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ORIGINAL ARTICLE

Aspergillus oryzae NRRL 35191 from coffee, a non-toxigenic endophyte with the ability to synthesize kojic acid

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Abstract *Aspergillus oryzae* NRRL 35191 was isolated as an endophyte from coffee leaves and found to produce kojic acid (KA) in culture. When inoculated into cacao seedlings (*Theobroma cacao*), *A. oryzae* grew endophytically and synthesized KA in planta. Cacao seedlings inoculated with *A. oryzae* produced higher levels of caffeine than non-inoculated ones. *Aspergillus oryzae* may be a useful endophyte to introduce to cacao since it grows non-pathogenically and induces the caffeine defense response that may make the plant more tolerant to insects and pathogens.

Keywords *Aspergillus oryzae* · Cacao · Coffee · Endophyte · Kojic acid

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Introduction

Penicillium and *Aspergillus* (Eurotiales: Trichocomaceae) are cosmopolitan fungi capable of synthesizing a multitude of secondary metabolites with a broad range of functions (Cole and Schweikert 2003; Keller et al. 2005). Some of these metabolites are known to play a role in the habitats in which these fungi occur and influence whether they are considered pathogens, endophytes, or opportunistic organisms. At times, the delineation between these categories is vague and may be only temporal or circumstantial. For instance, an endophyte can grow asymptomatically or cause disease depending on the developmental stage of a plant or its physiological status (White and Morgan-Jones 1996). Meanwhile, opportunistic organisms can exploit weakened plants to cause disease (Kay et al. 2002; Klich 2007; Leger et al. 2000). Moreover, some researchers, such as Schulz and Boyle (2005), have suggested that these organisms exist on a continuum from saprophyte to endophyte to pathogen.

Aspergillus oryzae (Ahlburg) Cohn is known to produce kojic acid (KA; 5-hydroxy-2-hydroxymethyl-γ-pyrone; $C_6H_6O_4$), a metabolite with insecticidal, antibacterial, and antifungal properties (Alverson 2003; Burdock et al. 2001; Dowd 1999, 2002; US Environmental Protection Agency

1997). This microorganism has been used for hundreds of years in the food industry for the preparation of many oriental foods, such as miso, sake and shoyu, and is generally regarded as safe (Bentley 2006).

Several reports indicate fungal endophytes have the potential to control pathogens in vitro or in planta when either naturally abundant or upon inoculation (Arnold et al. 2003; Cao et al. 2005; Clarke et al. 2006). Although many of these endophytes produce bioactive secondary metabolites (Guo et al. 2008), activation of plant defense mechanisms seems to account for the biological control action (Van Wees et al. 2008). An extensive number of endophytes have been found associated with tropical plants, including coffee and cacao, with potential application as biological control agents (Arnold et al. 2003, Rubini et al. 2005; Vega et al. 2010).

We report the isolation of *Aspergillus oryzae* NRRL 35191, a non-toxigenic endophyte from coffee with the ability to synthesize KA. Moreover, we discuss the role of KA and increased caffeine levels found in *Theobroma cacao* seedlings inoculated with *A. orzyae*.

Materials and methods

Fungal isolation and identification

Aspergillus oryzae NRRL 35191 was isolated as an endophyte from coffee leaves (*Coffea arabica* L.) collected in Chinchiná, Caldas, Colombia (5º00′N, 75º36′W), as part of a survey of fungal endophytes in coffee (Vega et al. 2010). See Vega et al. (2006) for endophyte isolation methods. The isolate has been deposited in the ARS Culture Collection in Peoria, Illinois (NRRL 35191). DNA was extracted from mechanically broken conidia and mycelium of *A. oryzae* NRRL 35191, purified using phenol extraction of proteins and binding to a silica matrix, and the ITS and partial large subunit rDNA were amplified and sequenced using published procedures (Serra and Peterson 2007).

