**Annex C - Pre- and post-harvest strategies to mitigate mycotoxin contamination in nuts**

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1. The crops
   1. Peanuts and tree nuts

Several nut crops are subject to mold attack and mycotoxin contamination in pre- and post-harvest. Many studies make it clear that *Aspergillus* species are frequent in nuts, Sections *Flavi* and *Nigri* being the predominant aspergilla (Palencia et al., 2010; Rodriguez et al., 2012; Palencia et al., 2014; Picot et al., 2016). In the literature, the most frequent *Aspegillus* section *Flavi* species isolated in nuts are *A. flavus* and *A. parasiticus*. Both species cause disease on very diverse plant species (from peanuts to tree nuts), and they affect seeds produced above ground and below ground. Each host-parasite interaction has unique features and, for these reasons the crop phenology and infection cycle of these two species are illustrated separately for each crop included in this review. *A. flavus* and *A. parasiticus* can potentially lead to aflatoxin (AF) contamination in all production chain, if preventive measures are not established (Cotty and Jaime-Garcia, 2007). Due to the significant health risks associated with the presence of AFs in nuts, a great number of publications is available concerning this topic, most of them focused on mitigation strategies for reducing contamination in pre and post-harvest in the most widespread nut crops.

* + 1. Peanuts phenology

Peanut (*Arachis hypogaea L.*) is characterized by aerial flowers and underground fruit development (pods). Seed-bearing pods grow below the soil surface, so they are in direct contact with *Aspergillus* soil populations and the seeds can frequently be invaded by these fungi before harvest.

The growth stage description in peanut was designed by Boote in 1982 (Boote, 1982). According to the author, the scale is based on visually observable vegetative (V) and reproductive (R) events which are further subdivided into individual steps. The determination of the vegetative growth stage is based on the number of developed nodes on the main axis of the peanut plant, beginning with the cotyledonary node as zero. While, determination of reproductive stages is based upon visually observable events related to flowering (R1), pegging (R2), fruit growth (R3 and R4), seed growth (R5 and R6), and maturity (R7 and R8) (Boote, 1982). In 1998, following the introduction of the general BBCH scale, a specific BBCH scale has been developed for peanuts. In the derived BBCH scale the phenological development process of the peanut plant is described by periods (principal growth stages from 0 to 9) and steps (secondary growth stages from 00 to 99) (Munger et al, 1998). The principal growth stage are: germination (0), leaf development (1), formation of side shoots (2), main stem elongation (3), inflorescence emerge (5), flowering (6), development of fruits and seeds (7), ripening of fruits and seeds (8) and senescence (9) (Forestry, 2001).

The flowering (R1) commences 30-40 days after planting depending upon genotypes and environment. From about 5 to 7 days after aerial fertilization of the flowers above ground, the peg begins rapid geotropic elongation and starts to penetrate the soil (R2). Following soil penetration the pegs develop into mature pods underground and a peanut fruit is formed (R3-4). It takes about 60 days from the time of fertilization to full maturity (R6). Harvest maturity is reached at 110-170 days from planting (Torres et al., 2014).

* + 1. Tree nuts phenology

The general growth pattern of pistachio (*Pistacia vera L.*) and almond (*Prunus dulcis*) trees is typical of deciduous woody plants, which alternate from a period of active growth under warm seasonal conditions to a dormant, leafless phase under cold, winter conditions. The phenological development process of both tree nuts include the following growth stages: sprouting and bud development (0), leaf development (1), shoot development (3), inflorescence emergence (5), flowering (6), development of fruit (7), maturity of fruit and seed (8) and senescence, beginning of dormancy (9) (Forestry, 2001).

Both nut crops are cultivated under different climate conditions but especially in subtropical and temperate zones. In particular, pistachio is a biennial-bearing and wind-pollinated tree with alternating in fruiting. Areas suitable for pistachio production have long, hot, dry summers and moderate winters. High humidity through the growing season promotes fungal diseases that subsequently overwinter on both male and female trees and reinoculate the tree the following season (Ferguson et al., 2005). Pistachio is a dioecious crop, meaning that the male flowers are borne on one tree and the female flowers on another. The pistachio tree is deciduous, so it loses most of its leaves by the end of November and fall dormant through the following March to reduce exposure of sensitive growing tissue to unfavourable conditions. In order to break out of its dormant state, the pistachio tree requires fulfilment of a chilling requirement (Benmoussa et al., 2017). The chilling period is then followed by heat requirements from April until harvest (Kebour et al., 2013). Pistachio tree have extensive root systems allowing them to mine the soil deeply, and to survive harsh climates without irrigation. The lateral axillary inflorescence buds on one year-old wood begin to swell in late March. Results shown that the duration of bud break is longer in males, which may take up to two months (February and March) (Kebour et al., 2013). The pistachio is a semidry stone fruit consisting of a single kernel (seed) enclosed in a thin, bony shell (endocarp), which is surrounded by the hull. Throughout the balance of April and May the nut shell, but not the seed, enlarges. Through this period the nut shell is soft and vulnerable to insect attack and the splitting that appears to be a result of rain. In June the nut shell hardens, and from late June through early August the seed enlarges until it fills the shell. Through late August and September the enlarged nut pushes on the surrounding shell to cause a natural split.

