Review

Biological control strategies of mycotoxigenic fungi and associated mycotoxins in Mediterranean basin crops

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Summary. Fungi that belong to the genera *Aspergillus*, *Fusarium*, and *Penicillium* pose serious phytopathological and mycotoxicological risks at pre-harvest and post-harvest stages, as well as in processed food products because they can produce several mycotoxins. Mycotoxins pose a serious problem for animal and human health and have a significant economic impact worldwide. The Mediterranean basin is a large geographical region with a temperate climate supporting the cultivation of a wealth of field and greenhouse crops with a high risk of mycotoxin contamination. The most important mycotoxins that occur in the Mediterranean basin are aflatoxins (B1, B2, G1 and G2) in dried fruits and nuts, ochratoxin A in grapes and raisins as well as trichothecenes and fumonisins in cereals. A variety of chemical, biological and physical strategies have been developed to control the mycotoxigenic pathogens; to minimize mycotoxin production at pre- or post-harvest level; to contribute to decontamination and/ or detoxification of mycotoxins from contaminated foods and feeds; or to inhibit mycotoxin absorption in the gastrointestinal tract. Biological control using microbial antagonists either alone or as part of an integrated control strategy to reduce pesticide inputs, has emerged as a promising approach for control of mycotoxins in crops, both pre- and post-harvest. Several organisms including atoxigenic Aspergilli, yeasts, bacteria and fungi have been tested for their ability to reduce both fungal infection and mycotoxin contamination. For instance, atoxigenic fungal strains are being used widely to prevent pre-harvest aflatoxin contamination of crops such as peanuts, pistachios, maize, and cottonseed in several parts of the world including the Mediterranean area. Recent advancements in the use of biocontrol strategies have led to registration of commercial products with increased practical applications for the benefit of growers in several countries.

Keywords: aflatoxin, fumonisin, deoxynivalenol, patulin, ochratoxin, atoxigenic.

Introduction

The Mediterranean basin is a large geographical region with a temperate climate comprising several countries that belong to the southern part of Europe and parts of Asia and Africa. It supports a diversified agricultural system comprised of a wealth of field and greenhouse crops. The Mediterranean countries are the main suppliers of many fresh products to Europe. The major products of this area include vegetables (solanaceous crops, cucurbits and brassicas), legumes (beans, peas, peanuts), citrus fruits, pome fruits (apples, pears), stone fruits (apricots, peaches, nectarines, plums, cherries), nuts (almonds, pistachios, hazelnuts, walnuts), cereals (maize, rice, wheat) as well as tubers and roots from temperate and subtropical plants. The basin is also famous worldwide for some special products specific to this area e.g. olives and olive oil, grapes (table and wine) and soft fruits (figs, strawberries, raspberries). Figs, raisins, apricots and dates are major dried fruits produced in this region. Because of the suitable environmental conditions, these host plants are very often infected with serious plant pathogens and pests. Both pathogens and saprophytes can additionally synthesise toxic secondary metabolites, which may lead to the accumulation of mycotoxins in the colonised tissues (Logrieco *et al.*, 2003).

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Mycotoxins are toxic low molecular weight natural products (secondary metabolites) produced by filamentous fungi grown on several crops. These toxic molecules significantly influence the quality and safety of food and feed products. Mycotoxins are capable of causing disease and death in humans and other animals. Mycotoxicoses are examples of "poisoning by natural means" and thus, are considered analogous to the pathologies caused by exposure to pesticides or heavy metal residues. Mycotoxins in plant products and in processed food and feed have a significant economic impact and pose a serious problem for animal and human health (IARC, 1993). Because of their pharmacological activity, some mycotoxins or their derivatives can also be used as antibiotics or other kinds of drugs and some others have also been implicated as chemical warfare agents (Bennett and Klich, 2003). Mycotoxin contamination is now one of the most insidious challenges to food safety. The importance of these compounds is evident from the constant international attention paid to them due to their harmful impacts on animal and human health and the economy.

The most prevalent toxigenic fungi belong to the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria*. These fungi pose serious phytopathological and mycotoxicological risks at preharvest and postharvest stages, as well as in processed food products (Castoria *et al.*, 2008). *Fusarium* and *Alternaria* species are often classified as 'field fungi', because they require high moisture content in the substrate for growth and mycotoxin synthesis (>20%) whereas the 'storage fungi', mainly species of *Aspergillus* and *Penicillium*, can also grow well at lower moisture contents. Thus, *Fusarium* and *Alternaria* usually pose a high mycotoxicological risk at pre-harvest level or in freshly harvested products that are drying, whereas toxigenic species of *Aspergillus* and *Penicillium* represent a higher risk for food and feed products in storage or other kind of processing (Logrieco *et al.*, 2003). Approximately, 400 secondary metabolites with toxigenic potential are produced by more than 100 fungi and the Food and Agriculture Organization (FAO) has estimated that as much as 25% of the world's agricultural commodities are contaminated with mycotoxins, leading to significant economic losses. The most important mycotoxins associated with human and veterinary diseases include aflatoxins (AF), ergot akaloids, fumonisins (FUM), deoxynivalenol (DON) and other trichothecenes,

ochratoxin A (OTA), patulin, zearalenone and citrinin. Mycotoxins exhibit four basic kinds of toxicity: acute (often causing deterioration of liver or kidney function), chronic, mutagenic, and teratogenic. Naturally occurring aflatoxins were classified as being carcinogenic to humans (Group 1) according to International Agency for Research on Cancer (IARC). Most of the developed countries have adopted regulations stipulating the maximum permissible levels for many important mycotoxins in foods and feeds (Bennett and Klich, 2003; Kabak and Dobson, 2009).

The severity of crop contamination (i.e. cereals, maize, peanuts, grapes) varies from year to year based on weather and other environmental factors. Aflatoxin contamination, for example, is usually worst during drought years; plants are weakened and become more susceptible to insect damage and other insults. Mycotoxins can enter the food chain in the field, during storage, or at later points. Mycotoxin problems are exacerbated whenever shipping, handling, and storage practices are conducive to mold growth. The economic consequences of mycotoxin contamination are profound. Crops with unacceptable levels of mycotoxins often have to be destroyed. Alternatively, contaminated food crops are sometimes diverted into animal feed. Giving contaminated feeds to susceptible animals can lead to reduced growth rates, illness, and death. Moreover, animals consuming mycotoxin-contaminated feeds can produce meat and milk that contain toxic residues and biotransformation products (Bennett and Klich, 2003).

The most important mycotoxins that occur in the Mediterranean basin are aflatoxins (B1, B2, G1 and G2) in dried fruits and nuts (figs, pistachios, hazelnuts, peanuts), ochratoxin A in grapes and raisins as well as trichothecenes and fumonisins in cereals. Aflatoxin contamination of dried fruits and ochratoxin A contamination of raisins are mostly widespread in the southern and eastern parts of the basin (African and Asian countries). In the northern part of the basin (European countries) trichothecenes and fumonisins (cereals) as well as ochratoxin A in grapes and wine are the most prevalent mycotoxins. However, the type and level of the different mycotoxins vary in each country as well as in different regions of each country depending mainly on environmental conditions (temperature, humidity etc). Additionally, mycotoxin contamination is also associated with pest and pathogen damage e.g. it is well documented that the highest OTA levels occur when the pest and dis-

ease control in vineyards give insufficient plant protection (Battilani *et al.*, 2006; Ozay and Ozer, 2008).

