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Amatoxin and phallotoxin composition in species of the genus *Amanita* in Colombia: A taxonomic perspective

N. Vargas [a,b], A. Bernal [a], V. Sarria [c], A. Franco-Molano [b], S. Restrepo [a,*]

[a] Laboratorio de Micologia y Fitopatologia, Universidad de Los Andes, Colombia
[b] Laboratorio de Taxonomía y Ecología de Hongos, Universidad de Antioquia, Colombia
[c] Departamento de Química, Universidad de Los Andes, Colombia

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ABSTRACT

Some species in the genus *Amanita* have a great variety of toxic secondary metabolites. They are characterized macroscopically by having a white spore print and free gills, and microscopically by the presence of a divergent hymenophoral trama. Some species of *Amanita* present in Colombia were chemically characterized by analyzing their toxin composition using HPLC. Samples were collected in oak (*Quercus humboldtii*) and pine (*Pinus radiata*) forests. Twelve species were recovered, *Amanita fuligineodisca, Amanita xylinivolva, Amanita flavoconia, Amanita rubescens, Amanita bisporigera, Amanita muscaria, Amanita humboldtii, Amanita sororcula, Amanita brunneolocularis, Amanita colombiana, Amanita citrina*, *Amanita porphyria* as well as two unreported species. Results showed that most of the analyzed species have α -amanitin in concentrations ranging from 50 ppm to 6000 ppm. Concentrations of α-amanitin in the pileus were significantly greater than in the stipe. Phalloidin and phallacidin were only present in *A. bisporigera*. Chromatographic profiles are proposed as an additional taxonomic tool since specific peaks with similar retention times were conserved at the species level.

1. Introduction

Fungi in the genus *Amanita* form ectomycorrhizal associations with plants, stimulating their growth and defense against diverse sources of stress (Sauter and Hager, 1989). They can also produce some of the most toxic compounds in the world. The genus is characterized by producing a whitish spore print, gills that are free from the stipe and the presence of a universal veil. Microscopic characteristics include the divergent hymenophoral trama and amyloid or inamyloid spores (Bas, 1969).

A prominent characteristic of most *Amanita* species is the presence of highly toxic compounds. Recent reviews have discussed the occurrence, chemistry and toxicology of these peptides (Karlson-Stiber and Persson, 2003; Vetter, 1998 and Li and Oberlies, 2005). Chemically, the structures of the compounds isolated from *Amanita* species characterized to-date can be subdivided into the following six categories: peptides, amavadin, isoxazoles, simple aminoacids, sterols and ceramides. Some *Amanita* species produce the most powerful peptides (toxins) known as cyclopeptides that contain a sulfur-linked tryptophan unit and some unusual hydroxylated amino acids (Li and Oberlies, 2005). According to Enjalbert et al. (1993) the distribution of the cyclopeptides can vary along the basidiocarp, in some cases the pileus being the most toxic part. These peptides considered the major toxins from *Amanita*, are classified into three groups: amatoxins, phallotoxins and virotoxins.

Amatoxins (slow-acting poisons) are 10–20 times more toxic than phallotoxins and virotoxins (quick acting poisons). It has been concluded that amatoxins are

* Corresponding author.
E-mail address: srestrep@uniandes.edu.co (S. Restrepo).

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probably responsible for fatal human poisonings (Li and Oberlies, 2005). The virotoxins are the most recently discovered toxins in *Amanita* and have only been found in *Amanita virosa*. Toxicological studies have focused on the famous death cap *Amanita phalloides* which was one of the earliest toxic mushrooms identified and in which phallotoxins were found for the first time. In addition, several other *Amanita* species, including *Amanita bisporigera, Amanita verna* and *A. virosa* have been found to produce phallotoxins and amatoxins as well. The specificity and sensitivity of the high-performance liquid chromatography (HPLC) allows the development of rapid and very sensitive assays for the main amatoxins (amanitin α, β and γ), and the chief phallotoxins, (phallisacin, phallacidin, phallisin, phalloidin and phalloin (Enjalbert et al., 1993). The major importance in the identification of toxins in *Amanita* is the fact that Amatoxins, including α- and β-amanitin, are specific inhibitors of eukaryotic RNA polymerase II and are thus important molecular biological tools in addition to being potent natural toxins. Functional amatoxins cannot be artificially synthesized (Wieland and Faulstich, 1991). Phallotoxins also play a role in the molecular biology laboratory, as they bind to actin and stabilize it in the filamentous F-actin form.

