



## Mycotoxins from Beehive Pollen Mycoflora

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### Authors' contributions

*This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.*

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### ABSTRACT

**Aims:** The present study was aimed to search for metabolites produced by fungal strains with antifungal activity against phytopathogens.

**Study Design:** The pollen of four beehives each from two apiaries from two locations in Argentina (Balcarce and Mar del Plata) was studied. Nine bee pollen samples were randomly collected from each beehive.

**Place and Duration of Study:** Study was undertaken during 2011 on the Organic Chemistry Department and Biodiversity and Experimental Biology Department, School of Exact and Natural Sciences, University of Buenos Aires.

**Methodology:** All of the 133 isolated strains were tested for antifungal activity. The most bioactive strains, *Myrothecium verrucaria*, *Nigrospora sphaerica* and a levuliform mycelia, were selected for the isolation and identification of compounds responsible for the antifungal activity.

**Results:** Seven mycotoxins, roridin A, verrucarrin A,  $\alpha$ - $\beta$ -dehydrocurvularin, phomalactones A, B, C and D, were identified spectroscopically and were the responsible the antifungal activity.

**Conclusion:** Mycotoxins were isolated from beehive pollen mycoflora. This is the first report of the production of these mycotoxins, which are not currently targets for the analysis of pollen or honey products.

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## 1. INTRODUCTION

Bee pollen consists of plant pollens, the male reproductive cells of flowers collected by the worker bees that are combined with plant nectar and bee saliva. This product contains photochemical that are particularly rich in carotenoids, flavonoids and phytosterols [1], and nutrients including free amino acids, sugars and mineral elements [2]. The beneficial properties of bee pollen transform this commodity into an increasingly regular ingredient of the human diet, leading to a constant increase in its consumption. But this fact also converts it into a suitable source for the growth of a variety of microorganisms. Fungi successfully colonize the terrestrial environment and efficiently utilize solid substrates by growing over their surfaces and penetrating their matrices [3]. For this reason, a search for natural bioactive microbial products of pollen from beehives as a new source for antifungal metabolites was investigated [4,5,6].

## 2. MATERIALS AND METHODS

### 2.1 Bee Pollen Sampling

Pollen samples were taken from beehives in Mar del Plata, Buenos Aires (B apiary, 4 beehives, N=36) and Balcarce, Buenos Aires (F apiary, 4 beehives, N=36). They were collected from pollen traps and stored frozen in sterile glass containers until analysis. Bee pollen samples were then randomly collected divided and distributed aseptically into sterile flasks to form a solid mass.

### 2.2 Selection of Fungal Strains

A dilution method was applied [7] where 0.4 g aliquots of pollen were placed in 10ml of sterile water and vortexed for 1 min. One-tenth serial dilutions were made and the samples were vortexed for 30 seconds each time. The 2<sup>nd</sup> dilutions (200 µl) were seeded into Petri dishes containing malt extract agar (15g agar, 20g malt extract, 20g glucose and 2g peptone) with antibiotics (0.25g of streptomycin and 0.125g chlortetracycline). The procedure was performed in triplicate and the Petri dishes were incubated in darkness at 25°C. Colonies developing on the plates were sub cultured for subsequent identification. A total of 78 isolates from the B apiary and 55 from the F apiary were obtained. All the strains were tested for antifungal activity and the most active strains, *Myrothecium verrucaria* (LM 278), a levuliform mycelia (LM 336) and *Nigrospora sphaerica* (LM 314) were further analyzed. These strains were classified by M. A. Rodriguez and A. M. Godeas and deposited at the BAFC culture collection (Fungal collection - School of Exact and Natural Sciences, University of Buenos Aires).

### 2.3 Culture

A well-grown agar slant of each fungus was used to inoculate four 250ml erlenmeyer flasks containing 100ml of malt extract medium (30g malt extract and 5g peptone per liter). The fermentation was carried out at 25°C for 15 days (stationary phase) under static conditions. The optimal fermentation and harvest conditions were determined in previous small scale experiments and agreed with previous reports on secondary metabolite production [8].

