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SHORT COMMUNICATION

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An insect parasitoid carrying an ochratoxin producing fungus

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Abstract The insect parasitoid *Prorops nasuta* has been introduced from Africa to many coffee-producing countries in an attempt to control the coffee berry borer. In this paper, we report on the sequencing of the ITS LSU-rDNA and beta-tubulin loci used to identify a fungus isolated from the cuticle of a *P. nasuta* that emerged from coffee berries infected with the coffee berry borer. The sequences were compared with deposits in GenBank and the fungus was identified as *Aspergillus westerdijkiae*. The fungus tested positive for ochratoxin A production, with varying levels depending on the media in which it was grown. These results raise the possibility that an insect parasitoid might be disseminating an ochratoxin-producing fungus in coffee plantations.

Introduction

Parasitoids have been long used in biological control programs aimed at controlling important insect pests. One of the most widely used methodologies involves a concept known as classical biological control, whereby a search for natural enemies is conducted in the area of origin of the pest, with the objective of identifying possible agents for introduction in new areas colonized by the pest, where natural enemies are not present or are ineffective. One aspect of this methodology, which is rarely discussed, is the possible presence of plant pathogenic fungal spores on the parasitoid cuticle. For example, Nemeye et al. (1990) conducted a laboratory study aimed at determining the possible phytosanitary risk of introducing *Heterospilus coffeicola* (Schmiedeknecht) (Hymenoptera: Braconidae), a parasitoid of the coffee berry borer [*Hypothenemus hampei* (Ferrari); Coleoptera: Curculionidae], into coffee-producing countries. Due to the difficulty of rearing this particular parasitoid, they used a closely related species, *Heterospilus prosopidis*, and showed that it was able to carry either *Colletotrichum coffeanum* F. Noack, the causal agent of coffee berry disease, or *C. gloeosporioides* (Penz.) Penz. & Sacc., the causal agent of mango anthracnose (the species was not reported with certainty). They extrapolated from their laboratory study and concluded that extreme caution must be taken to avoid introducing a serious plant pathogen into a country in an attempt to control the coffee berry borer. Similarly, Crowe (1963) reported the presence of uredospores of *Hemileia vastatrix* Berkeley & Broome, the causal agent of coffee rust, on two platygasterid biological control agents, *Leptacis kivuensis* Risbec and *Synopeas* sp. It is believed that the presence of the leaf spot fungus *Cercospora eupatorii* Peck, which is native to the United States, into Australia came as a consequence of introducing *Procecidochares utilis* Stone (Diptera: Tephritidae) from Hawaii for weed biocontrol (Dodd 1961).

For the past 3 years, we have been studying fungal endophytes in coffee plants. In July of 2003, we received a sample of coffee berries from Chinchiná, Caldas (Colombia), from which we were planning to isolate fungal endophytes. Much to our surprise, we noticed the presence of the coffee berry borer parasitoid *Prorops nasuta* (Waterston) (Hymenoptera: Bethylidae) shortly after receiving the berries. *P. nasuta* is endemic to Central Africa (Waterston 1923; Hargreaves 1935; Le Pelley 1968) and

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has been introduced on 13 occasions to many coffee producing countries (e.g., Mexico, India, Indonesia, Peru, Sri Lanka, Brazil, Colombia, Honduras, Guatemala, and Ecuador) in an attempt to reduce losses caused by the coffee berry borer. The parasitoid was introduced to Colombia in 1991 (Bustillo et al. 1996) and has become established (Quintero et al. 1998). As part of our survey of fungal endophytes on coffee, we decided to sample the parasitoid to determine what fungal spores it might be carrying.

Materials and methods

To assess for the presence of fungal spores on the cuticle of *P. nasuta*, the specimen was washed in sterile distilled water and an aliquot was plated on yeast malt agar (YMA). The same was done for a coffee berry borer specimen isolated from the same batch of berries. One of the fungi isolated from both specimens was an *Aspergillus* species (IBL 03035=NRRL 35193 for the *Prorops* isolate and IBL 03036=NRRL 35197 for the coffee berry borer isolate). These were individually re-isolated as pure cultures in YMA, followed by DNA extraction and sequencing of the ITS-lsu rDNA and beta-tubulin loci (see Peterson et al. 2005 for molecular methodology). The two *Aspergillus* isolates were grown in YMA, potato dextrose agar, or yeast extract plus sucrose at 25°C, and the media was sampled at 35 days for ochratoxin A (OTA) using the method of Bragulat et al. (2001). *A. ochraceus* G. Wilh. (provided by J. White, Rutgers University, NJ, USA) grown in the three different media and kept at 25°C was used as a positive control. An OTA (Sigma-Aldrich Co., St. Louis, MO) standard curve was prepared and used for quantification.