A variety of chemical, biological and physical methods have been developed to control mycotoxigenic pathogens and to minimize mycotoxin production at pre- or post- harvest stages; to contribute to decontamination and/or detoxification of mycotoxins from contaminated foods and feeds; or to inhibit mycotoxin absorption in the gastrointestinal tract. Despite the fact that specific control measures can reduce mycotoxin formation in different commodities, the complete elimination of mycotoxin contaminated foods and feeds is currently not realistically achievable. This article reviews research trials and developments in the biological control of both fungal infection and mycotoxin formation for different major crops of the Mediterranean region (Table 1).

Biological control of plant pathogens

The use of many of the available physical and chemical methods for the detoxification of agricultural products contaminated with mycotoxins is restricted due to problems concerning safety issues, possible losses in the nutritional quality of treated commodities, coupled with limited efficacy and cost implications (Kohl *et al.,* 2011). Additionally, the development of fungicide resistance in many fungal pathogens as well as rising public concern over risks associated with pesticide use has led to a significant interest in the development of alternative nonchemical and environmentally friendly methods of pest and disease control. Biological control using microbial antagonists either alone or as part of an integrated control strategy to reduce pesticide inputs, has emerged

as a promising approach for control of aflatoxins as well as some other mycotoxins in both pre- and post-harvested crops (Table 1). Several organisms including bacteria, yeasts and non-toxigenic *Aspergillus* fungi have been tested for their ability to reduce mycotoxin contamination. Despite the fact that the use of biocontrol agents has often been successful in the lab, their commercialization largely depends on whether they can consistently control mycotoxigenic fungi in different locations and cultivars and reduce mycotoxin levels below the legislative limits.

Microorganisms as biocontrol agents have a relatively narrow spectrum of activity compared to most chemical fungicides. Biological control of pre- or postharvest diseases is complex and involves a number of biological, environmental and economic variables. New biocontrol products, in addition to efficacy towards the targeted plant pathogen, must be safe and cost-effective (Nunes, 2002; Kohl *et al.,* 2011). Despite all these problems, a number of successful commercial products have been on the market in different countries worldwide to control mycotoxigenic fungi, including Aspire (*Candida oleophila* strain 182; Ecogen Inc., Langhorne, PA), Bio-Save 10 and 11 (*Pseudomonas syringae* strains ESC10 and ESC 11; EcoScience Corp., Worcester, MA), AF36 (atoxigenic strain of *A. flavus*; Cotty, 1992), and Afla-Guard (atoxigenic strain of *A. flavus* NRRL 21882; Dorner and Lamb, 2006).

Biological control of *Aspergillus flavus*, *A. parasiticus* and aflatoxins in nuts and maize

Several *Aspergillus* species are among the most notorious plant pathogens because of the production

Table 1. Examples of biological agents that control mycotoxigenic fungi and mycotoxins.

Targeted mycotoxigenic fungus	Targeted mycotoxin	Biocontrol agent
A. flavus, A. parasiticus	Aflatoxin	Atoxigenic *Aspergillus* strains
Aspergillus spp.	Aflatoxin	Lactic acid bacteria
Aspergillus spp.	Aflatoxin	Fungal strains that detoxify AF
A. carbonarius, A. niger	Ochratoxin A	Phyllosphere yeasts (e.g. *Aureobasidium pullulans*)
F. verticillioides	Fumonisin	*Bacillus subtilis, B. amyloliquefaciens, Kluyveromyces* sp.
F. graminerarum, F. culmorum	DON	*Fusarium equiseti*, yeasts, *Bacillus* spp., *Pseudomonas* spp.
Penicillium expansum	Patulin	*A. pullulans, P. syringae, Candida sake, Pantoea agglomerans*

of the carcinogenic mycotoxins called aflatoxins (AF). AF were isolated and characterized after the death of more than 100,000 turkeys (turkey X disease) in the UK, attributed to the consumption of mold-contaminated peanut meal. The four major AF are called B_1, B_2, G_1, and G_2 based on their fluorescence under UV light (blue or green) and the relative chromatographic mobility during thin-layer chromatography. AFB_1 is the most potent natural carcinogen known (Squire, 1981) and is usually the major aflatoxin produced by toxigenic *Aspergillus flavus* strains that occur worldwide in soil and air. AF contamination is usually a significant problem in tropical or sub-tropical areas and can occur during crop development when the crop is either damaged (e.g., by insects) or stressed by heat and drought, and after maturation when the crop is exposed to high moisture and high temperature either before harvest or in storage (Bennett and Klich, 2003). In the Mediterranean area, peanuts, pistachios and maize are the major crops contaminated with AF. AF outbreaks have occurred in the basin, such as in 2003, when environmental conditions (very hot and dry for several months) during maize cultivation in northern Italy led to AF levels 5–70 times higher than during the years 1995–2000. This outbreak led to milk contamination by AFM_1 produced by dairy cattle that consumed contaminated maize and to the dumping of thousands of tons of contaminated milk. As the global climate changes to hotter and drier, the possibility that all of Southern Europe may face a constant threat of AF contamination increases dramatically (Logrieco and Moretti, 2008).

A number of environmental and agronomic factors influence pod and seed infection of peanuts by AF-producing fungi, and consequently the aflatoxin contamination of peanuts at the pre-harvest stage. These factors depend largely on the field location as well as the year or season within the same field. Some environmental conditions are particularly favourable to fungal growth in combination with AF contamination of peanuts. In many cases though, it is possible to design appropriate agricultural practices and apply biocontrol agents (mainly atoxigenic *Aspergillus* strains) that help to reduce AF contamination in peanuts.

The pistachio is another nut that is quite often highly contaminated with AF. The major problem with pistachios is the "early split" that appears at the pre-harvest stage. The shell of the pistachio partially splits, usually at least a month before maturity and harvest. The hull covering the shell usually remains intact, protecting the kernels from infection by fungi and insects. However, in some cases, depending on the environmental conditions and the cultivar, the hull ruptures in a percentage of pistachios (early splitting), a condition conducive to infection with the AF producing fungi *A. flavus* and/or *A. parasiticus*. The aflatoxin content in single "early split" pistachio nuts often exceeds 20 ppb and sometimes exceeds 1000 ppb (Codex Alimentarius Commission, 2002; Bonjar, 2004).