Preliminary studies in Agaricales in Colombia documented high levels of diversity (Halling and Ovrebo, 1987; Halling, 1989a; Halling, 1989b; Horak and Halling, 1991; Singer et al., 1995; Franco, 1999) including the description of several new species, most of them with ectomycorrhizal association. In Colombia, macromycetes have traditionally been studied using morphological tests as the base for a taxonomical description but no chemical studies have been carried out in order to determine toxin composition and relations among species based on chemical profiles. Here, we report the use of a chromatographic method to determine the nature and concentrations of amatoxins and phallotoxins from fruiting bodies of species for which chemical composition has never been assessed and introduce chromate-chemical profiles for Colombian *Amanita* spp. taxonomy.

2. Materials and methods

2.1. Fungal collection

Fruiting bodies of the genus *Amanita* were collected from March 2007 through June 2008 from different localities in six departments in Colombia: Antioquia, Boyacá, Santander, Tolima, Cundinamarca and Valle del Cauca. Collected fungi were associated with *Quercus humboldtii* and *Pinus radiata.* All fruiting bodies were documented, and macroscopic and microscopic descriptions were made. Color designations given within parentheses are from Kornerup and Wansher (1978) or from The Online Color Auction Chart. All measurements of microscopic structures were made in 3% KOH and other cross sections were treated with Melzer's reagent. After documentation, the fruiting bodies were dried and packaged in plastic bags, stored at the ANDES Herbarium (Universidad de los Andes, Bogotá, Colombia) or HUA Herbarium (Universidad de Antioquia) and registered in the Specify database. Chemical and molecular analysis from herbarium specimens were made from *Amanita colombiana* and *Amanita humboldtii.* According to the phenotypic characters analysis we assigned the specimens into putative species. We selected one or two representative specimens from each species to carry out the chromatography.

2.2. Toxin extraction

One gram of the pileus and stipe tissues were weighted, frozen in liquid nitrogen and then ground to powder. Amatoxins and phallotoxins were extracted by sonication according to a previously described protocol (Enjalbert et al., 1996) with some modifications. Briefly, after the tissues were ground to powder, the toxins were extracted with 0.5–4 mL of extraction solution (methanol-water, 0.01 M, - HCl 5:4:1 (*v/v/v*)); the extraction sample volume was adjusted per sample weight; the sample was sonicated for 2 min using a Vibracell model, the sample was immersed in ice to avoid heating. Then, the extract was centrifuged at 10,000 g per 10 s and 4 C. The supernatant was recovered and subsequently the pellet was washed with extraction solution, to obtain a second supernatant. Each sample solution was filtered through a Millipore (0.2 μm) filter. 10 μl of the liquid culture media MMN where *Amanita rubescens* was grown were also filtered.

2.3. HPLC analysis

Five μl of filtrated solution was injected into the chromatograph. Toxins were separated by reversed-phase liquid chromatograph using an Agilent series 1200 system. Separations were carried out in a C18 HPLC Column (125 × 4 mm U.D., particle size 5 UM, Agilent Technologies). The pattern of mobile phases and the elution profiles were followed as described by Zhang et al. (2005). Briefly, the mobile phase (A) was 0.02 M aqueous ammonium acetate-acetonitrile (90:10, *v/v*) and phase (B) was 0.02 M aqueous ammonium acetate-acetonitrile (76:24, *v/v*). pH of solvents was adjusted to 5.0 with glacial acetic acid. The solvents were degassed by sonication prior to use. The elution profile consisted of four isocratic steps: (1) 0–15 min, phase A 100%, phase B 0%, (2) 15–40 min, phase A 95%, phase B 5%, (3) 40–50 min, phase A 20%, phase B 80% (4) 50–60 min, phase A 0%, phase B 100%. The flow rate was 1 mL/min. A curve was obtained with injections of each toxin at different concentrations: 5, 10, 20, 30, 120 and 200 ppm. The correlation regression coefficients of both curves were greater than 0.999. Identification of the toxins was based on retention times (Rt) and the coinjection of standards (donated by Dr. Heinz Faulstich, Max-Planck-Institut (Germany)). A blank extraction was also injected. One gr of the pileus context and stipe context were used in order to analyze the toxin composition from the two species with the parasite.

2.4. Statistical analysis

Chemical profiles using presence or absence of chromatographic peaks in the zone between 0 and 5 min were analyzed by Principal coordinates analysis (PcoA).