 ml of bacterial inocula. 15 min after inoculation, 4 cavities of 6 mm diameter were dug out in the agar medium. Three of them were filled with 0.1 ml of culture sterile filtrate, aqueous extract or extracted mycotoxin and the fourth one was used as control. After 24 h incubation at 37°C or 30°C to avoid too quick bacterial growth, the antibacterial effect was determined by measurement of the inhibition zone diameters. The effects of filtrate quantities varying from 0.02 to 0.1 ml poured into the cavities and of temperature (17, 25, 30, 34 and 37°C) were also tested on the antibacterial activity.

### Table 1. Culture media used in this study.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition (for 1 l distilled water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>130 g malt extract</td>
</tr>
<tr>
<td>MEA</td>
<td>20 g malt extract, 1 g peptone, 20 g glucose, 15 g agar</td>
</tr>
<tr>
<td>CYA</td>
<td>10 mL concentrated Czepeck, 1 g K₂HPO₄, 5 g yeast extract, 30 g sucrose, 15 g agar</td>
</tr>
<tr>
<td>YES broth</td>
<td>20 g yeast extract, 100 g sucrose</td>
</tr>
<tr>
<td>YES agar</td>
<td>20 g yeast extract, 100 g sucrose, 15 g agar</td>
</tr>
<tr>
<td>TYG</td>
<td>3 g tryptone, 3 g yeast extract, 3 g glucose, 1 g K₂HPO₄, 15 g agar</td>
</tr>
</tbody>
</table>
Table 2. Toxicity of Penicillium aurantiogriseum and P. viridicatum culture filtrates to different bacteria (average of 3 determinations, 3 replicates).

<table>
<thead>
<tr>
<th>The bacteria**</th>
<th>Diameters of inhibition zones (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aurantiogriseum*</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>00</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>00</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>13</td>
</tr>
<tr>
<td>Lactococcus diacetylactis</td>
<td>00</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>10</td>
</tr>
</tbody>
</table>

*Fungal culture on YES Broth for 10 d at 25°C; filtration through Millipore membrane; 0.1 mL of filtrate used.

**Bacteria cultivated on TYG, incubation at 37°C for 24h.

Figure 1. Effect of the volume of fungal culture filtrate on the antibacterial activity after 24 h at 30°C (three replicates).

Figure 2. Effects of filtrate volume and temperature

RESULTS AND DISCUSSION

Effect of bacterial species

B. subtilis was the most sensitive to both P. aurantiogriseum and P. viridicatum culture filtrates, with inhibition zone diameters of 13 and 20 mm, respectively (Table 2). S. typhimurium was only slightly sensitive to P. aurantiogriseum filtrate (10 mm). All other bacteria were totally insensitive to both filtrates. B. megaterium has been reported to be sensitive to culture filtrates of Penicillium italicum and Penicillium digitatum (Faid and Tantaouei-Elaraki, 1989) and more than 12 other different Penicillium species (Carlton et al., 1976). Among 154 Penicillium isolates tested by Samane et al. (1991), about 63% were shown to have culture filtrates toxic or very toxic to B. megaterium. However, none of the Penicillia involved belonged to the species P. aurantiogriseum and P. viridicatum. Both sensitive and insensitive bacterial groups included at least one Gram positive and one Gram negative species. Thus, there seems to be no influence of the cell wall structure on the sensitivity of the bacteria to the fungal culture filtrates studied.

Effects of filtrate volume and temperature

The antibacterial effect on B. subtilis and S. typhimurium increased together with the amount of filtrate used (Figure 1). All other bacteria remained insensitive independently of the amount of culture filtrate poured. The effect of incubation temperature on the antibacterial activity of fungal culture filtrates was determined with 0.1 ml filtrate on B. subtilis (Figure 2). The diameter of inhibition zone slightly varied between 17 and 34°C. At 37°C, the inhibitory effect decreased significantly for both fungal species, probably because of an intense metabolism of B. subtilis at its optimal growth temperature.

Comparative effect of culture filtrates and extracts

The antibacterial effects observed with fungal YES broth filtrates were higher than those produced by the corresponding YES broth extracts. After 24 h incubation at 37°C, average inhibition zone diameters measured were 20.1 and 13.3 mm, respectively, with P. aurantiogriseum, and 30.3 and 24 mm, respectively, with P. viridicatum. The active principles could not be sufficiently soluble in chloroform, ethanol and water to remain totally in the final extract.
Table 3. Toxicity of culture filtrates (0.1 mL) of two Penicillium obtained after cultivation on three media 10 days at 25 °C to B. subtilis cultivated 24 h at 30°C on TYG (average of 3 determinations, 3 replicates).

<table>
<thead>
<tr>
<th>Fungal culture media</th>
<th>Diameters of inhibition zones (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aurantiogriseum</td>
</tr>
<tr>
<td>MEA</td>
<td>11.5</td>
</tr>
<tr>
<td>CYA</td>
<td>14.5</td>
</tr>
<tr>
<td>YES agar</td>
<td>13.3</td>
</tr>
</tbody>
</table>

**Effect of culture media on antibacterial activity of chloroformic extracts**

For P. aurantiogriseum, the highest inhibition zone diameter was observed with CYA culture extract (14.5 mm) while MB medium was associated with the lowest effect (11.5 mm) (Table 3). However, for P. viridicatum, YES culture extract was the most efficient (24 mm) and CYA culture extract the weakest (13.3 mm).

**Identification of mycotoxins**

Three different toxins were identified in P. aurantiogriseum cultures: penicillic acid, terrestic acid and aurantiamine. They have been reported by other authors (Mills et al., 1995), together with many other mycotoxins (Kozakiewicz, 1992a). In this work, penicillic acid and aurantiamine were detected in YES Agar, MB and CYA, while terrestic acid was found together with penicillic acid in MEA and alone in YES Broth. Mills et al. (1995) have also found penicillic acid and aurantiamine in CYA. However, they have detected terrestic acid in YES agar. Four mycotoxins were found to be produced by P. viridicatum: penicillic acid, terrestic acid, brevianamide A and xanthomegnin.

In MEA, all four toxins were synthetized, while terrestic acid alone was detected in YES broth and penicillic acid in YES agar. None of the four metabolites was found in CYA. Previous works have reported penicillic acid (Kozakiewicz, 1992b; Mills et al., 1995), brevianamide A (Kozakiewicz, 1992b; Mills et al., 1995; Müller and Boley, 1993) and xanthomegnin (Carlton et al., 1976; Kozakiewicz, 1992b; Mills et al., 1995; Müller and Boley, 1993) to be produced by P. viridicatum. Using CYA, Mills et al. have found both brevianamide A and xanthomegnin, together with other mycotoxins: penicillic acid, penitrem A, vioxanthin and viomellein. P. viridicatum is known to produce also ochratoxin A, a metabolite toxic to many animals (Håggblom and Ghosh, 1985).

**Effect of toxins purified by TLC**

Three different substances taken from TLC plates and redissolved in water were tested for antibacterial activity on B. subtilis incubated 24 h at 30°C. According to their respective Rfs, those substances were penicillic acid, terrestic acid and aurantiamine.

**REFERENCES**