* + 1. Infection cycle of *Aspergillus flavus* in nuts and plant pathogen interaction
       1. Peanuts

Toxigenic species of *Aspergillus* sections *Flavi* (*A. flavus* and *A. parasiticus*) and *Nigri* (*A. niger*) are the main colonizers of peanut (Barros et al., 2003). *A. flavus* and *A. parasiticus* are soil borne pathogens, and they can infect peanut pods and seed in the soil before harvest or after digging during curing/drying in the field as well as during storage and processing. Both species can also cause aerial infection of flowers and above-ground parts of the plant. Nonetheless, the infection mainly occurs by direct contact of the pod wall with the contaminated soil during pod development and maturation (Horn et al., 1995). The important role of the soil in the disease cycle and epidemiology of *Aspergilli* infection in peanut is well documented. The main sources of inoculum for the colonization of peanuts are the soil borne propagules of *A. flavus* and *A. parasiticus* (Horn et al., 1994). There are evidences that both speciesinvade peanuts as soon as the plant emerges from soil, then disseminate throughout the plant(Pitt et al., 1991).The pod wall is generally resistant to penetration of saprophytic fungi, even though damaged, over mature and physiologically affected pods are more susceptible of *A. flavus* infection. Insect damage exacerbates AF contamination in peanuts. Mites and the lesser cornstalk borer (LCB), *Elasmopalpus lignosellus*, are often associated with injury to peanut pods prior to harvest, i.e., pod scarification and pod penetration. Lynch et al. (1991) studied the relationship between injury by the LCB and invasion of peanut pods and seeds by *A. flavus* under laboratory and field conditions. Results demonstrated that pod injury by LCB has a significant effect on the percentages of pods and seeds contaminated with *A. flavus*; which was significantly greater for LCB-scarified pods (pods=55.6%; seeds=27.0%) than for uninjured pods (pods=17.7%; seeds=3.6%) (Lynch and Wilson, 1991). Despite the important role of insect damage as enhancing factor for *Aspergilli* colonization and subsequent invasion of peanut tissues, peanut can be invaded by *A. flavus* without obvious damage.

A helpful approach to mycotoxin prevention is to minimize its production by monitoring and controlling the environment. Environmental conditions and substrate composition are well known to greatly influence the rate of fungal growth and AF production in food commodities (Aldars-Garcia et al., 2017). Water deficit, elevated temperature (T) and insect activity are the main stress factors promoting aspergilli infection in several nuts both in the field and during storage. Further to environmental conditions, change of intrinsic substrate parameters over the course of kernel ripening, mainly moisture content (water activity, *aw*) and pH, can influence the ability of aflatoxin-producing fungi to infect the fruit. For most nuts, the maturity period has been identified as the most conducive stage for fungal growth (Georgiadou et al., 2012a; Torres et al., 2014).

In peanuts, soil moisture stress acts by reducing the natural defense mechanisms of the plant as well as by inhibiting competitor microorganisms of *Aspergillus* spp., such as rhizospheric bacteria, and promotes the growth of *Aspergillus* species (Torres et al., 2014).

The influence of different combinations of T and *aw* on *A. flavus* growth and aflatoxin B1 (AFB1) production on almond based medium was recently investigated (Gallo et al., 2016). The authors reported that a wide range of aw (from 0.90 to 0.99) and T (from 20° C to 37° C) permit the growth of *A. flavus.* With regard to AF production, the author found that 28° C and 0.96 aw were the optimal conditions for the highest production of AFB1. Recently, (Picot et al., 2016) conducted a field study to determine the most conductive *in planta* conditions to *A. flavus and A. parasiticus* infection in almond. The study showed that for both fungal species, AF levels were significantly higher when inoculation occurred at the beginning of the summer, i.e. closer to the hull split, being the mean amount of AFs higher in kernels inoculated with *A. flavus* than in kernels inoculated with *A. parasiticus* regardless of the kernel stages. Under laboratory conditions, AF production by both *A.flavus* and *A.parasiticus* significantly decreased on mature kernels with very low kernel moisture (aw = 0.60-0.75) incubated under dry conditions. Additionally, the inability of fungi to grow and produce AFs on mature kernels was entirely restored when kernels were incubated under high humidity conditions. The results of this study suggest that as almond dry out in the tree over summer, AF production is less likely to occur in the absence of sufficient moisture. However, the micro-climate in the orchard is sufficient to create moisture conditions conductive for AF production even in dry kernel.

