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Distribution and incidence of atoxigenic *Aspergillus flavus* VCG in tree crop orchards in California: A strategy for identifying potential antagonists, the example of almonds

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ABSTRACT

To identify predominant isolates for potential use as biocontrol agents, *Aspergillus flavus* isolates collected from soils of almond, pistachio and fig orchard in the Central Valley of California were tested for their membership to 16 atoxigenic vegetative compatibility groups (VCGs), including YV36, the VCG to which AF36, an atoxigenic isolate commercialized in the United States as biopesticide, belongs. A surprisingly large proportion of isolates belonged to YV36 (13.3%, 7.2% and 6.6% of the total almond, pistachio and fig populations, respectively), while the percentage of isolates belonging to the other 15 VCGs ranged from 0% to 2.3%. In order to gain a better insight into the structure and diversity of atoxigenic *A. flavus* populations and to further identify predominant isolates, seventeen SSR markers were then used to genetically characterize AF36, the 15 type-isolates of the VCGs and 342 atoxigenic isolates of the almond population. There was considerable genetic diversity among isolates with a lack of differentiation among micro-geographical regions or years. Since isolates sharing identical SSR profiles from distinct orchards were rare, we separated them into groups of at least 3 closely-related isolates from distinct orchards that shared identical alleles for at least 15 out of the 17 loci. This led to the identification of 15 groups comprising up to 24 closely-related isolates. The group which contained the largest number of isolates were members of YV36 while five groups were also found to be members of our studied atoxigenic VCGs. These results suggest that these 15 groups, and AF36 in particular, are well adapted to various environmental conditions in California and to tree crops and, as such, are good candidates for use as biocontrol agents.

1. Introduction

Almonds and other tree crops such as pistachios and figs are important crops in California due to their high value. Each year 70% of the total almond production is exported, mainly to Western Europe and Pacific Asia (Almond Board of California, July 2013 Position Report). These tree crops are occasionally contaminated with aflatoxins, a group of difuranocoumarin-derived mycotoxins produced by members of the *Aspergillus* section *Flavi*, mainly *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) are the main aflatoxins, with AFB_1 being the most common and the most potent genotoxic and carcinogenic of the aflatoxins (IARC, 2002). Because of their carcinogenicity, aflatoxins are subject to strict regulations in most countries of the world (van Egmond et al., 2007). The EU relaxed its tree nut aflatoxin standard from 4 ng/g to 10 ng/g in 2009 (Commission Regulation (EC) No 1881/2006 amended by Commission Regulation (EU) No 165/2010), while the U.S. Food and Drug Administration (FDA) maintains a 20 ng/g threshold for peanut product in food (Food and Drug Administration, 2005) and feed (Food and Drug Administration, 1994). The lower tolerance for aflatoxins in the European Union is a serious concern for US exporters since 115 tree-nut loads have become rejected at the border over the last five years according to the RASSF (Rapid Alert System for Food and Feed Safety) portal put in place by the European Commission (RASFF, 2016).

In almonds and pistachios, pyralid moths such as the navel orangeworm (NOW, *Amyelois transitella*) greatly contribute to the

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development of aflatoxin contamination through nut damage and vectoring of propagules of *A. flavus* (Palumbo et al., 2014; Picot et al., 2016). Control of these moths, mainly through chemical management practices, has resulted in reduced levels of aflatoxin on the crops (Campbell et al., 2003; Schatzki and Ong, 2001). Alternatively, a biocontrol method has also been successful in reducing levels of aflatoxin contamination in other crops including cotton, maize and pistachios, raising the interest of the almond and fig industries in California. This approach relies on the application of indigenous atoxigenic isolates of *A. flavus* that competitively exclude aflatoxin-producing strains (Cotty et al., 2008; Dorner and Lamb, 2006). Currently, two atoxigenic isolates of *A. flavus* are registered in the United States as biopesticides, namely AF36 and AflaGuard (Cotty et al., 2008; Dorner and Lamb, 2006). The atoxigenic *A. flavus* AF36 has been developed as a biocontrol agent for preventing aflatoxin contamination of cottonseed (Antilla and Cotty, 2002, 2004). Currently, AF36 is used in commercial cotton production in Arizona, California, and Texas and in corn production in Texas and Arizona. In addition, field trials testing AF36 started in 2002 for figs and pistachios and in 2007 for almonds in Californian research orchards. AF36 has been registered for pistachios since January 2012 and it is now commercially used in California and Arizona pistachio orchards.

One prerequisite to successfully develop such biocontrol strategies relies on the knowledge of the diversity and structure of *A. flavus* populations (Grubisha and Cotty, 2010). Several tools have been used to study the population diversity and structure of *A. flavus* including the identification of vegetative compatibility groups (VCG) (Bayman and Cotty, 1991; Mauro et al., 2013; Pildain et al., 2004) and the use of molecular methods such as pyrosequencing (Das et al., 2008) or microsatellites (Grubisha and Cotty, 2010). Assignment of isolates to VCG is laborious as well as time consuming given the large number of VCGs within *A. flavus* populations and given that each VCG may be represented by few individuals (Barros et al., 2006; Bayman and Cotty, 1991; Novas and Cabral, 2002; Pildain et al., 2004). Highly variable molecular markers such as simple sequence repeat (SSR) markers can provide a powerful and complementary tool to study population structure within a species due to the high degree of discrimination, random distribution throughout the genome and absence of selecting forces (Lowe et al., 2005).