## 2.4 Extraction Procedure, Mycotoxin Isolation and Identification

A liquid-liquid extraction procedure using ethyl acetate resulted in the best recovery for the isolated compounds because allowed the extraction of a higher number of different metabolites. The same procedure was performed for each culture. The fermentation broth was filtered and the filtrate (400 ml) was partitioned three times with 200 ml of ethyl acetate. The crude organic extract was evaporated *in vacuo* and was subjected to preparative-scale thin-layer chromatography (Merck, silica gel 60F) leading to the isolated compounds. While the crude extract (50.0 mg) of strain *M. verrucaria* yielded two compounds from preparative-scale thin-layer chromatography (CH<sub>2</sub>Cl<sub>2</sub>: ethyl acetate, 1:1), roridin A (**1**, 2.9 mg, 5.8%) and verrucaric acid (**2**, 4.9 mg, 9.8%),  $\alpha$ - $\beta$ -dehydrocurvularin (**3**, 2.0 mg, 2.5%) was obtained from the crude organic extract of LM336. The chromatographic separation (ethyl acetate: methanol, 95:5) of the crude organic extract of *N. sphaerica* (198.1 mg) led to the isolation of phomalactone (**4**, 10.1 mg, 5.1%), 6-(1-propenyl)-3,4,5,6-tetrahydro-5-hydroxy-4H-pyran-2-one (**5**, 2.0 mg, 1.0%), 5-[1-(1-hydroxybut-2-enyl)]-dihydrofuran-2-one (**6**, 1.9 mg, 1.0%) and 5-[1-(1-hydroxybut-2-enyl)]-furan-2-one (**7**, 1.0 mg, 0.5%).

The isolated mycotoxins were identified spectroscopically by Nuclear Magnetic Resonance (NMR) and Mass Spectrometry. NMR spectra were recorded on a Bruker Avance II operating at 500.13 MHz for <sup>1</sup>H and at 125.13 MHz for <sup>13</sup>C. Electrospray-High accuracy Mass Spectra were measured on a Bruker micrOTOF Q II (Bruker Daltonics, Billerica, MA, USA).

## 2.5 Antifungal Activity

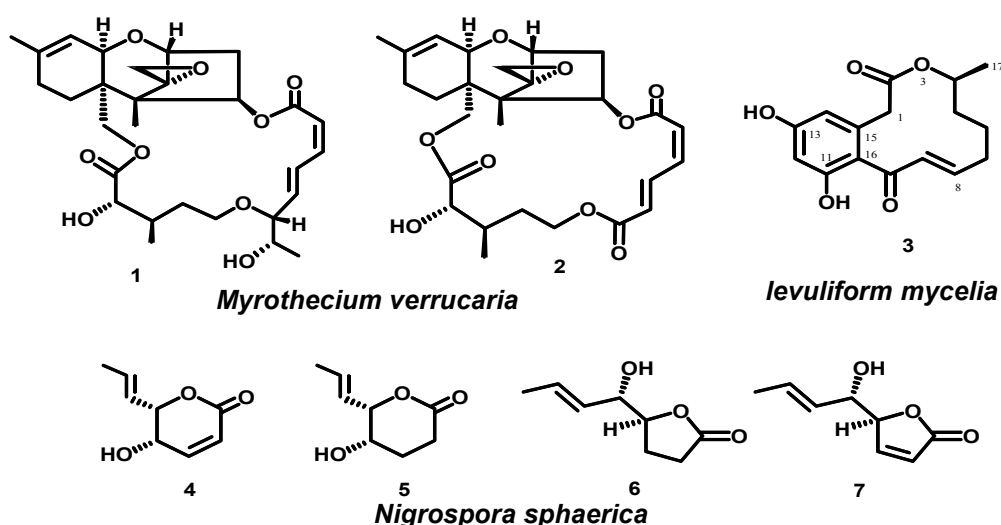
Direct bioautography of thin-layer chromatography was employed as the method for detecting fungitoxic substances [9]. A concentration level of 25  $\mu$ g/spot of each compound was assayed. The test was performed against *Fusarium lateritium* (FL), *Fusarium virguliforme* (FV) and *Botrytis cinerea* (BC). Benomyl, a systemic benzimidazole fungicide, was used as a test compound. The assay was repeated in triplicate.

## 3. RESULTS

The search for new sources of bioactive metabolites with antifungal activity led to the study of fungal strains isolated from bee pollen. Three strains, *Myrothecium verrucaria*, a yeast-like mycelia (LM336) and *Nigrospora sphaerica*, which showed the highest bioactivities, were chosen from a total of 133 isolates from two apiaries located in Mar del Plata and Balcarce, Buenos Aires. After an exhaustive extraction of the *M. verrucaria* culture, a crude organic extract was subjected to a preparative-scale thin-layer chromatography leading to the isolation of the mycotoxins, roridin A (**1**) and verrucaric acid (**2**) (Fig.1).