Results

The sequences for the two strains, which were identical at both loci, were compared to deposits in GenBank. BLAST results on the beta-tubulin sequences indicated 98–100% sequence similarity with *A. ochraceus* records AY819975, AY819976, and AY819974, isolates identified as *A. westerdijkiae* Frisvad & Samson by Frisvad et al. (2004). Other BLAST hits on sequences from authentic *A. ochraceus* isolates (AY819970, AY819971, and AY819973) displayed 93–94% sequence similarity, and our isolates were identified as *A. westerdijkiae*. Before the description of the new species (Frisvad et al. 2004), *A. westerdijkiae* isolates would have been identified as *A. ochraceus*. The DNA sequences for the ITS-lsu rDNA and the beta-tubulin for NRRL 35193 (*Prorops* isolate) are accessioned in GenBank as DQ250527 and DQ250528, respectively, and as DQ250529 and DQ250530 for NRRL 35197 (coffee berry borer isolate). Both isolates in our study had the *A.westerdijkiae* phenotype (Frisvad et al. 2004), and produced OTA in all media tested, although at much lower levels than the *A. ochraceus* sample (Table 1).

Table 1 Ochratoxin A production (μg OTA g^{-1} media±STD. dev.) 35-day post-inoculation by *A. ochraceus* (control) and two *A. westerdijkiae* isolates from *P. nasuta* and the coffee berry borer (*CBB*)

	YMA	PDA	YES
A. ochraceus	4.6±1.10	6.1±1.26	26±2.60
A. westerdijkiae from *Prorops*	0.7±0.04	0.6±0.10	1.1±0.35
A. westerdijkiae from CBB	0.6±0.42	0.9±0.04	3.5±0.56

Fungi were grown in yeast malt agar (*YMA*), potato dextrose agar (*PDA*) or yeast extract plus sucrose (*YES*). Means shown are for four replicates

Discussion

A. westerdijkiae has been reported from surface-disinfected green coffee beans from India, from green coffee beans in Venezuela, and from coffee samples from Brazil, India, Indonesia, Kenya, and Venezuela (Frisvad et al. 2004). All these coffee-related isolates are OTA producers (Frisvad et al. 2004). Several other species in the genus *Aspergillus* are known to produce OTA, a known contaminant in many agricultural commodities, including coffee (Bennett and Klich 2003). Similarly, some *Penicillium* species are OTA producers, including some endophytic species in coffee (Vega et al. 2006). The finding that a parasitoid of a very important agricultural pest of coffee is carrying an ochratoxin-producing fungus is significant, in large part because it could contribute to the spread of OTA-producing fungi in coffee. Ochratoxins have been reported in many agricultural commodities, and the European Union (2005) has recently established maximum OTA levels in coffee.

P. nasuta females enter coffee berries infested with the coffee berry borer and deposit a single egg in a larva or pupa, of which there can be approximately 74 per berry (Bustillo et al. 1998). If the parasitoid is carrying *A. westerdijkiae* spores on its cuticle, these could be deposited inside the coffee berry, and these spores could serve as an inoculum for contaminating the coffee seeds. Similarly, coffee berry borers that are not parasitized by *P. nasuta* could emerge from the berry carrying fungal spores on its cuticle, thus, moving them within the coffee agroecosystem. Pérez et al. (2003) reported 40 fungal species in 22 genera on the cuticle of the coffee berry borer, including various *Aspergillus* species. Vega and Mercadier (1998) reported the presence of *A. ochraceus*, *A. flavus* Link, and *A. niger* Tiegh. in field collected coffee berry borers. Several endophytic *Aspergillus* species have also been isolated from coffee plants (Vega et al., unpublished data). If there is coffee berry borer damage to the berry and seeds, these could be screened out during harvesting and processing to reduce the possibility of having fungus-contaminated material in the final product. However, this option would be costly.

Our results point at the possibility of exporting toxigenic fungi together with parasitoids in classical biological control schemes. Nevertheless, it is important to observe that regardless of whether the parasitoid is carrying a toxigenic fungus, the fungus might already exist in the field

in the country where the parasitoids would be introduced. Furthermore, parasitoids might not become established or might cause low parasitism rates, thus, not playing an important role in transferring the fungus into the coffee berry or to the coffee berry borer. In addition, the fact that an insect pest or biocontrol agent is carrying a toxigenic fungus does not necessarily imply that the fungus will become established in the commodity or that fungal metabolites will be produced. There is clearly a strong need for detailed research on the associations between toxigenic fungi carried by insect pests and biocontrol agents, and whether these have any effect at all on fungal infection rates in agricultural commodities.

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