* + - 1. Tree nuts

Infected orchard litter and overwintering fruit mummies (unharvested nuts that remain on trees) may act as inoculum sources for the spread of *Aspergillus* spp. spores in tree nuts (Payne, 1998; Donner et al., 2015). Aspergilli typically grow on soil and plant litter of the orchard floor and, spores are air dispersed and reach all plant parts (Doster and Michailides, 1994). In general, intact nuts are well protected during the growing season against Aspergilli infection because their seeds are enclosed in a shell/seed coat. In late summer, spores can infect pistachios on the tree, especially early-split nuts (hull and/or shell split) (Doster et al., 2014).

In addition, various insect pests may contribute to fungal colonization and subsequent AF contamination. As in the case with pistachios and almond, the key role of insect feeding-damage in facilitating pre-harvest fungal infection is well documented under field and laboratory conditions (Picot et al., 2016). A study on Greek pistachios, revealed higher AF contamination from orchards with heavier level of insect infestation (Georgiadou et al., 2012a). The most common insect pest reported in pistachios in Italy are *Hylesinus vestitus*, that attack buds and branches, *Rhynchota Heteroptera*, like *Carpocoris spp.* or *Nezara viridula*, with adults feeding on fruits, *Teleiodes decorella*, whose larvae developed on buds and leaves, but can also feed on fruits and *Coelioides* spp., mainly *C. rubber*, able to cause occasionally fruit damages (Dinatale et al., 2007). Whitaker et al. (2010) conducted a study in which 12,000 g of almond samples were divided in five grades (high quality, insect damaged, mold damaged, mechanical damaged, and other defects), and found that nuts from the insect damaged grade accounted for 76.3% of the total almonds containing AFs (Whitaker et al., 2010). Among the principal insect pests of almonds, the navel orangeworm (NOW), *Amyelois transitella*, gives the greatest contribution to the invasion of aflatoxigenic *Aspergillus* spp in California and, it is one of the most studied pests in this crop (Campbell et al., 2003; Palumbo et al., 2014). During their development, larvae of NOW feed upon the meat of these nuts causing physical damage and carrying fungi spores (Beck, 2013). The NOW eggs are typically laid on maturing almonds just prior to or at initiation of hull split, which occurs approximately four to six weeks before harvest. However, AFs are also recovered from undamaged kernels, suggesting that *Aspergillus* spp. are able to penetrate through an intact shell, infect the kernel, and produce AFs. Therefore, it is now accepted that insect damage is not mandatory for infection, but it remain as a key risk factor influencing pre-harvest contamination in this crop.

The most common *Aspergillus* spp. found decaying pistachios in Italy (Dinatale et al., 2007), Spain (Fernane et al., 2010) and California (Doster et al., 2014) is *A. niger*, which is much common than the aflatoxin-producing *A. flavus* and *A. parasiticus*. Black aspergilli acquired interest since 1994, when their ability to produce ochratoxin A (OTA) was reported (Abarca et al., 1994). In the literature, the presence of potentially OTA-producer species and OTA have been detected in several substrates including grapes and their derivatives (Battilani et al., 2003), pistachios and peanut seeds (Barros et al., 2003). *A. niger* and *A. carbonarius* were isolated also in Greek pistachios with kernel necrotic spots (Georgiadou et al., 2012b). However, a survey conducted on Italian pistachios in 2007 revealed that the presence of *A.* Section *Nigri* was not a main concern both because strains were not identified as *A. carbonarius* and seeds were found free from ochratoxins. A study conducted in pistachios in Spain showed that contamination with *A.* section *Nigri* and *A. flavus* was 40% and 30%, respectively. Further, OTA-producers in the *A. niger* aggregate (54.4% of the isolated strains) were mostly present in the same samples where aflatoxin-producing *A. flavus* were detected (70.8% of the isolated strains) with a correlation coefficient of 0.61. The correlation of the two toxigenic species would suggest co-occurrence of both mycotoxins in such samples. However, no correlation among OTA and AF contents was detected in the 50 pistachio samples analysed. AFs were detected in five samples (10% of total), whereas only one sample out of 54 pistachio samples showed OTA contamination at a level of 0.67 µg kg -1 (Fernane et al., 2010). Considering the frequent simultaneous presence of toxigenics *A. flavus* and *A. niger* in peanuts in Argentina, a similar experimental study was conducted on fungal interactions and associated production of AFB1 and ochratoxin A (OTA) in this crop. The author suggested that when environmental conditions are favorable, the interaction between the two strains could result in an inhibition in *A. flavus* development and AFB1 production, and in a stimulation of OTA production (Barberis et al., 2012). However, despite several surveys have been conducted on the natural occurrence of aflatoxin- and ochratoxin-producing fungi in peanuts, the information available about the co-occurrence of AFs and OTA in this crop is still not sufficient.

* + 1. Infection cycle of *Fusarium* spp in nuts and plant pathogen interaction

In the published studies, information on *Fusarium* spp. contamination and related mycotoxins in nuts is very limited in comparison with *Aspergillus* spp. Most data refer to AF and OTA occurrence in nuts and dried fruits (Abdulkadar et al., 2000; Heperkan, 2006; Cheraghali and Yazdanpanah, 2010). Some information is available on the occurrence of emerging *Fusarium* mycotoxins (i.e. beauvericin (BEA) and enniatins (ENNs)) in nuts (Tolosa et al., 2013).