A number of biological (i.e. bacteria, yeasts, atoxigenic *Aspergillus* strains) and chemical control agents (i.e. fungicides) have been reported to inhibit growth of aflatoxigenic fungi and subsequent AF biosynthesis. Isolates of *A. flavus* vary greatly in AF production, with some producing copious amounts and others none. One of the most successful patented approaches (Cotty, 1992; Cole, 1994) is the application of competitive, atoxigenic strains of *A. flavus* or *A. parasiticus* that has clearly been shown to reduce the AF contamination of agricultural products such as peanuts (Dorner *et al.*, 2003), pistachios, rice, maize (Dorner *et al.*, 1999), and cottonseed (Cotty, 1994). The effect is believed to be mediated mainly through competition for substrate and through the potential production of inhibitory metabolites. It is well established that AF contamination of crops can be suppressed by atoxigenic biocontrol strains. The application of non-aflatoxigenic strains of *A. parasiticus* in field soils has been reported to result in lower AF levels in peanut crops, with levels decreasing from 531, 96 and 241 μg kg⁻¹ in untreated soils to levels of 11, 1 and 40 μg kg⁻¹ for treated soils in three consecutive years (1987, 1988, and 1989), respectively (Dorner *et al.*, 1992). Cotty (1994) discovered that the application of non-aflatoxigenic strains of *A. flavus* (on grain seeds) to soils of cotton fields was very effective for controlling AF production in cottonseed. In another 2-year study, Dorner *et al.* (1998, 2003) found that the application of atoxigenic strains of *A. flavus* and *A. parasiticus* on soils of peanut fields at different doses reduced AF contamination of peanuts by 74.3–99.9%. In another study, a non-aflatoxigenic strain of *A. flavus* reduced AF contamination by 80 to 95% in maize (Brown, 1991). In Greece, Tsitsigiannis *et al.* (2010) are screening large numbers of *Aspergillus* strains from pistachio orchards from different parts of the country in order to isolate native non-aflatoxigenic *Aspergillus* strains for further tests in reducing afla-

toxin levels in pistachios. The use of native atoxigenic isolates also alleviates some of the concerns about safety and environmental impacts that might be of greater concern for introduced organisms.

Several studies have shown that, in order to be effective in reducing levels of AF contamination, atoxigenic strains of *Aspergillus* spp. must be applied in a method and at a time that allows successful competition with AF-producing *Aspergillus* strains. In theory, the atoxigenic strain is applied once per growing season when the overall *A. flavus* inocula levels in the field are low, thereby providing the applied strain the time and preferential exposure to be established in the crop, as well as a subsequent advantage when competing for crop resources with the toxigenic strains (Cleveland *et al.*, 2003; Cotty and Melon, 2006). Two atoxigenic *A. flavus* strains, AF36 and NRRL 21882, are currently being used in USA to minimize AF contamination in crops. AF36 has been registered as a biopesticide by the U.S. Environmental Protection Agency and is being used for the management of aflatoxin producing fungi in cotton fields whereas NRRL 21882 is being used to prevent AF contamination in peanut fields (Chang and Hua, 2007). Afla-guard is composed of hulled barley coated with conidia of the atoxigenic strain of *A. flavus* (NRRL 21882) that does not produce AF, cyclopiazonic acid, or known AF biosynthetic precursors (Dorner, 2010). Large-scale tests of peanuts showed that applications of Afla-guard reduced aflatoxins by 85.2% in farmers' stock peanuts and by as much as 97.5% in shelled, edible grade peanuts (Dorner and Lamb, 2006).

Advances in biological control have been aided by recent advances in molecular biology, genetic engineering and microbial genomics coupled with the discovery of the very broad catabolic potential present within the microbial world (Bata and Laztity, 1999; Karlovsky, 1999). *A. flavus* has no known sexual stage; consequently, most studies on its genetic variability have been made mainly by characterizing isolates based on vegetative compatibility group (VCG). Complementary nitrate-nonutilizing (*nit*) mutants are commonly used to identify compatible isolates. VCG analysis becomes cumbersome for genetic analysis involving large populations because it requires pairing each new mutant strain with a representative of each VCG determined for that population while eliminating isolates that are self-incompatible. In an effort to explain the atoxigenicity of some *Aspergil-*

lus strains, Donner *et al.* (2010) compared genetic loci across the 68 kb AF biosynthesis gene cluster among 18 atoxigenic and two aflatoxin-producing vegetative compatibility groups (VCGs) from Nigeria and an atoxigenic VCG used commercially in North America. Five of the atoxigenic VCGs had large deletions (37–65 kb) extending from the teleomeric side of the aflatoxin biosynthesis cluster. The remaining 12 atoxigenic VCGs, including the VCG used for aflatoxin management in North America, contained all the AF pathway genes, but with defects. Two observations support the long-term persistence of atoxigenicity within *A. flavus*: first, a comparison of pathway genes revealed more changes in atoxigenic than in aflatoxin-producing isolates compared to the AF-producing strain NRRL 3357; and second, several non-synonymous changes are unique to atoxigenics. Atoxigenic VCG diversity was assessed with phylogenetic analyses. Although some atoxigenics share relatively recent ancestry, several are more closely related to aflatoxin producers than to other atoxigenics (Donner *et al.*, 2010).

Besides biological strategies using atoxigenic strains, there are numerous reports regarding the antifungal properties of various lactic acid bacteria, which can exhibit activities against several *Aspergillus* species and a broad range of other mycotoxigenic fungi. These lactic acid bacteria have a long history of use in food and have been regarded as safe. These bacteria produce antimicrobial compounds, e.g., lactic acid, acetic acids, hydrogen peroxide, bacteriocins, and low-molecular-weight proteinaceous compounds, during carbon source metabolism, and they compete with other species by acidifying the environment and rapidly depleting nutrients (De Muynck *et al.*, 2004; Kabak and Dobson 2009). For instance, earlier work reported the antifungal activity of a *Lactobacillus casei* strain that inhibited both growth and aflatoxin production in *A. parasiticus* (El-Gendy and Marth, 1981). Coallier-Ascah and Idziak (1985) also reported that aflatoxin production by *A. flavus* was significantly reduced in the presence of *Streptococcus lactis* cultures, while antifungal activity from a *Leuconostoc mesenteroides* strain was reported, but no specific substance was identified (Suzuki *et al.*, 1991). Another isolate from a silage inoculant, *Lactobacillus casei pseudoplantarum,* has also been reported to inhibit production of AFB_1 and AFG_1 by 80 and 92%, respectively (Gourama and Bullerman, 1997). In another study, Corsetti *et al.,* 1998 reported

antifungal activity from *Lactobacillus sanfrancisco* CBI which was caused by the formation of short-chained fatty acids, including caproic acid, which inhibited spoilage moulds from the genera *Monilia*, *Aspergillus*, *Penicillium*, and *Fusarium*. In addition the inhibition of aflatoxins by metabolites such as benzoic acid, cyclo (Gly-L-leucyl) methylhycantoin, and nevalonactone produced by specific lactic acid bacteria has been reported (Kabak *et al.*, 2006).

In addition to the use of biocontrol agents in the field or at post-harvest level, a number of different fungal or bacteria cultures have been shown to detoxify AFB_1. Fungal taxa such as *Trichoderma* sp., *Phoma* sp., *Rhizopus* sp., *Sporotrichum* sp. ADA, *Sporotrichum* sp. SF, and *Alternaria* sp. have been shown to degrade AFB_1 by 65 to 99% in 5 days at 28±2°C (Shantha, 1999). Some strains of *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* have been shown to degrade AFB_1 in milk by fermentation (Karlovsky, 1999). *Flavobacterium aurantiacum* can significantly remove AFB_1 from a liquid medium and a variety of food products including milk, peanuts, and corn without leaving toxic by-products. *F. aurantiacum* strain NRRL-B-184 removed 79.9–98.9%, 92.6–99.8%, and 88.7–100% AFB_1 from phosphate-buffered saline, peanuts and red pepper, respectively within 48 h. In another study by D'Souza and Brackett (2000), the divalent cations Ca^{+2} and Mg^{+2} were shown to stimulate AFB_1 degradation by *F. aurantiacum*. However, the bright orange pigmentation of this microorganism limits its use in food or feed fermentations (Line, 1994; Kabak *et al.*, 2006).

