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Production of Toxic Metabolites by *Penicillium italicum* and *P. digitatum* Isolated from Citrus Fruits

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ABSTRACT

Ninety-six mold isolates were obtained from naturally rotten citrus fruits. Among them, forty were identified as *Penicillium italicum* and twenty-four as *P. digitatum*. Twenty-four isolates of the former and twenty of the latter were tested for toxigenesis. They were first grown on Yeast Extract Sucrose (YES) broth for ten d at 22°C. Then, after mycelium removal, the cultures were sterilized by Millipore filtration and the toxicity of the sterile filtrates tested by four different bioassays; i.e. a bacterial test with *Bacillus megaterium*, a plant test with *Lepidium sativum*, a test with the brine shrimp *Artemia salina* and the chick *(Gallus domesticus)* embryo test. In *P. digitatum*, 95% of the filtrates were toxic to *B. megaterium*, 100% caused strong inhibition of seed germination in *L. sativum*, 75% showed acute toxicity to the brine shrimp and 65% were toxic to the chick embryo, while the figures for *P. italicum* filtrates were about 96%, 71%, 87%, and 42%, respectively. The results observed with the four different tests didn't always correlate.

Food spoilage by molds is a natural problem occurring more or less frequently depending upon the nature of foods and the environmental conditions. In addition to the economic consequences of mold growth, some fungal species have been shown to produce mycotoxins on foods, which may constitute a potential health hazard for the consumer.

Since the early 1960's, many kinds of food products have been investigated for contamination by toxigenic molds and mycotoxins. However, even though citrus fruits are known to be frequently molded, little data have been published on the potential ability of the molds involved to produce toxic metabolites. Among the fungi that grow on citrus fruits as post-harvest contaminants, *P. italicum* and *P. digitatum* are the most common all over the world. They cause, respectively, the so-called blue mold-rot and green mold-rot. The spoilage can occur during commercial shipments as well as in packaging houses and retail markets *(4,9)*.

A *P. italicum* strain isolated from orange was toxic to ducklings and rats *(7)*. This strain was shown to produce a mycotoxin identified as 5,6-dihydro-4-methoxy-2H-pyran-2-one *(6)*. Also two different diketopiperazines, known metabolites of *Aspergillus ustus*, were produced in low yield by *P. italicum* in liquid medium and on unsterilized orange peel, but the toxicity of these alkaloids was not specified *(16)*.

The objective of this study is to screen *P. italicum* and *P. digitatum* isolated from citrus fruits for toxic metabolites by using biological tests.

MATERIALS AND METHODS

Isolation and identification of molds

The molds were isolated from about 100 visibly rotten oranges and tangerines collected in Rabat (Morocco) at the wholesale market and small shops. The streaking method on Malt Agar or Potato-Dextrose-Agar plates was used. Fungal spores were collected directly from the rots and spread on the solid media. The plates were incubated at 20-22°C for 10 d. The isolates were then streaked again on the same media in order to purify them and to keep only those which likely belong to *P. italicum* or *P. digitatum* species. Before identification, the isolates were stored, if necessary, on Malt Agar slants at 4°C. The identification was done on the basis of the appearance of the molds when grown on citrus fruits and on Agar media as well as the microscopic examination of their characteristics according to Pitt *(14)*.

Screening for toxigenesis

Stationary cultures of the molds were made in flasks containing 50 ml of Yeast Extract Sucrose (YES) Broth (20 g Yeast Extract and 100 g sucrose in 1000 ml distilled water). The cultures were incubated at 22°C for 10 d, and filtered first through filter paper (Whatman #4) to remove the mycelium, and then through Millipore membrane (HA type, pore size 0.45 μm) for sterilization. The sterile filtrates obtained were then submitted to four different biological tests for screening of toxic metabolites.

Bacterial test

Ten milliliters of Tryptone-Yeast-Glucose Broth (TYG) were inoculated with a *Bacillus megaterium* strain from our laboratory's collection. After an incubation of 18 h at 37°C, 0.2 ml of this culture were added and mixed vigorously with 16 ml of melted TYG Agar which was then poured into a Petri dish of 90 mm diameter. Then, cavities of 6 mm diameter were made by the means of a small tube in the medium when solidified (no more than 10 cavities per plate). Each cavity was then filled up with 0.25 ml of one of the sterile filtrates. Each filtrate was tested in triplicate, and a control was made in every plate with non-inoculated YES medium. The plates

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were pre-incubated at 5°C for 45 min to allow for diffusion of potentially toxic metabolites before the bacteria start growing *(17)*, and then incubated at 37°C for 24 h. The diameter of any subsequent zones of inhibition was measured, and an average of 3 measurements was calculated for each filtrate.