* + 1. Cropping system

Many approaches have been applied to prevent AF contamination in the most significant nut crops along the entire production chain. Evidences suggested that the best way to alleviate AF contamination is to apply a holistic approach to control the growth of aflatoxin-producing fungi during farming, harvesting, postharvest, and processing. A good example of a rational approach for the prevention of AFs in peanuts has been recently reviewed by (Torres et al., 2014). The article summarizes the main factors influencing AF contamination in the whole supply chain of peanuts. And, for each of these factors a review on the current prevention practices is provided. The authors divided current management practices in pre-harvest, harvesting and post-harvesting strategies. The pre-harvest control takes all relevant environmental and agronomic factors into account, such as peanut cultivars, crop rotation, soil type, water activity, chemical and biological control of AF-producing fungi; the optimum harvest time and timely drying of peanuts are reported as the main factors during harvesting; while segregation, moisture control and other storage conditions are the main factors resulting in the AF contamination after harvesting peanuts.

Despite the fact that AF contamination of tree nut crops has become a growing international food safety concern during the last decades, studies on prevention strategies are very limited in comparison with other food products. In 2011, a study was conducted on AF contamination of Greek pistachio nuts (variety *P. vera* cv *Aegina*) (Georgiadou et al., 2012a). The study followed all production and processing steps from field to storage and determined the conditions and/or handling practices that mainly affect AF production. The results show that higher levels of AF were detected at maturity and in orchards with heavy insect infestations. Furthermore, legal limits were not exceeded in a well irrigated and cultivated, clean orchard without insect infestation where hot-air drying and storage under controlled conditions of T and relative humidity were used. Regarding almonds, current management practices to reduce AF contamination are mainly designed by the U.S. Department of Agriculture (USDA) and related boards, because of the large importance of this crop in the country (Campbell et al., 2003; Picot et al., 2016).

Pre-harvest control of AF contamination in nuts is based on the use of good agricultural practices (GAPs) designed to avoid conducive conditions to aspergilliinfection. These practices have been identified by taking into consideration various environmental and agronomic factors that influence susceptibility of the plant to aflatoxin-producing fungi infection.

* + - 1. Managing crop residues and crop rotations

The native habitat of *Aspergillus* spp. is soil, as well as decaying vegetation, hay, and grains undergoing microbiological deterioration. Therefore, a primary consideration must be to ensure orchard sanitation practices to reduce the sources of fungi inoculum, including mummy nuts, crop debris and pests.

In peanuts, there are evidences that continued cultivation of the plant on the same land can increase the population of *A. flavus* and *A. parasiticus* in the soil (Horn et al., 1995). However, the influence of crop rotation on AF contamination can vary widely according to the environmental conditions (Torres et al., 2014). Regarding tree nuts, adverse micro-climate conditions in the orchard can enhance fungal growth. Correct management of the orchard floor is of particular importance because the fruit is often picked up off the soil surface where it is left to dry for few days after being knocked from trees. Therefore, the orchard floor is assumed to be an important source of contamination and it should be in the best possible condition especially at the time of harvesting. Canopy density as well as canopy size can have large impact on humidity, light interception and quality of orchard floor. Dense canopy lets very little light to reach orchard floor under tree (higher yield, cooler temperatures) while sparse canopy lets much more light reach orchard floor under tree (lower yield, warmer temperatures). Research funded by the Almond Board of California (ABC) demonstrate that at the end of drying period, the moisture content under almond tree in 2% higher compared to in drive row (ABC, 2014).

* + - 1. Water stress

Drought stress has been closely associated with AF contamination in peanuts and pistachio nuts. In peanuts, water deficit increases susceptibility to fungal invasion, since it decreases the moisture content of the pod and kernels and greatly lowers the physiological activity of the peanut. It is shown that prolonged water deficit during seed filling period associated with elevated soil temperatures (>22°C) enhances AF production. For this reason, it is important to guarantee an appropriate water supply to the plant until harvest time. In particular, the last 3 to 6 weeks of the growing season are considered the most critical for peanuts development (Cole et al., 1995). It is documented that the soil water holding capacity may influence the AF accumulation in peanuts seeds. Soils with high water holding capacity, such as heavier soils, may cause a lower drought stress and, therefore, minimize the fungal colonization (Torres et al., 2014).

Regarding tree nuts, it has been observed that under heat or drought stress the fruit may develop a hull cracking of the external shell known as “early split” that facilitate *Aspergilli* infection (Georgiadou et al., 2012b)

* + - 1. Biological control agents (BCAs)

Biological control of toxigenic fungi is the most effective strategy to prevent AF production under pre-harvest and post-harvest conditions in several agronomical commodities, including nuts. This technique is based on the use of several organisms including bacteria, yeasts and non-toxigenic strains of *A. flavus* and/or *A. parasiticus* which act through competitive exclusion of naturally occurring toxigenic strains. Other organisms including rhizobacteria and yeasts have been tested for the control of AF contamination in peanuts. Evidences have shown that these antagonistic microorganisms successfully colonized the root and suppressed the growth of *A. flavus* in the vicinity of roots. The enzyme activities of *Trichoderma spp.* were reported to be effective in suppressing the growth of aflatoxigenic fungi and the production of AF under laboratory and greenhouse conditions (Gajera and Vakharia, 2012; Navya et al., 2015).