Biological control of *Aspergillus* spp. and ochratoxins in grapes

Grapes are important for Europe, as European wine production represents about 70% of world production and thus is an important export commodity. Berry rot, known as sour rot or *Aspergillus* rot or black rot, is mainly caused by black Aspergilli which are very common in vineyards. Berry rot is particularly severe in the warmer grape-producing southern areas of Spain, Italy, France and Greece. Black *Aspergillus* spp. appear to be secondary invaders that infect berries only after they have been damaged by pre-harvest rain, other fungi, insects, mechanical impact or other factors (Leong *et al.*, 2006). Soil and vine debris on soil are the primary sources of black *Aspergillus* spp. in vineyards (Kazi *et al.*,

2008). Ochratoxin A (OTA) was first detected as a wine contaminant in 1996 and the role of *Aspergillus* section Nigri and *A. carbonarius* in OTA production discovered in Europe in 1999 (Abrunhosa *et al.*, 2001; Battilani *et al.*, 2001, 2003; Cabanes *et al.*, 2002; Serra *et al.*, 2003; Tjamos *et al.*, 2004). OTA is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties (Battaglia *et al.*, 1996) and EU has established a maximum level of 2.0 μg kg^{-1} (ppb) (European Commission 2007) in order to protect consumers. After the first detection of OTA in wine in several countries (Zimmerli and Dick, 1996), subsequent studies showed that *A. carbonarius* is predominantly responsible for OTA contamination of grapes, wine and vine fruits and detected OTA contamination of grapes and derived products such as raisins (Stefanaki *et al.*, 2003; Tjamos *et al.*, 2004), grape juices and wines (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000; Soufleros *et al.*, 2003; Stefanaki *et al.*, 2003). Analyses of wine samples throughout Europe have shown that there is a gradient in OTA concentration with a decrease from red, to rose and to white wines (Visconti *et al.*, 1999; Majerus *et al.*, 2000; Soleas *et al.*, 2001). Some parts of Southern Europe are more prone to contamination with the toxigenic *Aspergillus* species and OTA. OTA has also been found in much higher concentrations (max 53 μg kg^{-1}) in dried vine fruit than in wine suggesting that *A. carbonarius* can dominate the drying vine fruit ecosystem. Additionally, in Greece a tendency of increasing OTA contamination in red dry wines from the northern to southern parts of the country was observed (Tjamos *et al.*, 2006). There is a significant lack of knowledge in Europe on conducive pre-harvest climatic conditions and their relationship with risk of OTA contamination in grapes, and regarding the fate of OTA in wine production (Battilani *et al.*, 2006).

Black Aspergilli, always present in the vineyard, colonize the berries at early veraison and surveys conducted in different countries have revealed that *Aspergillus carbonarius* and *A. niger* are the predominant Aspergilli in vineyards (Serra *et al.*, 2003; Battilani *et al.*, 2006; Bejaoui *et al.*, 2006; Perrone *et al.*, 2006; Tjamos *et al.*, 2006). However, population studies of the black Aspergilli showed that although *A. carbonarius* was never dominant, its isolates were strong producers of OTA and *A. niger* was only occasionally the source of OTA in wine grapes (Serra *et al.*, 2003; Battilani *et al.*, 2004; Tjamos *et al.*, 2004).

Biological control against *Aspergillus* rot in grapes has been tested by several research groups. The efficacy of microbial antagonists in combination with reduced rates of fungicide applications was also evaluated. The search for natural competitors has focused on the epiphytic organisms on leaves or fruits. Experimental results showed that phyllosphere yeast isolates are able to control sour rot caused by *A. carbonarius* in wine-producing vineyards. Guerzoni and Marchetti (1987) recorded the various genera of yeasts isolated from grape berries of different varieties, healthy and with sour rot symptoms. The most frequently occurring yeasts in the case of the infected berries were: *Candida krusei*, *Kloeckera apiculata*, *Metschnikowia pulcherrima*, *Saccharomycopsis vini*, *Saccharomycopsis crataegensis* and *Issatchenkia occidentalis*. In the case of healthy berries the frequently isolated yeasts were: *Brettanomyces* spp., *Zygosaccharomyces* spp., *Pichia* spp., *Aureobasidium pullulans*, *Rhodotorula* spp., *Cryptococcus* spp., and *Geotrichum candidum*. The yeasts *Aureobasidium pullulans*, *Rhodotorula* spp. and *Cryptococcus* spp. were the species with the highest incidences in most grape varieties.

Among the organisms reported to be effective were *A. pullulans* (Lima *et al.*, 1997), *Metschnikowia fructicola* (Karabulut *et al.*, 2003), and various other yeasts (Wilson *et al.*, 1991; Zahavi *et al.*, 2000; Bleve *et al.*, 2006) and *Bacillus subtilis* (Pusey, 1989). *In vitro* experiments showed that some *A. pullulans* strains are highly suppressive to the rot caused by *A. carbonarius* on detached and wounded wine grape berries; i.e., under conditions that are favorable to the fungal pathogen. These strains are able to degrade OTA to the less toxic compound ochratoxin-a *in vitro*. In the few berries that were infected by *Aspergillus* sp. and were pretreated with the biocontrol agents, OTA contamination was lower than in infected non-treated berries (De Felice *et al.*, 2007).

Zahavi *et al.*, 2000, reported that *Candida guilliermondii*, strain A42 was effective in reducing incidence of decay caused by *Botrytis cinerea* and *A. niger* in wine-producing vineyards for two consecutive years. Schena *et al.*, 1999 reported the activity of *A. pullulans* isolates against *A. niger* on table grapes along with their inhibitory activity against *B. cinerea* and *Rhizopus stolonifer*. The isolates used were L-47, known for its effectiveness, and two new isolates from a total of 41. These three isolates were applied in detached berry tests at three different concentrations of $10^6, 10^7$ and 10^8 cells per ml and evaluated after five days. Complete inhibition of *Aspergillus niger* on table grapes was achieved by all three isolates of *A. pullulans* with the concentration of 10^8 cells mL^{-1}. The concentration of 10^7 cells mL^{-1} of L-47 gave 100% inhibition, while the other two were 5–60% effective. A concentration of 10^6 cells mL^{-1} was not satisfactory. Bleve *et al.*, 2006, also identified several isolates of different species with antagonistic activity against *A. niger* and *A. carbonarius* on artificially wounded, infected berries.