Kojic acid extraction from fungal culture

An amount of 100 mL of yeast extract sucrose (YES) medium (2% yeast extract and 20% sucrose) was inoculated with 1×10^7 spores mL^{-1} suspension (in DI water) of *A. oryzae* NRRL 35191 and placed in a shaker at 100*g* for 7 days at 25°C. The broth was filtered through glass wool and the filtrate was centrifuged at 10,000*g* for 5 min. The pH of the supernatant was adjusted to 3.5 and extracted 3 times with 15 mL ethyl acetate. The organic phase was evaporated to dryness under vacuum.

Seedling inoculation with *A. oryzae*

A pod from a self-compatible Amelonado cocoa tree that was hand pollinated in a Rutgers University greenhouse was cracked open, the seeds cleaned of mucilage (scraped off), and the seed coat removed using a scalpel. Seeds were immersed in 70% ethanol for 1 min and then submerged in a 10% bleach solution for 1 h, followed by rinsing (3 times) with sterile autoclaved water. Seeds were then placed in vermiculite in flats. After 3 weeks, 20 cacao seedlings were selected based on uniformity (plant height 20–25 cm, 8–10 leaves). Ten were used as controls and the other ten were inoculated with 0.5 mL of 1×10^7 spores ml^{-1} *A. oryzae* NRRL 35191 suspension in 0.01% Tween 80, applied to the leaves with a brush. Control seedlings were treated similarly but without the spore suspension. The inoculated seedlings were enclosed in a plastic bag for 1 week and kept at 25°C in a 12-h daily photoperiod (80 μmol m^{-2} s^{-1}). Three weeks after inoculation, seedlings were surface sterilized by rinsing with tap water, immersed in 70% ethanol for 1 min and a 10% bleach solution for 1 h. The seedlings were then rinsed three times with sterile autoclaved water, and small square sections (0.5–1.0 cm) of leaves, stem, and roots from both inoculated and control plants were plated on potato dextrose agar to assess for the presence of *A. oryzae* NRRL 35191. Plates were kept at 25°C under 20 μmol m^{-2} s^{-1} continuous light. The remaining plant parts were used for kojic acid and caffeine analysis.

Kojic acid and caffeine analysis

The control and inoculated seedling material (0.5–1 g) were ground in liquid nitrogen and extracted with 150 mL methanol, stirring overnight. The methanolic extract was filtered and concentrated to dryness under vacuum. The sample was redissolved in 0.5 mL methanol and derivatized with Tri-sil reagent (Pierce, Rockford, IL, USA) and analyzed by GC-MS (HP 6890 GC, HP5973 MSD). A KA commercial standard (Sigma-Aldrich, St. Louis, MO, USA) was converted with Tri-sil to its trymethylsilylated derivative and injected in the GC-MS (Column DB-1; the initial temperature of 70°C was held for 2 min, then ramped up to 240°C at 10°C min^{-1}, and held for 30 min; MS Quadrupole detector set at 280°C and ionization energy of 70 eV, and mass scan range from 40 to 550 amu at 1.97 scans s^{-1}). KA was identified by matching the retention time of the derivatized standard with the samples as well as by comparison of their mass spectra.

Caffeine (1,3,7-trimethylxanthine) was analyzed by grinding 0.5–1 g leaves in liquid nitrogen and stirring overnight with 70% ethanol. After filtration, the ethanol extract was concentrated in vacuo at 35°C and the pH raised to 10 with 14 N ammonium hydroxide. The sample

was partitioned three times with chloroform, and the chloroform fractions combined and dried in vacuo at 35°C (Aneja and Gianfagna 2001). The sample was redissolved in 0.5 mL of methanol and injected into a GC/MS under the same conditions as mentioned above for KA. Caffeine was identified and quantified by comparing the mass spectra and retention time of the sample to a commercial standard from Sigma-Aldrich.