Initial studies on biocontrol strategy in peanuts began in 1987 in the United States using a naturally occurring strain of *A. parasiticus* (NRRL 18991) (Dorner et al., 1992). The effectiveness of pre-harvest biocontrol strategies in this crop has been demonstrated in several years of laboratory and field studies (Dorner et al., 2003; Dorner and Lamb, 2006; Chiuraise et al., 2015). Consequently, during the past years several bio-competitor agents have been registered as biopesticides for the use in peanuts and their commercial use was done in few countries such as USA, Australia and Argentina (Dorner, 2004; Torres et al., 2014). Additional evidence demonstrated that not only the application to soil of nontoxigenic strains of *Aspergillus* reduces levels of pre-harvest AF contamination in peanuts, but it also has a carryover effect, reducing contamination during storage (Dorner and Cole, 2002). In 2015, the efficacy of a native non-aflatoxigenic *A. flavus* (AFCHG2) strain applied to the soil was evaluated under field trials in Argentina (Chulze et al., 2015). Treatments resulted in significant reductions of the incidence of toxigenic isolates of *A. flavus* and *A. parasiticus* in soil and peanuts.

Naturally occurring atoxigenic isolates of *A. flavus* have been used successfully to reduce AF contamination in pistachios. The *A. flavus* strain AF36, which belong to the VCG YV36, has been extensively used as a biocontrol agent in commercial corn and cotton fields to reduce AF contamination. A recent study, evaluated the efficacy of this atoxigenic strain in California pistachio orchards. Four-year application of the AF36 strain resulted in increasing the proportion of VCG YV36 within the *A. flavus* population in the soil as well as a reduction in AF contamination of pistachio nuts. Reductions in percentages of samples contaminated with AF from treated orchards ranged from 20 to 45%, depending on the year (Doster et al., 2014). Nevertheless, the optimal time and irrigation to maximize the application of AF36 product have not been determined yet.

It has been demonstrated that the use of biological control agents during pre-harvest carries beneficial effects through the value chain from the field where AF contamination begins to storage (Dorner, 2008). Moreover, in 2010 the first report was published demonstrating that a strain of marine bacterium *Bacillus megaterium* could be used as a biocontrol agent against postharvest fungal disease caused by *A. flavus* in peanuts (Kong et al., 2010).

* + - 1. Host resistance

Breeding for host-plant resistance to *Aspergillus* species and/or pests plays a significant role in preventing AF contamination in nuts. Identifying sources of resistance is a complex process that includes: (i) direct selection for resistance to fungus and mycotoxin occurrence, and (ii) indirect selection for resistance to biotic/abiotic stresses or morphological traits that impede or delay fungal introduction or growth (Torres et al., 2014).

Since the late 1960s, several attempts have been carried out leading to the development of resistant peanut genotypes, which were eventually released as improved germplasm in some peanut-producing countries. Earlier research on genetic improvement at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) were mainly focused on *in vitro* seed resistance to *A. flavus* colonization duringpost-harvest, which was by then considered as a priority in the peanuts production chain (Mehan et al., 1987). When subsequent field studies revealed that pre-harvest contamination was also common, efforts were broadened to include breeding high-yielding varieties with resistance to seed infection and AF production (Mehan et al., 1991). As a consequence, several resistant lines to pre-harvest AF contamination were identified in this crop.

In peanuts, considerable effort has been made also in understanding the molecular mechanisms of host resistance to *A. flavus* infection (Guo et al., 2008; Guimaraes et al., 2012; Senakoon et al., 2015; Wang et al., 2016). Guo et al. (2008) identified the resistance-related genes (iso ara h3 and LEA 4) in peanuts against *A. parasiticus* infection, and then developed a microarray to identify candidate genes that confer resistance to *A. flavus* infection (Guo et al., 2011). Wang et al. 2016 used RNA-seq for global transcriptome profiling of post-harvest seed of resistant (R) and susceptible (S) peanut genotypes under the fungus infection stress. The study results suggested that AF production activated many genes to a much higher level in R than in S genotype (Wang et al., 2016). Finally, biotechnological approaches to increase host-plant resistance through the use of anti-fungal and anti-mycotoxin genes were also considered. This approach received a major boost with the successful establishment of peanut regeneration and transformation protocols (Sharma and Vanamala, 2000), and led to the transformation of popular peanut cultivar JL 24 with a rice chitinase gene to help prevent invasion by fungal pathogens.