The yeast-like fungus *A. pullulans* is one of the most widespread and well-adapted saprophytes, both in the phyllosphere (Blakeman and Fokkema, 1982) and in the carposphere. *A. pullulans* has a high tolerance to desiccation and irradiation (Elad *et al.*, 1994; Kohl *et al.*, 1995) and has been considered as a possible biological control agent of postharvest diseases on table grapes (Schena *et al.*, 1999). Castoria *et al.* (2001), in experiments with grapes, showed that strain LS-30 of *A. pullulans* reduced *A. niger* infections by approximately 86% after storage at 20°C for six days. According to their experiments, competition for nutrients seems to be a possible mechanism of action of *A. pullulans*. Isolate LS-30 showed production of extracellular β-1,3-glucanase, but not chitinase, *in vivo* and *in vitro*. It does not seem to produce any kind of antibiotic substance nor to adhere to the hyphae of the pathogenic fungus. It has been described by other investigators that a possible mechanism of action of antagonists of postharvest pathogens is competition for nutrients, but it seems that high rates of extracellular β-1,3-glucanase production contribute to the possibility of the yeast to control post-harvest pathogens, considering that these enzymes cause disruption of the cell wall (as a carbon source).

Dimakopoulou *et al.* (2008) isolated another efficient *A. pullulans* strain (Y-1) and demonstrated that in detached berry tests (Figure 1) as well as in field experiments in Greece, it was as effective as the commercial fungicide mixture fludioxonil + cyprodinil in controlling sour rot, *A. carbonarius* infection and the consequent OTA contamination in must (Figure 2). The *A. pullulans* isolate Y-1 was applied twice, one week before veraison and two weeks before harvesting. It is noteworthy that neither the fungicide nor the yeast isolate reduced the percentage of *A. niger* infection on berries; however, a decrease in sour rot incidence and OTA level was observed in these treatments compared to the untreated control berries. This discrepancy between the population density of

A   **B**
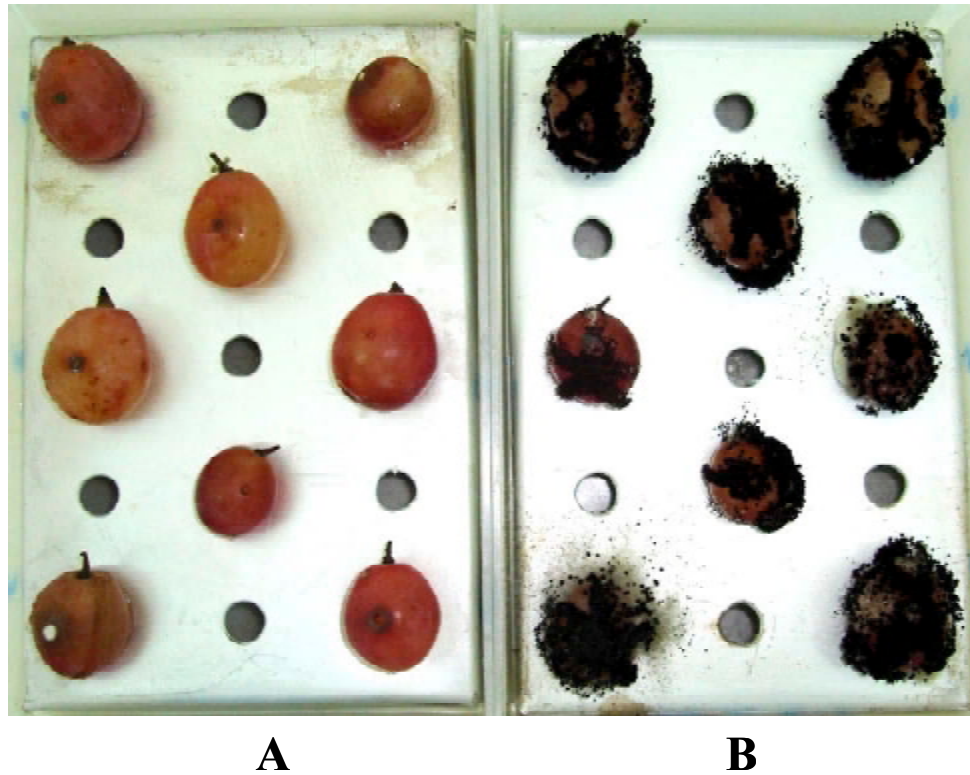
Figure 1. Biological control of *Aspergillus carbonarius* using the epiphytic yeast *Aureobasidium pullulans* on grape berries (cultivar "Fraoula"). **A**. Protection of berries treated with *Aureobasidium pullulans* strain Y1 (immersion in yeast suspension of 10^7 cfu mL^{-1}) and 24h later infection with *Aspergillus carbonarius*. **B**. Control berries infected with *Aspergillus carbonarius* (25 µl of 10^4 conidia mL^{-1} per berry) (Dimakopoulou *et al.*, 2008).

A. niger, sour rot incidence and OTA level can be explained by the fact that *A. niger* is considered as a minor contributor of sour rot and OTA contamination (Tjamos *et al.*, 2004); the main species is *A. carbonarius*. Future work will focus on evaluating the antagonist's ability to colonize berries either externally or internally by using molecular markers (Dimakopoulou, personal communication). Better understanding of its ecology may lead to an optimized formulation, time and mode of application; in order to be effective, Biological Control Agents (BCAs) must survive on the plant surface and consequently remain active against target pathogens during periods favourable to plant infection (Lo *et al.*, 1998).

A. pullulans, in addition to being an effective epiphytic biocontrol microorganism, has been reported as an endophyte, found in plant parts without symptoms. Endophytic organisms usually develop within the tissues of the host without causing symptoms and often produce substances with antifungal or antibacterial activity. There are several cases where the existence of endophytic fungi or bacteria leads to induced resistance of the host. The endophytic organisms compared with epiphytic ones have advantages because they develop in conditions of reduced nutritional requirements and to some extent protected, but at the same time they can provide protection to the host. Schena *et al.* (2003) obtained 51 endophytic isolates of *A. pullulans* from cherry fruit flesh in order to assess their effectiveness to control post-harvest rots of cherry and table grapes. From the 51, two were selected, 533 and 547, which significantly reduced (32–80% in sour cherries and 59–64% in table grapes) post harvest infection by *Botrytis cinerea*. In particular it was demonstrated that 547 was able to survive under field conditions, proliferate in storage at cold temperatures and penetrate and colonize the flesh of the cherry or grape fruit if the application was made at the time of flowering.
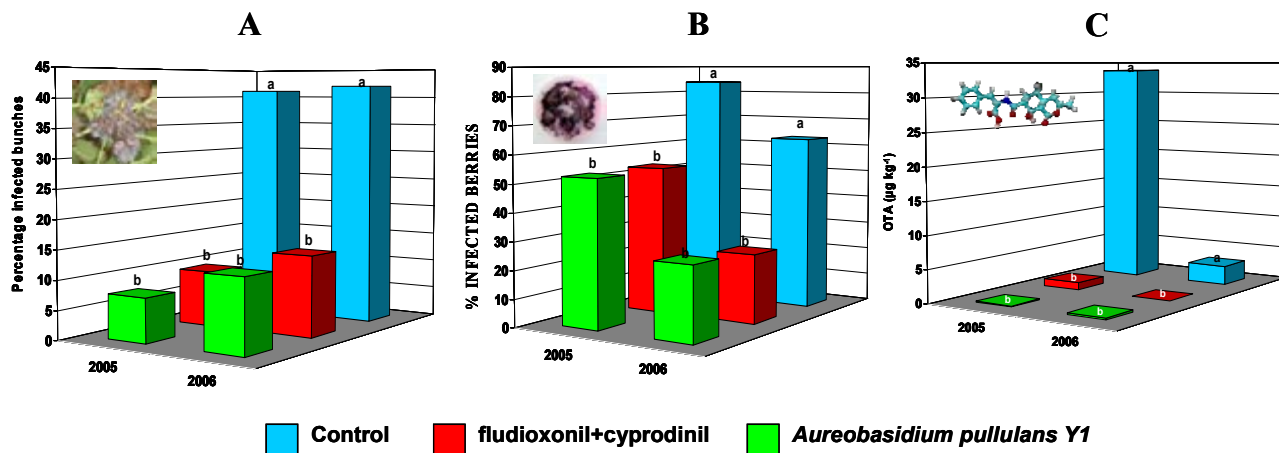
Figure 2. Effect of fludioxonil+cyprodinil and *Aureobasidium pullulans* Y1 treatments on **A)** Sour Rot Incidence; **B)** *Aspergillus carbonarius* populations in grape berries and C) on ochratoxin A content in must. Bars represent the mean percentages for 2005 and 2006 and letters on the top of each bar indicate the results of the Duncan's test (P<0.05); values with different letters in each year are statistically different (Dimakopoulou et al., unpublished data; Dimakopoulou et al., 2008).

