



Short communication

## Aflatoxins produced by *Aspergillus nomius* ASR3, a pathogen isolated from the leaf-cutter ant *Atta sexdens rubropilosa*



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### ABSTRACT

*Aspergillus* spp. cause economic impacts due to aflatoxins production. Although the toxicity of aflatoxins is already known, little information about their ecological roles is available. Here we investigated the compounds produced by *Aspergillus nomius* ASR3 directly from a dead leaf-cutter queen ant *Atta sexdens rubropilosa* and the fungal axenic culture. Chemical analyses were carried out by high-resolution mass spectrometry and tandem mass spectrometry techniques. Aflatoxins B1 and G1 were detected in both the axenic culture and in the dead leaf-cutter queen ant. The presence of these mycotoxins in the dead leaf-cutter queen ant suggests that these compounds can be related to the insect pathogenicity of *A. nomius* against *A. sexdens rubropilosa*.

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### Introductory remarks

Fungus-farming ants cultivate a symbiont fungus as their primary food source. A group of fungus-farming ants started to feed the symbiont fungus with plant material in about 8–12 million years ago and they are known as leaf-cutter ants (Schultz and Brady, 2008). Leaf-cutter ants of the genera *Atta* and *Acromyrmex* cultivate the fungus *Leucoagaricus gongylophorus* (Licht et al., 2014) and are considered as dominant herbivores of the New World tropics (Schultz and Brady, 2008). An adult colony of *Atta* spp. can have millions of ants, which collect large amounts of plant material to feed their mutualistic fungus (Hölldobler and Wilson, 1990). Leaf-cutter ants are important for the ecosystem equilibrium in temperate areas of America and can also cause agricultural losses when they collect plant material from crops (Weber, 1966).

A new leaf-cutter ant colony starts with a queen ant that is fecundated during the nuptial flight. Most of the queen ants are predated and a small number succeed in starting a new colony (Weber, 1966). Leaf-cutter ants are associated with antibiotic-producing bacteria to protect their colonies (Santos et al., 2004; Haeder et al., 2009; Seipke et al., 2011; Schoenian et al., 2011). Fungus-farming ants possess evolved glands to feed symbiont bacteria

hosted on their exoskeleton and, in turn, bacteria produce antimicrobial compounds (Currie et al., 2006). Ant-symbiont bacteria are considered a promising source of antibiotics, the natural products dentigerumycin and selvamicin are novel antifungal agents produced by ant-symbiont bacteria (Oh et al., 2009; Van Arnem et al., 2016).

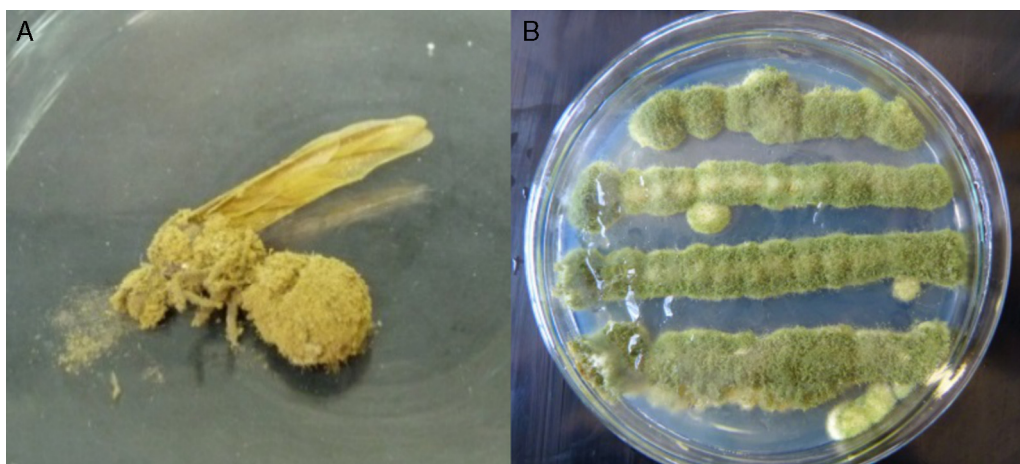
Fungal pathogens also play a role in this multilateral symbiotic system. The well-known specialized fungal garden pathogen *Escovopsis* spp. can destroy colonies of fungus-farming ants (Currie et al., 1999). Other fungi from the genera *Aspergillus*, *Fusarium*, and *Trichoderma*, can also parasitize leaf-cutter ants colonies (Poulsen et al., 2006; Pagnocca et al., 2012). *Aspergillus nomius*, for instance, produces insecticidal natural products such as nominine and aspernomine (Gloer et al., 1989; Staub et al., 1992). The main goal of this work was to identify the major secondary metabolites produced by *A. nomius* ASR3 after its proliferation in an *Atta sexdens rubropilosa* queen ant.