New varieties of almonds have also been developed which are resistant to insect attack. Plant breeding is developing almonds with better shell integrity and an improved suture seal that prevent infestation of the nut kernel by insects (Campbell et al., 2003). However, despite great efforts the level of resistance reached through breeding and genetic engineering is still not sufficient to protect nut crops from AF contamination under all field conditions.

* + - 1. Pest and disease control

Insect damages are well known to cause significant losses and to be conducive of toxigenic *Aspergillus* spp. infection in several food commodities, including nuts. Several studies detected higher AF contamination in orchards with heavy level of insect infestation indicating a positive correlation between AF contamination and insect infestation, including pistachios (Cotty and Jaime-Garcia, 2007; Georgiadou et al., 2012b), almonds (Picot et al., 2016) and peanuts (Torres et al., 2014). Therefore, it can be expected that field application of insecticides can reduce the risk of mycotoxin contamination associated with insects.

Several chemical compounds are known to be effective against aspergilli infection in nuts as well as in controlling pest infestation. However, the increase of resistance to synthetic agents by *Aspergillus* spp. is an additional concern and has stimulated the search for new antifungal agents to control AF contamination in nuts. Moreover, as regard fungicides, reported results are not conclusive regarding the efficacy of their application under field conditions.

* + 1. Harvest management

Several good practices can be applied to prevent AF contamination at harvest stage, including (i) proper harvesting time, (ii) quickly drying of seed, (iii) reduction of mechanical damage of kernels and, (iv) removal of impurities. Identify the optimum physiological maturity of the plant and consequently the right harvesting time is important, as both too mature and very raw kernels influence the grain safety and quality. Rain just prior or during harvest can complicate moisture control efforts, and since rain can increase the risk of mold growth, it’s important to adjust harvest accordingly.

Drying is one of the most common and cost-effective techniques/tool used to reduce AF production in nuts post-harvest. Different drying techniques have been applied to control the growth of aflatoxin-producing fungi in nuts, including typical (i.e. sun-drying, natural ventilation, heated ventilation and stirred ventilation drying) and advanced drying techniques (i.e. hot air-, infrared and superheated steam drying) (Chiewchan et al., 2015). Evidences demonstrated that high percentage of kernel invasion by *A. flavus* in peanuts occur in slowly-dried pods as compared to rapidly-dried pods. In general, rapid drying of nut products should be ensured because little opportunity for fungal invasion occurs when the moisture content is lowered rapidly and steadily. Furthermore, drying process should be continued until a product reaches the safe storage moisture. Safe moisture should be maintained also after drying, as inappropriate storage and transport conditions can reactivate the fungi and cause AF contamination.

* + - 1. Peanuts

Peanut harvesting is a 2-stage process consisting of digging and drying prior to picking. Harvest maturity (R8) in peanuts is reached at 16–24 weeks after crop emergence, with minor fluctuations depending on variety, planting time, seasonal conditions and location. Among the mentioned factors, T has the greatest influence on ripening time. It has been demonstrated that premature and delayed harvesting after physiological maturity can increase the risk of AF contamination. In many crops, the influence of delayed harvest on AF contamination is more severe when crops are affected by rain just prior to or during the harvest (Milani, 2013). Therefore, harvest earlier than normal is considered a good preventive action when the risk of AF contamination is high (Battilani, 2010). The moisture content of peanut kernels at digging ranges from 30 to 50%. The seed moisture content must be reduced to 10.5% or below before seeds can be graded and marketed. After digging, peanuts are partially cured for 2–5 days and then mechanically separated from the plant prior to drying to reduce the moisture content from 18% to 10%. The period following digging is crucial for AF production because substrate conditions are inductive for fungal activity. Thus, soon after digging, peanut must be rapidly dry to or below *aw* of 0.83 (Dorner, 2008; Torres et al., 2014; Chiewchan et al., 2015). Research indicated that the maximum substrate moisture content for safe storage is respectively 9% and 7% in unshelled and shelled peanuts, whit 70% environmental relative humidity and temperature of 25-27 C° (Diener and Davis, 1977). Mechanical damage to pod shells is also a major contributing factor for AF contamination. Research indicates that damaged grains deteriorate more rapidly during storage and they are much more susceptible to fungi infection. It is therefore recommended to minimize mechanical damages during threshing as well as during handling, transportation and loading of stocks into the warehouse (Pattee and Young, 1982).

* + - 1. Pistachios

Maturity period (July and August) can be considered as the most critical stage for AF contamination in pistachios. This is probably related to high moisture content of the fruit at this stage; the moisture at harvest is between 32 and 38% (Aktas and Polat, 2007), whereas *aw* remains very high until harvesting, around 0.99 (Mahoney et al., 2014). Furthermore, matured pistachios soften and can be physically damaged, due to the cracking of the hull, becoming vulnerable to the attack of aflatoxin-producing fungi. Evidences demonstrated that the amount of AFs detected is higher at later maturity stage (Georgiadou et al., 2012a). A study conducted in four Greek pistachios orchards, show that, at early maturity stage, a small amount of AF (B1:3.1 µg/kg) was detected only in one orchard included in the study. At the following stage of maturity (thirteen days later) AFs were detected in all orchards. AFB1 was the dominant one among the four types of aflatoxin in every stage, followed in order by aflatoxin G1 (AFG1), aflatoxin B2 (AFB2) and aflatoxin G2 (AFG2).