Plant test

Seeds of the garden cress *Lepidium sativum* were carefully selected and placed on sterile hydrophilic cotton in Petri dishes. The filtrates to be tested were diluted with sterile distilled water to a ratio of 1 to 5 and poured aseptically on the seeds. Each Petri dish received 2.5 ml of a diluted filtrate. Two controls were done under the same conditions, respectively with sterile distilled water, and YES medium diluted to the same ratio as the filtrates. This dilution was necessary because of the inhibitory effect on seed germination observed when undiluted YES medium was used. The plates were covered to avoid dehydration, and kept at 28-30°C for 3-4 d. The germination of the seeds, and the growth of the plant were compared to the controls and scored according to the method described by Payen et al. *(13)*. Each trial was done in triplicate.

Brine shrimp nymph test

Dried eggs of the brine shrimp *Artemia salina** were used. Half ml of egg powder was soaked in 1 L of sea water in a glass bottle and aerated with bubbling air. After 36 h at 30°C, the nymphs were collected with a pipet using a narrow light ray in a dark room to attract the nymphs. Then 0.5 ml portions of sea water containing about 30 nymphs each were poured into watch glasses, which were placed in Petri dishes with wet cotton to avoid dehydration; 0.25 ml of each sterile filtrate was added to the nymph suspensions. Each experiment was done in triplicate. Also 3 controls were made under the same conditions; i. e. with sterile distilled water, sterile YES medium and sea water. The plates were then kept at room temperature and the number of dead nymphs was counted after 2 h, 4 h, 6 h, 8 h, and 24 h. Dead nymphs were easily distinguished since they fell to the bottom of the watch glass. At the end of the experiment, the nymphs still alive, if any, were killed with formalin and total nymph number was determined.

Chick embryo test

Chick eggs *(Gallus domesticus)* were cleaned, disinfected with chlorinated water and alcohol, rinsed with water and then pre-incubated for 6 d at 37°C. They were then examined under a light spot in a dark room to remove clear eggs, which are nonfertile. Two small holes were then made with a sharp needle in the air sac of each egg. One of the holes was used to inject 0.25 ml of a sterile culture filtrate while the other was necessary for air evacuation during this operation. After the filtrate was injected, the holes were sealed with adhesive paper. Ten eggs were used for each filtrate, and also ten for each of the three controls; i.e. YES-injected eggs, eggs with holes pierced and sealed without injecting anything, and eggs without any treatment.

Then all the eggs were incubated at 37°C, and embryos mortality was checked every 48 h, until the hatching of the controls. The total number of dead embryos was recorded. Positive filtrates were tested twice to confirm their toxicity.

RESULTS AND DISCUSSION

Ninety-six molds were isolated from moldy fruits exhibiting blue and/or green rots presumably due to *Penicillium* species. Forty of these were identified as *P. italicum* and twenty-four as *P. digitatum*, while 32 isolates belonged to other unidentified species of *Penicillium* and other genera. That *P. italicum* is relatively more frequent than *P. digitatum* could be explained by the fact that the latter requires prior injury to the peel, which can occur either mechanically at harvest, during transportation and in storage, or succeeding *P. italicum* attack, whereas *P. italicum* spores can germinate and its hyphae can penetrate the fruit peel without prior injury *(12)*.

Twenty-four isolates of *P. italicum* (Table 1) and 20 of *P. digitatum* (Table 2) were investigated for toxicity.

Ninety-five percent of *P. digitatum* filtrates and 96% of *P. italicum* filtrates were toxic to *B. megaterium*, developing inhibition zones ranging from 1 to 10 mm width. Only two isolates, one of each species, showed no inhibitory effect on this bacteria. On the other hand, one isolate of *P. digitatum* developed an inhibition zone of 10 mm width, and one of *P. italicum* showed a 9 mm width zone. *B. megaterium* has been reported to be sensitive to some mycotoxins including *Penicillium* toxins *(17)*. However, since bacteria are also sensitive to antibiotics, one could not conclude on this basis alone that the isolates positive in this test necessarily contain mycotoxins. Moreover, *P. italicum* has been shown to produce penitaline, a water soluble antibiotic *(15)*.

TABLE 1. *Toxicity of 24* P. italicum *isolates to four different living organisms.*

# of Isolate	*B. megaterium* (a)	*L. sativum* (b)	*A. salina* (c)	Chick embryo (d)
1	3.9	2	92	0
3	4	2	98.6	0
4	3.25	2	27.7	20
5	3.4	0	87.4	0
6	3.5	2	40	0
7	3.4	2	91	0
8	1.9	2	97.5	40
9	2.25	2	89	0
11	2.25	2	97.4	10
13	4.25	2	100	60
14	4.25	2	100	60
29	3.5	0	97.4	20
31	4	0	64	0
35	0	0	37	30
37	1	0	97	30
38	3.4	2	100	0
39	2	0	70	50
41	2.25	2	79.6	30
46	2.5	2	81	0
47	3	2	100	10
28	9	0	86.7	40
30	5	2	88.5	20
50	5.5	2	100	50
54	6.5	2	100	50
Controls	0	0	0-2	0-20

(a):width of the inhibition zone (mm).
(b):inhibition of seed germination : score according to Payen *et al. (13)*.
(c):Percentage of dead nymphs after 24 h.
(d):Percentage of dead embryos after 21 d.