### Materials and methods

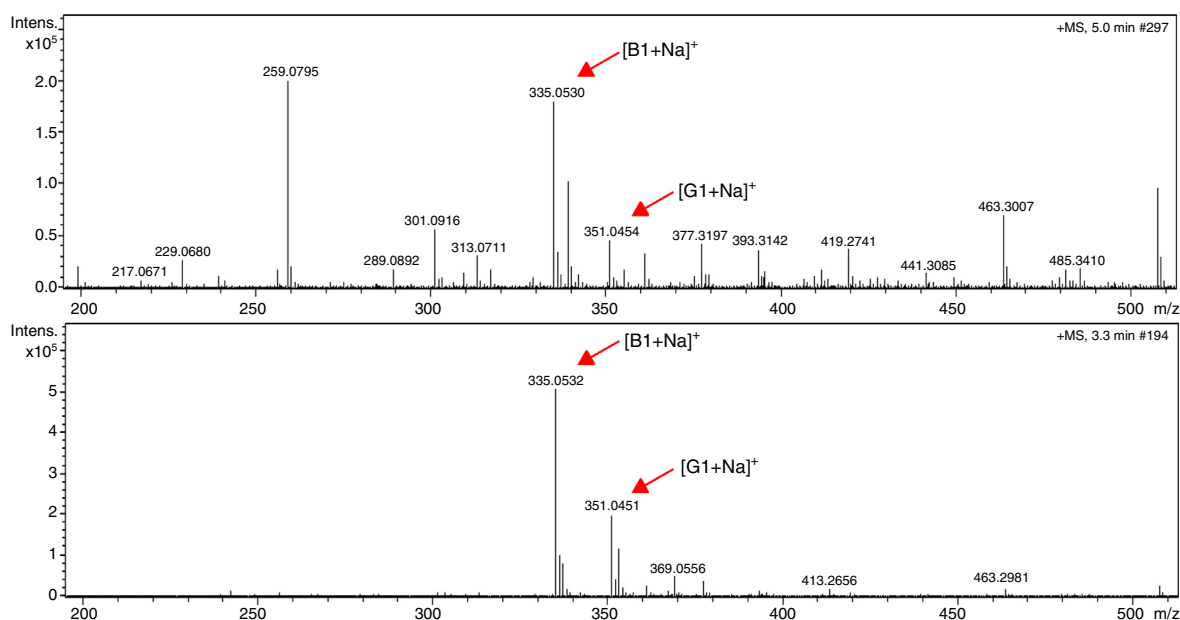
Fungal spores were collected from the body of an *A. sexdens rubropilosa* queen ant infested by a greenish-yellow fungus and were inoculated on Petri plates containing PDA medium. The plates were incubated at 30 °C until fungal growth and sporulation. Spores of the isolated fungus were preserved at –80 °C in cryotubes containing a 20% glycerol aqueous solution. The fungal strain ASR3

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**Fig. 1.** *Atta sexdens rubropilosa* queen infested by *Aspergillus nomius* (A), and *Aspergillus nomius* ASR3 isolated from the ant (B).



**Fig. 2.** ESI-HRMS spectra of ethyl acetate extracts of *A. sexdens* queen (A) and *A. nomius* ASR3 cultivated in PDA medium (B). The red arrows indicate the aflatoxins ions.

was identified by sequencing the ITS (internal transcribed spacer) region using the primers ITS-1 (5'TCCGTAGGTGAACCTGCGG3') and ITS-4 (5'TCCTCCGCTTATTGATATGC3'). The species (GenBank accession number KY986877) was identified by comparison (BLAST search) with sequences deposited at NCBI.

Spores of ASR3 were inoculated on 80 mm Petri dishes containing 25 ml of PDA medium. The fungal culture and the control (PDA only) were incubated for 14 days at 30 °C. After this period, the culture medium of each plate was collected and extracted with 60 ml of ethyl acetate. The infested queen ant was also extracted with ethyl acetate. Finally, the extracts were filtered and evaporated to dryness.

The crude extracts and authentic Sigma–Aldrich standards of aflatoxins B1 and G1 solutions in methanol/water (1:1 v/v) were analyzed by direct infusion into the electrospray (ESI) source of the microTOF Q II-ESI-TOF mass spectrometer (Bruker Daltonics). Formic acid was added in the samples before the ESI-MS acquisitions to improve the protonation. The data acquisition was performed at the positive ion-monitoring mode and the MS/MS spectra were obtained by collision of the selected ions with nitrogen gas. A solution of trifluoroacetic acid (TFA-Na<sup>+</sup>) at 10 mg ml<sup>-1</sup>

was used for internal calibration, the capillary voltage was set for 3.5 kV and the drying temperature was 180 °C.