The structures of these compounds were elucidated by 1D and 2D-NMR (COSY, HSQC, HMBC, NOESY) and HR ESIMS. All the spectroscopical data were in agreement with those reported previously [10] (Tables 1 and 3 supplementary material). These compounds are known trichothecenes previously isolated from this species [11,12]. The crude organic extract of LM336 was subjected to a preparative-scale thin-layer chromatography leading to  $\alpha$ - $\beta$ -dehydrocurvularin (**3**) (Fig. 1), identified spectroscopically on the basis of 1D and 2D-NMR and HR ESIMS. All the spectroscopical data were in full agreement with those reported previously [13,14] (Tables 1 and 3 supplementary material).  $\alpha$ - $\beta$ -dehydrocurvularin, first isolated and identified by Munro et al. [15] and Hyeon et al. [16], is produced by a number of

fungal species, especially members of the genus *Alternaria* which are potent plant pathogens [17,18,14]. Finally, four compounds were isolated from the preparative-scale thin-layer chromatography of an ethyl acetate extract of *N. sphaerica*: phomalactone (4), 6-(1-propenyl)-3,4,5,6-tetrahydro-5-hydroxy-4H-pyran-2-one (5), 5-[1-(1-hydroxibut-2-enyl)]-dihydrofuran-2-one (6) and 5-[1-(1-hydroxibut-2-enyl)]-furan-2-one (7) (Fig. 1). The spectroscopical data were in full accordance with those reported previously [19,20] (Tables 2 and 3 supplementary material). In 1969, Evans, et al. [21] first isolated phomalactone and 6 from *Nigrospora* sp. Afterward, several authors reported the production of phomalactone from different fungal strains such as *Phoma* spp. [22] and *Hirsutella thomponii* [23]. Compound 7 was also isolated from *Nigrospora saccari* [19]. Recently, compound 5 was isolated for the first time as a natural product together with phomalactone from *Xylaria* spp. [20]. It was notable that most of the isolated compounds were present in the extracts in high concentrations (0.5 – 10%).



**Fig. 1. Mycotoxins isolated from beehive's pollen**

These compounds were tested for antifungal activity against *Fusarium virguliforme*, responsible for sudden-death syndrome of soybean and other phytopathogens, *Fusarium lateritium* and *Botrytis cinerea*, as the crude extracts of the producing organisms were all active. All the tested compounds have an important activity (inhibition halos from 10 to 20 mm) and are responsible for the antifungal activity of the extracts (Table 1).

**Table 1. Antifungal activity of isolated compounds 1 to 4. Diameter of inhibition zone in mm (+/- 1)**

Compound	<i>Botrytis cinerea</i>	<i>Fusarium lateritium</i>	<i>Fusarium virguliforme</i>
<b>1</b>	12	10	15
<b>2</b>	12	12	17
<b>3</b>	ND	10	15
<b>4</b>	ND	20	17
Benomyl	15	25	20

N.D.: not determined

#### 4. DISCUSSION

The isolated compounds have been previously reported to possess different bioactivities. Roridin A and Verrucarrin A are inhibitors of pollen development in *Arabidopsis thaliana* [10]. The antifungal activity of roridin A was also reported against *Sclerotinia sclerotiorum* [24].  $\alpha$ - $\beta$ -dehydrocurvularin showed sporulation-suppressing activity and also suppressed fungal germination [16] and showed antibiotic activity against Gram-positive and Gram-negative bacteria [25], nematocidal activity [26] and phytotoxicity [14]. Phomalactone is a known antibiotic [14] and an important herbicide [19]. It showed a weak fungicidal activity against *Phytophthora infestans* [27] and antiplasmodial activity when tested against a chloroquine-resistant strain of *P. falciparum* [20]. None of the isolated mycotoxins were previously reported as antifungals against the tested phytopathogen fungal strains. There are only few reports on the isolation of metabolites from fungal strains from beehive pollen mycoflora. L-tenuazonic acid and other tetramic acids and Altertoxin I were isolated from *Alternaria brassicicola* and *Alternaria raphani* [5]. Terrestric acid and hydroxyaspergillilic acid were isolated from *Trichoderma koningii* [28]. Other reports on the occurrence of toxigenic fungi in commercial bee pollen focused on the evaluation of the mycobiota occurring in bee pollen and the potential ability of the isolated fungi to produce aflatoxins and ochratoxin A [3]. Recently, aflatoxin B1 production by *Aspergillus parasiticus* in commercial bee pollen of Greek origin was studied [29]. It is noteworthy that mycotoxins, which are compounds of known toxicity, are not involved in the primary search for pollen or honey product contamination but the results showed in this work would indicate that strains with the ability for a variety of mycotoxin production may be widely present in these products, which are increasingly consumed.

#### 5. CONCLUSION

Seven mycotoxins were isolated in high yields (0.5-10%) from the crude extracts from the bee pollen: roridin A, verrucarrin A,  $\alpha$ - $\beta$ -dehydrocurvularin, phomalactone, 6-(1-propenyl)-3,4,5,6-tetrahydro-5-hydroxy-4H-pyran-2-one, 5-[1-(1-hydroxibut-2-enyl)]-dihydrofuran-2-one and 5-[1-(1-hydroxibut-2-enyl)]-furan-2-one. The compounds 1-4 revealed antifungal activity against phytopathogenic fungi.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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