The objectives of this study were: (i) to determine the distribution and incidence of atoxigenic VCG among isolates collected from tree crops in California, and (ii) to further identify atoxigenic predominant groups for potential use as biocontrol agents using SSR markers. *Aspergillus flavus* isolates collected in almond, pistachio and fig orchard soils from the main crop-producing areas in the Central Valley of California were initially tested for complementation with 16 atoxigenic VCGs including YV36, to which AF36 belongs. To complement the results of the VCG analysis, 17 SSR markers (Grubisha and Cotty, 2009), were further used to genotype YV36, type isolates of the 15 VCGs, and 342 atoxigenic isolates collected from almond orchard soils. Moreover, deletions in the aflatoxin and cyclopiazonic acid gene clusters of the 342 atoxigenic almond isolates were monitored using a multiplex PCR method.

2. Materials and methods

2.1. Sample collection

Soil samples were collected from 28 commercial almond orchards in three regions of the Central Valley during September 2007, 2008, 2010, and 2011, as described in Donner et al. (2015). In total, 11 orchards were surveyed in the northern region (Colusa, Glenn, and Butte Counties), 9 orchards in the central region (Madera County), and 8 orchards in the southern region (Kern County, Fig. 1). The northernmost almond orchard was approximately 650 km away from the southernmost one. The orchards from the central region were approximately 350–400 km away from the orchards of the northern region and 200–250 km away from the orchards of the southern region (Fig. 1). Samples of soil, inflorescences, leaves, and nuts were collected from 38 pistachio orchards between 1990 and 2007 in predominantly 6 counties located in the central region (Merced, Fresno, Madera, Kings, Tulare Counties) and south region of the Central Valley (Kern County, Fig. 1), as described in Doster et al. (2014). Samples of figs, fig leaves, and soil were collected from 24 fig orchards between 1990 and 1999 in 3 counties located in the central region of the Central Valley (Merced, Madera, Fresno Counties, Fig. 1) in California (Fig. 1).

In each almond orchard, three soil samples were collected at each summit of an equilateral triangle of approximately 100 m per side. Each soil sample was composed of ten sub-samples of 4–8 g, taken from a depth of 3 cm. All samples were stored in a cold room at 4 °C until analyzed. The soil samples were then dried at room temperature (25 ± 1 °C) for 7 days, clumps were broken, and samples were hand mixed to allow homogenization. Samples from fig and pistachio orchards were obtained in various ways as part of previous studies (Doster and Michailides, 1994, 1998; Doster et al., 1996).

2.2. Strain isolation and identification

The *A. flavus* isolates from fig and pistachio orchards were already present in our culture collection at the Kearney Agricultural Research and Extension Center (Doster et al., 2014). Isolation of *A. flavus* populations in each almond soil sample was performed by distributing 0.2 g of the dried ground soil evenly on 10 Petri plates (9 cm in diameter) of Modified Rose Bengal Agar, MRBA (Cotty, 1994). The plates were incubated in the dark for 3 days at 31 °C. Colonies were identified by morphological criteria. No > 25 isolates per soil sample were subcultured on Czapek yeast agar (Klich and Pitt, 1988) and incubated in the dark for 7 days at 31 °C. *A. flavus* was assigned to S- or L-strains based on their colony characteristics, conidial morphology, and size of their sclerotia (Cotty, 1989). Identifications were confirmed by the color reaction on AFPA (*A. flavus* and *A. parasiticus* agar, Pitt et al., 1983). These isolates are maintained in glycerol at − 80 °C for long-term storage at the Michailides laboratory.

2.3. Mycotoxin analysis

The lack of ability of the *A. flavus* population from almond soils to produce aflatoxins (*i.e.* atoxigenic isolates) was determined by measuring the amount of aflatoxin produced in an artificial medium. Isolates were grown in Adye & Mateles fermentation medium (A&M, Mateles and Adye, 1965), using 22.4 mM urea as the sole nitrogen source and adjusted to pH 4.7 prior to autoclaving (Cotty and Cardwell, 1999). Two vials of 15 mL capacity containing 5 mL of A&M media per isolate were inoculated with 50 μL of approximately 2000 conidia. Following incubation (7 days, 31 °C), fungal cells were lysed by adding 3 mL acetone. After 1 h, one volume of water and 3 mL of dichloromethane were added and vial contents were mixed by inverting. The dichloromethane phase was allowed to separate, removed with a glass pipette and filtered (0.2 μm nylon membrane, VWR International, Radnor, PA) into fresh 4 mL vials. Extracts were evaporated to dryness and re-dissolved in 1 mL methanol and 1 mL of water before injection into HPLC. Extracts containing high concentrations of aflatoxins were diluted as appropriate. The mycelia were collected on previously weighed Whatman No. 4 filter paper, dried in a forced air oven at 46 °C for 7 days, and weighed to quantify fungal biomass.

Aflatoxins were quantified with an HPLC system (Hewlett Packard 1050) with an isocratic pump, autosampler, and fluorescence detector (Hewlett Packard 1046A). Aflatoxins were separated on Nova-Pak C18 column (length of 150 mm, inner diameter of 3.9 mm, and particle size of 4 μm) supplied by Waters (Massachusetts) and the column temperature was set at 25 °C (Pickering laboratories, CHX650). The mobile phase was a mixture of methanol:water (45:55, v/v) with a flow rate of 0.8 mL min^{-1}. A postcolumn photochemical reactor (Aura Industries,