The efficacy of biological control methods that rely on the use of a single BCA can be variable depending upon the pathogen's inoculum level and local environmental conditions (O'Neill *et al.*, 1996, Guetsky *et al.*, 2002). One potential way to reduce this variability and improve efficacy is to combine compatible biocontrol treatments that occupy different environmental niches and/or have different modes of action (Elmer and Reglinski, 2006). Therefore, a combination of the most efficacious yeast isolates in the *in vivo* detached berry test can be screened under field conditions.

Besides BCAs, Varga *et al.* (2000) reported the ability of various *Aspergillus* strains such as *A. fumigatus, A. japonicus,* and *A. niger* to degrade OTA in liquid YES media, with *A. niger* degrading OTA to ochratoxin-α and phenylalanine in solid media and liquid cultures. Additionally, *Acinetobacter calcoaceticus* has been shown to degrade 100% of OTA in ethanol minimal salts medium at an initial OTA concentration of 10 μg mL^{-1} at 30°C within 120 h (Hwang and Draughon, 1994).

Biological control of *Fusarium* spp. and associated mycotoxins in maize and wheat

The occurrence of *Fusarium* mycotoxins, particularly in cereal grains is of great concern in Mediterranean area, and their presence in processed feeds and foods is often associated with mycotoxicoses in humans or domesticated animals. The most common *Fusarium* mycotoxins are trichothecenes, zearalenone and fumonisins. Trichothecenes can cause a variety of toxic effects in animals including haemorrhagic syndrome in internal organs, immunosuppression (leukopenia) and disturbance of the nervous system, skin inflammation, digestive and blood disorders. Zearalenone (ZEA) is among the most widely distributed *Fusarium* mycotoxins in agricultural commodities and is often found at relatively high concentrations, especially in maize. ZEA is responsible for recurring toxicoses in livestock. ZEA has estrogenic effects causing reproductive disorders in farm animals (IARC, 1993).

Fumonisins are produced by a number of *Fusarium* species, notably *Fusarium verticillioides* (formerly *Fusarium moniliforme = Gibberella fujikuroi*), *Fusarium proliferatum, Fusarium nygamai,* as well as *Alternaria alternata* f. sp. *lycopersici* (Pitt, 1986; Marasas, 2001) and *Aspergillus niger*. Fumonisins B$_1$ (FB$_1$) and B$_2$ (FB$_2$) present the greatest mycotoxicological concern. Feeds contaminated by FB$_1$ cause altered hepatic and immune function in cattle, poor performance in poultry, leukoencephalomalacia in horses and pulmonary oedema in swine. Maize contaminated by FB$_1$ has also been associated with oesophageal cancer of humans in Africa, China, and the United States. The structural similarity of fumonisins with

sphingosines suggests a role for these toxins as depletion agents of the complex sphingolipids from biological membranes accounting for their toxicity and, perhaps, their carcinogenicity. The evidence that cultures of *F. verticillioides* and samples of FB_1 can promote liver cancer in rats, led to the classification of fumonisins as carcinogenic to animals and possibly to humans (Group 2B) (IARC, 1993). The major species with an economic importance is *Fusarium verticillioides*, which grows as an endophyte in both vegetative and reproductive maize tissues, often without causing any clear disease symptoms in the plant. However, if the appropriate fungal and plant genotype are present under favourable weather conditions and in the presence of wounds caused by insect damage, the fungus can cause seedling blight, stalk rot, and ear rot (Nelson, 1993). The fungus is present in virtually all maize samples (Marshall, 1983, Marasas *et al.*, 2001). The fungus is transmitted vertically and horizontally to the next plant generation via mainly plant debris and to a less extent by seed infection. Horizontal infection is the manner by which this fungus is dispersed in the field by rain and water and disease occurs through external local infection. Horizontal infection can be reduced by the application of certain fungicides and seed treatments can also greatly reduce seed transmission. The endophytic phase can result from vertical transmission but fumonisin contamination is mostly due to local and not endophytic infection (Plumlee and Galey, 1994). Although it is phytotoxic, fumonisin B_1 is not required for plant pathogenesis (Marasas, 1996; Desjardins and Plattner, 2000).

A biological control system using an endophytic bacterium, *Bacillus subtilis*, has been developed that shows great promise for reducing mycotoxin accumulation during the endophytic (vertical transmission) growth phase. It was shown that this isolate is able to occupy the same ecological niche as *F. verticillioides* within the plant, and it was postulated to act competitively to the pathogen. Additionally to *B. subtilis*, an isolate of *Trichoderma* gave also promising results in postharvest control of the development and toxin accumulation of *F. verticillioides* on maize in storage conditions (Bacon *et al.*, 2001).

Studies by Etcheverry *et al.* (2009) investigated the antagonistic activity of bacterial and yeast isolates (*Bacillus amyloliquefaciens* BA-S13, *Microbacterium oleovorans* DMS 16091, *Enterobacter hormomaechei* EM-562T, and *Kluyveromyces* spp. L14 and L16

isolates) on mycelial growth of toxigenic *F. verticillioides* and *A. flavus* strains. The experiments carried out on 3% maize meal extract agar at different water activities (0.99, 0.97, 0.95, and 0.93) showed that the antagonistic isolates *M. oleovorans*, *B. amyloliquefaciens* and *Kluyveromyces* sp. (L16) led to an increase of lag phase of growth and a decrease of the growth rate of all fungal strains *in vitro*. *In vitro* experiments using biocontrol and pathogenic strains in co-inoculated cultures in non-rhizospheric maize soil (centrally or sprayed inoculated) and *in vitro* infected maize (ears apex- and base- inoculated) showed that *B. amyloliquefaciens* significantly reduced *F. verticillioides* and *A. flavus* numbers in maize soil inoculated centrally whereas *Kluyveromyces* sp. L16 reduced *F. verticillioides* and *A. flavus* colonies in maize soil that was spray inoculated. Among the strains tested, *Kluyveromyces* sp. L16 was the most effective biocontrol agent limiting *F. verticillioides* infections on maize ears (Etcheverry *et al.*, 2009).