*From HOBBY, dose Aquaristik. Bonn, Federal Republic of Germany.

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TABLE 2. *Toxicity of 20* Penicillium digitatum *to four different living beings.*

# of Isolate	*B. megaterium* (a)	*L. sativum* (b)	*A. salina* (c)	Chick embryo (d)
40	0	2	77.5	0
45	3.6	2	47.8	20
80	3.25	2	5.8	0
81	3.5	2	57.5	40
82	2.5	2	60	20
83	3.6	2	78	20
84	10	2	100	50
85	3.5	2	100	40
86	3.5	2	78.5	60
87	5	2	100	60
88	2.65	2	67.5	40
89	3	2	55	50
90	6	2	48.5	60
91	2.5	2	37.5	50
92	2.5	2	88	30
93	3.25	2	90	30
94	4.8	2	57.6	20
95	5.6	2	90	50
96	2	2	87	30
97	2.5	2	48.7	20
Controls	0	0	0-7	0-20

(a):width of the inhibition zone (mm).
(b):inhibition of seed germination : score according to Payen *et al. (13).*
(c):Percentage of dead nymphs after 24 h.
(d):Percentage of dead enbryos after 21 d.

In the *Lepidium sativum* test, about 71% of *P. italicum* and 100% of *P. digitatum* filtrates showed strong inhibition of seed germination with a score of 2, according to the technique described by Payen et al. *(13)*, in spite of the dilution of the filtrates with distilled water to the ratio of 1 to 5, that was necessary to suppress the inhibitory effect developed in the controls by YES medium itself. *Lepidium sativum* seeds are known to be sensitive to mycotoxins when placed under germination conditions *(13)*. But again, the toxicity of our filtrates to a plant species does not provide evidence that they contain mycotoxins. For, even though numerous mycotoxins show a certain toxicity towards plants, other fungal metabolites could be phytotoxic without exhibiting any toxicity to man and animals *(11)*.

Fifteen out of 20 filtrates (75%) of *P. digitatum* and 21 out of 24 (87%) of *P. italicum* showed acute toxicity to the nymphs of the brine shrimp *Artemia salina*, causing more than 50% mortality in 24 h, while this never exceeded 7.7% in the controls with YES medium and 1.3% in sea water controls. 100% mortality was observed after 8 h with 2 filtrates of *P. italicum* and 1 of *P. digitatum*, and after 24 h with 6 filtrates of the former, and 3 filtrates of the latter species. This test has been used by numerous authors to detect the toxicity of fungal metabolites *(5,10,13)* both in fungal cultures and in contaminated foods.

In the chick embryo test, due to the limited capacity of the egg incubator, only 4 to 5 filtrates could be investigated each time, with a complete set of controls. Hence, in total nine sets of ten eggs per control were needed. Mortality generally ranged from 0 to 10% in the controls and only twice reached 20%. The filtrates that caused 30% mortality or more were considered as toxic, 13 out of 20 filtrates of *P. digitatum* (65%) and 10 out of 24 of *P. italicum* cultures (42%). The most toxic filtrates caused the death of 60% of the embryos. These comprised 2 filtrates of *P. italicum* and 3 of *P. digitatum* isolates. This test is widely employed *(1,2,3,8,10)* and a positive response to it is generally considered as good evidence of toxicity.

The data presented in this work show that *P. italicum* and *P. digitatum* can produce toxic metabolites to bacteria, plants and animals. However, it must be emphasized that the results observed with the four different tests don't always correlate. For none of these tests is strictly specific for mycotoxins. The bacterial test involving *Bacillus megaterium* could be positive to antibiotics while phytotoxins, not necessarily toxic to man and animals, could lead to a positive test with *Lepidium sativum*. Also, some of the filtrates tested could have no effect in a given test while they actually may contain a mycotoxin that is either in too small concentration to be detected or simply non-toxic to the organism involved.

Some *P. italicum* isolates (e.g. #11 and 38) showed toxicity to *A. salina* and were not toxic to the chick embryo whereas other (e.g. #35) were moderately toxic to *A. salina* but toxic to the chick embryo too (Table 1). In *P. digitatum* most of the isolates were toxic to both *A. salina* and the chick embryo, but the isolate #80 was non-toxic to *A. salina* while it showed relatively high toxicity to the chick embryo (Table 2). These observations provide evidence that in both species at least two different mycotoxins were involved.

However, further investigations are needed in order to determine the chemical nature of the toxic metabolites. It must also be stressed that the toxicity was shown after cultivation of the isolates in a rich laboratory medium under favorable conditions. Thus, one can not assume that the toxins could be produced on citrus fruits as well. Also, if that can occur, even though it is likely unthinkable that rotten fruits could be eaten as such, it would be interesting to investigate the fate of the toxins when such fruit are used to process juice, marmalade, or animal feed.

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