## Results and discussion

The fungus ASR3 was isolated from a queen of the leaf-cutter ant *A. sexdens rubropilosa*, which had died after the nuptial flight and its body was infested by a greenish-yellow fungus (Fig. 1). The isolated fungus was identified as *A. nomius* by a BLAST search of the ITS sequence from ASR3 strain, which revealed 99% of identity match with ITS sequence for *A. nomius* MW16 (GenBank accession number KF312149.1). This species has been described as pathogenic for social insects such as leaf-cutting ants (Poulsen et al., 2006) and termites (Chouvenec et al., 2012); however the compounds involved in the pathogenesis remain underexplored.

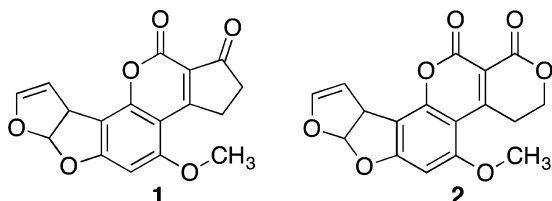
In order to identify the compounds possibly involved in the toxicity of *A. nomius* ASR3 against *A. sexdens rubropilosa*, the fungus was grown axenically in PDA Petri dishes and extracted with ethyl acetate; the dead queen ant was also directly extracted with ethyl acetate. The crude extracts were then analyzed by ESI-HRMS and ESI-MS/MS with no previous separation or other sample

**Table 1**  
Compounds produced by *Aspergillus nomius* identified using ESI-HRMS and ESI-MS/MS.

Compound	Axenic fungal culture		Queen ant		Fragment ions ( <i>m/z</i> ) <sup>a</sup>
	Molecular formula	[M+H] <sup>+</sup> (error (ppm))	Molecular formula	[M+H] <sup>+</sup> (error (ppm))	
Aflatoxin B1	C <sub>17</sub> H <sub>12</sub> O <sub>6</sub>	313.0712 (1.6)	C <sub>17</sub> H <sub>12</sub> O <sub>6</sub>	313.0711 (1.3)	298, 285, 270, 257, 241
Aflatoxin G1	C <sub>17</sub> H <sub>12</sub> O <sub>7</sub>	329.0661 (1.5)	C <sub>17</sub> H <sub>12</sub> O <sub>7</sub>	329.0658 (0.6)	311, 283, 243, 215

<sup>a</sup> Fragment ions are common for aflatoxins produced by *A. nomius* ASR3 and authentic standards. Protonated aflatoxins ions were observed after the addition of formic acid in the samples.

preparation. The major ions at *m/z* 335.0530 and *m/z* 351.0454 were present in both extracts, but absent in PDA control medium extract (Fig. 2). The high-resolution mass analysis suggested their identities as aflatoxin B1 (**1**) (C<sub>17</sub>H<sub>13</sub>O<sub>6</sub><sup>+</sup>, error 1.6 ppm) and aflatoxin G1 (**2**) (C<sub>17</sub>H<sub>13</sub>O<sub>7</sub><sup>+</sup>, error 1.5 ppm; Table 1). The fragmentations patterns at ESI-MS/MS for these ions were consistent with the literature data for **1** and **2** (Cavaliere et al., 2007; Sirhan et al., 2013; Tóth et al., 2013) and matched with authentic standards (Fig. S2–S5). These fragmentations involve losses of CO, CO<sub>2</sub>, H<sub>2</sub>O, and methyl radicals, which are common eliminations for lactones and aromatic methoxyl group (Crotti et al., 2009; Vessecchi et al., 2011; Demarque et al., 2016)



Aflatoxins are mycotoxins generally produced by *Aspergillus* spp. that have been described as toxic to humans, animals and insects (Matsumura and Knight, 1967; Kirk, 1971; Gacem and Hadj-Khelil, 2016). The carcinogenic effects of aflatoxins B1 and G1 are due to the CYP450 activation of the double bond in the ring A to an active epoxide, which covalently binds to the DNA (Niu et al., 2008). However, despite the knowledge about the production and biological activity of aflatoxins, little is known about the ecological functions of these natural products. It has been shown that some insects such as the earworm *Helicoverpa zea* are able to detoxify aflatoxins (Niu et al., 2009), while aflatoxins impair the proper development of other insects (Matsumura and Knight, 1967; Gunst et al., 1982). The high toxicity of aflatoxins and the detection in the dead leaf-cutter queen ant, suggest that these compounds may be involved with the pathogenicity of *A. nomius* ASR3 for the leaf-cutter ants *A. sexdens rubropilosa*.

#### Authors' contributions

LCAV and FSN contributed with the collection and identification of the leaf-cutter queen ant. EASJ and CRP contributed with the laboratory work, data analysis and drafted the paper. NPL contributed with mass spectrometry data acquisition and analysis. MTP supervised the laboratory work and contributed with critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

#### Conflicts of interest

The authors declare no conflicts of interest.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bj.2017.05.001.

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