Harvesting can be done mechanically or manually by covering the area under and round the tree with a type of protective hard cloth, shaking the tree branches by hand and packing the dropped nuts into sacks. After harvesting, the fruits are de-hulled (separation of the soft hull from the shell) in mechanical water de-hullers. During this process, trash and blank, not fully developed or low weight nuts are sorted by floating. De-hulled nuts are dried either mechanically, in hot-air dryers (65-70 °C for about 8-10 h), or naturally, under the sun (spreading pistachios on flat concrete roofs in a thin layer of 2-3 cm thickness for approximately 2-3 days), or under plastic tunnels to accelerate the drying process. Naturally drying lasts at least 4-5 days and exposes the fruits to insect and bird damages, as well as to adverse weather conditions. This procedure increases the risk of fungal growth and AF production, especially if the pistachio layer exposed to the sun is not thin (Georgiadou et al., 2012a). After drying, moisture content of pistachios is around 12-13%. Evidences show that the most effective preventative control during storage is to dry freshly harvested fruits as soon as possible to 6% of moisture content and then cool stored (Aldars-Garcia et al., 2015; Aldars-Garcia et al., 2017). Concerning water activity, 0.82 or lower *aw* is required for short-term storage and 0.7 or lower *aw* for long-term storage (Aktas and Polat, 2007).

* + - 1. Almonds

In July and early August, almond hulls begin to split open exposing the almond shell and allowing it to dry. Shortly before harvest, the hulls open completely. Harvesting starts from mid-August through October. Starting harvest too late increases worm damage and AF potential. Harvesting is typically done by shaking almonds off the trees by mechanical tree "shakers" and left them dry naturally for 10 days on the orchard floor. Near the end of sun-drying, the almonds are swept into neat rows to be prepared for the final step of harvesting which is the pick-up phase (Picot et al., 2016). This harvest practice facilitates colonization of almonds by *Aspergillus* spp. soil community. Harvested almonds, with their hull and shells intact, are stored in large stockpiles under tarps while waiting for processing. The impact of different tarp materials on stockpile conditions has been studied. Covering a stockpile with a tarp is necessary, but it can increase the humidity within the stockpile, causing an increase in moisture and a major risk of AF contamination. Due to this risk, the Almond Board of California (ABC) has identified few best practices for monitoring and adjusting the amount of moisture in stockpiled almonds: it is recommended that (i) almonds not be stockpiled if hull or kernel moistures exceed 12% and 6%, respectively; (ii) the shape and positioning of stockpiles should equally be evaluated when choosing where to stockpile almonds, as well as (iii) the use of effective tarps at minimizing temperature fluctuations, i.e. white-on-black tarp, with the white side facing upward (ABC, 2014).

* + 1. Post-harvest management

During post-harvest nuts are prone to quality deterioration and AF contamination due to improper handling, storage and processing practices. In post-harvest management, it is noticeable that hygienic conditions and environmental factors, such as T and humidity fluctuations as well as light and oxygen exposure, can affect aspergilli growth and AF contamination in stored nuts. The risk of AF contamination during storage is significantly reduced if these factors are optimized and maintained throughout the storage period, as any deviation from desirable conditions can enhance the growth of toxigenic molds.

* + - 1. Segregation

In peanuts, the first good practice for a correct post-harvest management is segregation of lots with visible moulds or with AF contamination higher than the legal limit which must be stored separately and not used for edible purposes (Torres et al., 2014). When peanuts are harvested, they contain a wide variety of foreign material (e.g. soil, rocks, sticks, immature shriveled pods, etc.) and loose shelled kernels (LSK) that cause safety and quality problems in storage and processing. Therefore, after lot segregation, peanuts undergo a screening/sorting process prior to storage. This is done primarily to remove foreign material as well as to separate damaged and discolored pods and/or kernels that may contain a higher concentration of AFs. There are evidences that removing these high-risk components before shelling or storing has the effect of reducing the amount of AF concentration in the final product up to 70% (Dickens and Whitaker, 1975; Dorner, 2008). The type of technologies used for sorting ranges from traditional manual screen, grains size and density separation, as well as electronic color sorting (ECS) (Udomkun et al., 2017). The effectiveness of ECS is widely accepted. However, as AFs can be found also in peanuts without external alterations, this method is not wholly effective. Thus, blanching followed by ECS is recognized as the best method for reducing AFs in shelled peanut lots. ECS after blanching removes aflatoxin-contaminated kernels from the blanched lot because slight discolorations in the kernel tissue that were not visible before the seed-coat removal become evident after removal (Torres et al., 2014).