Aflatoxin and ochratoxin analysis

Aspergillus oryzae NRRL 35191 was analyzed for the production of aflatoxins using an AgraStrip aflatoxin test (Romer Labs, Union, MO, USA), an immunochromatographic assay based on an inhibition immunoassay format with a detection limit of 4 ppb. This fungal isolate was also analyzed for the production of ochratoxins in YES media following the method described in Vega et al. (2006) using HPLC with a fluorescence detector.

Caffeine spiked plate assay

A. oryzae was inoculated on potato dextrose agar media spiked with caffeine at concentrations of 2.8, 28, 56 and 100 ppm. Three replicate plates were used for each concentration. Growth rate was measured in centimeters after 1 week.

Results

The DNA sequence from *A. oryzae* NRRL 35191 (GenBank # EF591304) was compared to sequences in GenBank using BLAST and differed from the sequence of the ex type culture of *A. oryzae* at only 3 out of 1,152 nucleotide positions. Thus, the coffee isolate warrants inclusion in the *A. oryzae/A. flavus* species group (Peterson et al. 2001). Because the ID gene of the *A. flavus/A. oryzae* complex is insufficiently variable to clearly distinguish the two taxa, the *omt12* locus (O-methyl transferase) was also amplified and sequenced as detailed by Geiser et al. (2000). The *omt12* locus from *A. oryzae* NRRL 35191 is identical with the *omt12* sequences for the *A. oryzae* lineage reported by Geiser et al. (2000) and has been deposited in GenBank (EF641268).

Distinguishing *A. oryzae* and *A. flavus* species from one another can be difficult since both species have the genes predicted to be involved in the aflatoxin synthetic pathway, but the aflatoxin pathway genes are only expressed in *A. flavus* (Keller et al. 2005). When grown in YES media, *A. oryzae* NRRL 35191 did not produce ochratoxin A or B or aflatoxins B1, B2, G1 and G2, but produced kojic acid, identified as its trimethylsilylated derivative with the following fragmentation pattern 45(11), 73(58), 75(12), 147(23), 271(100), 272(26), 273(11), $M^{+\cdot}$ 286(1) (Fig. 1).

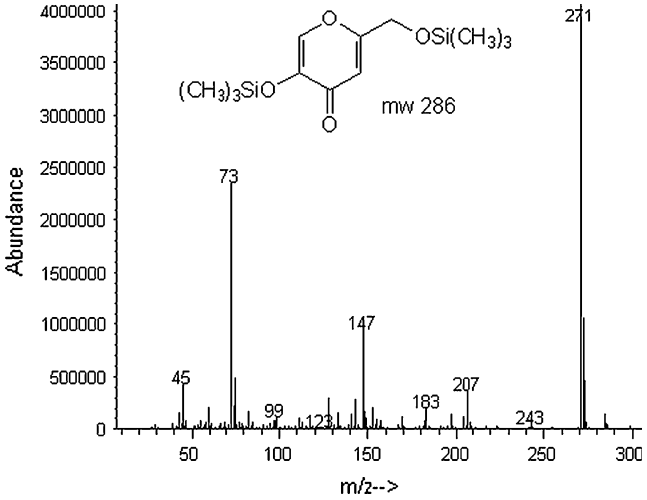

Fig. 1 Kojic acid identified by GC-MS as its trimethylsilylated derivative (M^{+}. 286)

It has been hypothesized that *A. oryzae* evolved from *A. flavus*, and may have lost certain features important for survival in the natural environment (Kurtzman et al. 1986). However, our discovery of an endophytic *A. oryzae* in coffee leaves, isolated from the wild, suggests that the same strain may indeed be capable of independent growth and reproduction.

A. oryzae NRRL 35191 was inoculated into ten cacao seedlings as a spore suspension and was recovered from the leaves from nine of the seedlings after surface sterilization (Fig. 2), although we were not able to isolate the fungus from the roots and stem tissues. *A. oryzae* could not be isolated from any of the control plants, but this is not surprising, since our greenhouse trees do not contain isolatable endophytes.