The species predominantly found associated with *Fusarium* head blight (FHB) of wheat and other small-grain cereals in the Mediterranean region are *F. graminearum* and its widespread teleomorph *G. zeae*, *F. culmorum* and *F. avenaceum* (Logrieco *et al.*, 2003). FHB causes severe damage to wheat and other cereals. The disease causes significant losses by reducing grain yield and quality in many wheat production regions in the Mediterranean basin, especially in areas with warm temperatures and high relative humidity or frequent precipitation during the heading and blossoming periods. The fungus survives and reproduces in crop residues and is dispersed by wind or rain to the developing wheat or barley. Wheat is most susceptible during the flowering growth stages, but some infection can still occur during kernel development. *F. graminearum* is known to produce two important mycotoxins, deoxynivalenol (DON) (a type of trichothecene) and zearalenone, which can contaminate the diseased grain. The mycotoxin DON can cause reduced feed intake and lower weight gain in animals at levels as low as 1–3 ppm. Vomiting and feed refusal can occur when levels of DON exceed 10 ppm. Humans are also sensitive to DON (Yuen and Schoneweis, 2007).

Biological control experiments showed that a strain of *Fusarium equiseti* (G9) has been consistently effective in controlling FHB and reducing DON formation (by more than 70%) on wheat (Dawson *et al.*, 2004). Yeasts of the genus *Cryptococcus* also are

very effective against FHB (up to 59% reduction) on durum wheat in the field (Khan *et al.*, 2001). Among the factors that influence the successful application and colonization of FHB antagonists in the field include the variable and sporadic arrival of pathogen inoculum on wheat heads over an extended period of head susceptibility, the phylloplane environment with significant fluctuations in temperature, moisture, and nutrient availability as well as the potential deleterious effect of UV light (Schisler *et al.*, 2002). In addition, four yeasts (NRRL Y-30213, NRRL Y-30214, NRRL Y-30215, and NRRL Y-30216) and a bacterium (*Bacillus* sp. NRRL β-30212) have been identified as excellent antagonists that are capable of suppressing FHB in cereals, particularly wheat and barley (Schisler, 2006). In another study, 4 out of 54 microbial strains that were isolated from wheat anthers and that utilized tartaric acid *in vitro,* were effective against *Gibberella zeae*, whereas only 3 out of 170 tested isolates that did not utilize tartaric acid were effective against this pathogen, showing the potential benefit of pre-screening candidate FHB antagonists for their ability to utilize tartaric acid (Khan *et al.*, 2001). Another report showed that three bacterial isolates, *Bacillus subtilis* H-08-02, *Bacillus cereus* L-07-01, and *Bacillus mycoides* S-07-01, inhibited *Fusarium graminearum* by 60, 52, and 55%, respectively (Fernando *et al.*, 2002), whereas *Lysobacter enzymogenes* strain C3 significantly reduced FHB severity in a particular wheat cultivar but had little or no effect on disease development on some other wheat cultivars. Treatment of heads of FHB susceptible wheat with a *Streptomyces* sp. reduced both FHB disease severity and associated loss in grain weight by approximately 50% under glasshouse conditions (Nourozian and Khodakaramian 2006). Khan and Doohan (2009) found that the use of *Pseudomonas fluorescens* strains MKB 158 and MKB 249 and *Pseudomonas frederiksbergensis* strain 202 reduced the severity of FHB disease symptoms caused by *F. culmorum* in wheat and barley grown under both greenhouse and field conditions, and *Pseudomonas* sp. AS 64.4 isolated from wheat anthers was as effective as the fungicide tebuconazole for controlling FHB disease severity under field conditions (Schisler *et al.*, 2006). Treatment with either of the two *P. fluorescens* strains (MKB 158 and MKB 249) also resulted in a 74 to 78% reduction in DON levels in wheat and barley grains in the *F. culmorum* - inoculated field trails (Kabak and Dobson, 2009; Khan and Doohan, 2009).

Transgenic strategies to ear mold/mycotoxin resistance are now feasible as well. These potentially include genetically increased fungal resistance, enhanced resistance to insect feeding, and detoxification/prevention of mycotoxins in the grain. An example of these strategies the transgenic maize expressing *Bacillus thuringiensis* (*Bt*) toxin that is already on the market and is targeted several maize insects. Studies have shown that *Bt* maize hybrids have the potential to reduce Fusarium ear rot levels in field-harvested grain, presumably by reducing feeding of *Bt* susceptible insects in ear tissues. The plant though will still be vulnerable to non-insect routes of entry to *Fusarium.* However, because of the European legislation these transgenic plants are not widely used in the Mediterranean region. A second strategy that has been carried out in several laboratories is to interfere with the ability of *Fusarium* to infect and colonize the maize ear. This could potentially be achieved by incorporation of resistance genes in elite varieties or by overexpression of specific antifungal proteins or metabolites, or by enhancement of the plant's own innate immune system in kernel tissues. The challenge though in these approaches is to achieve reproducible and stable maize lines with enhanced and durable ear mold resistance under field conditions. A third strategy aims to prevent mycotoxin biosynthesis, or to detoxify mycotoxins *in planta,* creating transgenic lines with genes that code for instance for enzymes that degrade fumonisins (Duvick *et al.*, 2001).

Biological control of patulin and ochratoxin producing fungi in pears and apples

Penicillium expansum, the blue mold that causes soft rot of apples, pears and other fruits, is recognized as one of the most common producers of the mycotoxin patulin. Patulin has been found as a contaminant in many mouldy fruits, vegetables, cereals, and other foods; however, the major sources of contamination are apples and apple products. Patulin occurs mainly in damaged fruits, although the presence of mould does not necessarily indicate the presence of patulin. Patulin is also regularly found in unfermented apple juice, although it does not survive the fermentation into cider products. Patulin is quite toxic at high concentration in laboratory settings, but evidence for natural poisoning is indirect

and inconclusive. Nevertheless, a provisional maximum tolerable daily intake has been established in many countries worldwide (Janisiewicz *et al.*, 2002; Bennett and Klich, 2003).

The use of fungicides or other chemicals is the most common procedure to prevent *P. expansum* soft rots at the postharvest stage, but legislation is becoming more and more restrictive. The use of biocontrol agents as an alternative tool has been investigated in several studies. Several epiphytic microorganisms isolated from fruits and leaf surfaces of apples have been screened for antagonistic activity against *P. expansum* (Ippolito *et al.*, 2000; Nunes *et al,.* 2007; Morales *et al.*, 2008; Robiglio *et al.*, 2011).

The biocontrol activity of the yeast *A. pullulans* on decay of apple fruit caused by *P. expansum* and *B. cinerea* and its ability to induce biochemical defense responses were investigated in apple tissues. In apple wounds, *A. pullulans* multiplied rapidly and controlled decay caused by either *B. cinerea* or *P. expansum*. At the end of the storage period, *A. pullulans* reduced the incidence of gray and blue mold of apples by 89% and 67%, respectively. In addition to controlling the disease, *A. pullulans* led to a transient increase in β-1,3-glucanase, chitinase, and peroxidase activities starting 24 h after treatment and reaching maximum levels 48 h and 96 h after treatment, indicating the likely basis of its biocontrol activity (Ippolito *et al.*, 2000).