GC-MS analysis of the seedling extracts revealed the presence of KA (0.31±0.01 $\mu g.g^{-1}$ fresh weight) in all ten of the inoculated seedlings, but KA was undetectable in each of the control seedlings. KA is not a natural plant product, but instead has been found only in fungi, so the

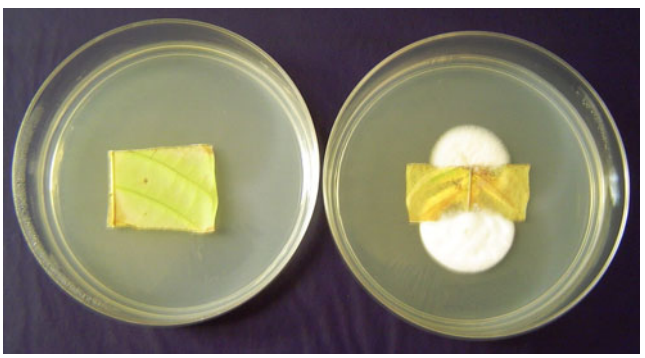

Fig. 2 *Aspergillus oryzae* growing out of a surface sterilized cacao leaf segment plated on potato dextrose agar (*right*) and control leaf (*left*)

source of the KA must be from the *A. oryzae* isolate. *A. oryzae* NRRL 35191-inoculated seedlings also produced caffeine at statistically significant higher levels (28± 3.2 μg g^{-1} fresh weight) than non-inoculated seedlings (5.3±2.6 μg g^{-1} fresh weight; *t* test, $t=5.476$, $P<0.0001$). Caffeine has broad insecticidal and fungicidal activity and has been shown to be part of the defensive mechanisms against pathogens in *T. cacao* and is often only detectable in leaves that have been infected by pathogens (Aneja and Gianfagna 2001).

Discussion

When *A. oryzae* NRRL 35191 was inoculated on a nutrient media supplemented with 28 μg $g^{-1}$of caffeine, a 15% reduction in growth rate (measured as colony diameter) was observed when compared to its growth in media containing 5.3 μg g^{-1}, which was the caffeine content found in control leaves. Although this may not represent the actual conditions found in planta, this assay, comparing the growth of *A. oryzae* on plates containing caffeine levels found in inoculated plants with *A. oryzae* growth on the lower levels of caffeine found in un-inoculated plants, does serve as an indication of the effectiveness of the plant response. While the increased caffeine content does not prevent growth of *A. oryzae*, it may be enough to prevent infections by other pathogens, or perhaps it may prime the plant's defense system against further attacks. Moreover, the induced caffeine levels may limit the growth of the endophyte preventing it from becoming a pathogen (Schulz and Boyle 2005). *A. flavus* grows as a saprophyte on a broad range of substrates but it is known as an opportunistic post-harvest plant pathogen that can cause disease in peanuts, cotton and corn (Cleveland et al. 2003; Leger et al. 2000). Although there are no reports of *A. oryzae* as a pathogen, we hypothesize that, in this study, the cacao plant may recognize the endophytic *A. oryzae* as a pathogen and respond by producing caffeine, which is inhibitory to fungal growth (Aneja and Gianfagna 2001). This hypothesis supports the idea of the endophytic continuum suggested by Schulz and Boyle (2005).

Due to the multitude of antipathogenic functions of KA, an endophytic non-toxigenic *A. oryzae* isolate that has the ability to produce KA may be useful as a protective agent against insects and pathogens. Although the mode of action of KA in cacao needs further investigation, it may involve both induction of plant defenses and direct antibiotic action against pathogens and insects. In addition, non-toxigenic *Aspergillus* isolates can reduce contamination of food crops by toxic isolates through competitive exclusion (Cleveland et al. 2003). With further affirmative testing, through the inoculation of cacao seedlings and/or seeds, this isolate of *A. oryzae* has the potential to be used for this purpose as well.

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