Among several bacterial isolates that have been tested, strain CPA-5 of *Pseudomonas syringae*, isolated from an organic orchard, was very effective against *Botrytis cinerea*, *P. expansum* and *Rhizopus stolonifer* at various concentrations on 'Golden Delicious' apple, and 'Blanquilla', 'Rocha' and 'Conference' pears. Additionally, under cold storage conditions and in semi-commercial trials, *P. syringae* (CPA-5) significantly reduced development of *P. expansum* and *B. cinerea* on 'Golden Delicious' apple, and 'Blanquilla' and 'Rocha' pears. Control of *P. expansum* obtained with CPA-5 at 10^8 cfu mL^{-1} on 'Golden Delicious' apple and 'Rocha' pear, was equal to the fungicide imazalil. Population ecology experiments showed that *P. syringae* CPA-5 increased more than 100-fold during the first 50 days after application, and then remained stable on apple fruits, and slightly decreased on pears, indicating the high capacity of this biocontrol agent to colonize wound surfaces of pome fruits under cold storage conditions (Nunes *et al.*, 2007).

In another study, Morales *et al.* (2008) evaluated the effect of two biocontrol agents (*Candida sake* CPA-

2 and *Pantoea agglomerans* CPA-1) on *P. expansum* development and patulin accumulation in cold storage and ambient storage. A cell suspension of a combination of *C. sake* or *P. agglomerans* with a *P. expansum* conidial suspension was applied on wounded apples. Both BCAs controlled blue rot and patulin accumulation during cold storage. *C. sake*-treated apples showed a significant reduction of *P. expansum* growth. On the other hand, *P. agglomerans*-treated apples were lower in patulin accumulation. However, BCA treatment could not control blue rot and patulin accumulation during further storage at room temperature (Morales *et al.*, 2008).

Preliminary assays *in vitro* and *in vivo* on the combination of some nutrients with *Candida sake* strain CPA-1 showed that combination of the antagonist with ammonium molybdate was most effective for reducing blue mold decay caused by *P. expansum* on apples and pears. In laboratory trials, improved control of blue and gray molds was obtained with the application of ammonium molybdate (at concentrations of 1, 5, 10, and 15 mM) alone or in combination with *C. sake* at 2×10^6 or 2×10^7 cfu mL^{-1} on "Blanquilla" pears stored at 20°C. In semi-commercial trials, the efficacy of *C. sake* at 2×10^6 cfu mL^{-1} on reducing *P. expansum* and *B. cinerea* decay was enhanced more than 88% with the addition of 5 mM ammonium molybdate in the 1999–2000 season. In these two seasons, the performance of *C. sake* at 2×10^6 cfu mL^{-1} with the addition of ammonium molybdate was similar to or greater than that of *C. sake* at 2×10^7 cfu mL^{-1}. Similar control of blue mold was obtained on pears stored under low oxygen conditions. The preharvest application of ammonium molybdate did not reduce postharvest blue mold decay. The population of *C. sake* on pear wounds significantly decreased though in the presence of ammonium molybdate 1 and 5 mM at 20°C and 1°C. Studies have shown that ammonium molybdate affects metabolic processes in several organisms. The basis of its biological activity was reported to be its ability to inhibit acid phosphatase, which interferes with phosphorylation and dephosphorylation, one of the most important processes of cell regulation (Teixidó *et al.*, 1998b; Nunes *et al.*, 2002).

Since infection of fruits by postharvest pathogens often occurs in the field prior to harvest it would be a great advantage for the farmer to apply biocontrol agents before harvest, which would reduce initial infection and then remain active and suppress the pathogens in storage. Leibinger *et al.* (1997), using

mixtures of yeasts and bacteria for control of apple postharvest diseases, obtained a level of control of *P. expansum* and *B. cinerea* that was similar to that obtained with a fungicide. For this strategy to be successful, biocontrol inocula need tolerance to environmental stresses, especially high temperature, low water activity (*a*w), low nutrient conditions, and UV light, for effective establishment and disease control (Deacon, 1991). Physiological studies with *C. sake* CPA-1 demonstrated that growth conditions can be modified so that specific endogenous compounds such as sugar alcohols and trehalose accumulate in cells, resulting in improved viability over a wider range of relative humidity with a retained biocontrol efficacy. Accumulation of low- (glycerol and erythritol) and high- (arabitol and mannitol) molecular-weight sugar alcohols occurs in many fungi grown under conditions of environmental stress. Intracellular accumulation of these polyols reduces cytoplasmatic water activity (*a*w) and enables enzymes to remain active during periods of water stress (Teixidó *et al.*, 1998b).

Conclusions

It is well established that the preferred strategy for reducing the concentration of mycotoxins in foods and animal feeds is the prevention of mycotoxin formation during pre-harvest and/or post-harvest stages of the various susceptible crops. Several biocontrol agents have been tested in laboratory and field experiments to effectively reduce colonization and mycotoxin contamination by *Aspergillus*, *Fusarium* and *Penicillium* species in the Mediterranean basin, an area with significant problems with mycotoxins in several crops. In this respect, atoxigenic fungal strains are being widely used to prevent pre-harvest AF contamination of crops such as peanuts, pistachios, maize, and cottonseed by *Aspergillus* spp. in several parts of the world including the Mediterranean area. Recent advancements in the use of biocontrol strategies involving atoxigenic strains has led to registration of commercial products with increased practical applications for the benefit of growers. On the other hand, genetic and molecular approaches aimed at preventing mycotoxin biosynthesis have not yet reached commercial application in the field and require substantial further development. More studies are required to evaluate the potential efficacy of various biological agents, including studies focusing on the dose, formulation and timing of the applications as well as molecular studies that elucidate impacts of the biocontrol agents in alterations of plant physiology or plant innate immune system. Additionally, all the biocontrol agents or GMOs that are planned to be developed for the control of mycotoxigenic fungi have to pass all the necessary safety tests and stages in order to be safe for the consumers.

Prevention strategies at the post-harvest stage can only be effective for those mycotoxins that are formed during this stage of the food production. Natural fungal contamination that occurs pre-harvest can only be minimized post-harvest by application of processing techniques which will minimize subsequent entry into the food and feed chain where possible by inhibition, detoxification or degradation of the mycotoxin. Many scientists are working on this area trying to discover appropriate and cost effective formulations that can effectively detoxify or decontaminate food products.

We also need to point out that in addition to biocontrol methods, key management tools and traceability procedures must be followed that will facilitate effective preservation of stored commodities with minimum loss in quality and quantity. These measures include a) accurate and regular moisture measurements to ensure safe thresholds are not breached b) efficient and rapid drying of wet commodities for medium and long term storage in disinfected and hygienic silos or warehouses that are free of pests and fungal inocula c) traceability during storage and transport for processing d) representative sampling and effective diagnostic tools that can be used to monitor and quantify mycotoxins rapidly (Magan and Aldred, 2007).

Current research needs to utilise the existing sound control measures in order to develop integrated strategies that can counteract the effects of mycotoxigenic fungi and prevent the production of the associated mycotoxins in human and animal food chains.

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