* + - 1. Moisture control

Substrate moisture content and temperature dictate the extent of mold contamination in stored nut commodities. Mold growth on nuts during storage is mainly associated with high moisture content of the kernel as well as elevated relative humidity surrounding the commodity (Dorner, 2008). Therefore, maintaining safe and constant moisture level during storage till processing or consumption is necessary to reduce the risk of contamination. Primary sources of elevated moisture are inappropriate storage warehouse (e.g. inadequate protection from rain/leaky roofs and/or condensation due to insufficient aeration and ventilation systems), presence of high-moisture foreign materials and high initial moistures of the nuts before storing.

* + - 1. Cleaning

Proper cleaning is essential to reduce the risk of contamination. It is recommended to clean the warehouse storage facilities and handling equipment before loading of new peanuts stocks. Moreover, it is essential to monitor peanuts in storage on a routine basis to check for evidence of mold contamination.

Recent results demonstrated that high pressure treatment (HPP) had a significant inhibitory effect on *A. flavus* growth in stored peanuts. HPP is an emerging non-thermal technology that can inactivate food spoilage microorganisms improving food safety. The study shows that the HPP treatments at 800 MPa and 600 MPa for 10 min inhibited *A. flavus* growth in peanuts, and also reduced AF accumulation during 30-days storage period (Huang et al., 2014).

* + - 1. Air gas composition and storage containers

Recent studies indicate that storage of peanuts under controlled atmosphere offers superior safety over traditional storage under normal air conditions. Hermetic packaging and storing of peanuts is more useful in maintaining constant moisture as well as controlled atmosphere composition compared to traditional jute and/or woven polypropylene bags. Jute bags are highly porous and can easily absorb moisture, and so foster the rapid growth and multiplication of aflatoxigenic molds. While polypropylene bags are non-absorptive, they tend to trap heat inside. Hermetic storage works on the principle of drastic elimination of oxygen in conjunction with an increase in CO2 within the storage atmosphere which is achieved by the respiration of insects, fungi and grain (Quezada et al., 2006). Since mycotoxin-producing molds are oblige aerobes, the fungal development ceases when the oxygen level decreases to 1% (Villers et al., 2006). Moreover, it has been reported that increased levels of CO2 in storage containers results in reduction of AF production (Moseley et al., 1971). Sudini (2015) demonstrated that hermetic storage of peanuts using triple layer “Purdue Improved Crop Storage (PICS)” bags retard significantly mold development and subsequent AF accumulation (Sudini et al., 2015).

* + - 1. Antioxidants, Phenolic Compounds, and essential Oils

To overcome the development of fungal resistance as well as residual toxicity posed by chemical fungicides/synthetic additives, a wide range of plant-based natural compounds with bioactivity against molds have been identified as alternative agents to control AF contamination in nuts both in pre- and post-harvest. The effect of isothiocyanates (ITCs), which are reported as some of the most potent antimicrobials from plant origin, was confirmed also in nuts (Otoni et al., 2014). Evidences shown that the treatment with gaseous dispersion of ITCs generated by the enzymatic hydrolysis of glucosinolates (GLCs) present in oriental (*Brassica juncea*) and yellow (*Sinapis alba*) mustard flours reduced *A. parasiticus* growth in several nuts products, including peanut, walnut, cashew, hazelnut, almond and pistachio. The reduction of AFB1, B2, G1 and G2 ranged from 83.1% to 87.2% using the oriental mustard flour, whereas employing the yellow flour the mean reduction observed ranged from 27.0 to 32.5% (Hontanaya et al., 2015). Other evidences exist on the *in vitro* antagonistic effects of aqueous extracts of mentha (*Mentha pulegium*), senna (*Cassia senna*), basil (*Ocimum basilicum*), thyme (*Thymus vulgaris*), and safflower (*Carthamus tinctorius*) on the growth of *A. flavus* isolated from pistachios (Omidpanah et al., 2015). Several studies have revealed the fungicidal activities of essential oils (EOs) as well as derived compounds on *Aspergillus* section *Flavi* populations in stored peanuts. Adjou Euloge (2013) demonstrated the fungal growth and mycotoxin inhibitory properties of the essential oil from fresh leaves of Sweet Fennel (*Ocimum gratissimum*) in stored peanuts (Adjou et al., 2013). This EO was found to be effective against several *Aspergillus* and *Fusarium* strains. The extract exhibited a pronounced antifungal activity against the growth of *A. ochraceus* and *F. oxysporium* compared with *A. flavus* and *A. parasiticus*. The Minimal Fungicide Concentration (MFC) was recorded to be 8.0 μl/ml for A. flavus and *A. parasiticus*, 6,5 μl/ml for *A. ochraceus*, and 6.0 μl/ml for *F. oxysporium*. Furthermore, several marine natural products, which are well known as potently active metabolites, have been screened for their use as bioactive agents against toxigenic fungi. In particular, the antifungal activity of crude extracts obtained from marine sponges was tested against aflatoxin producing fungi. The results indicate that extract obtained from the sponge *Amphimedon* spp. and *Monanchora arbuscula* were effective antifungals against *A. flavus* isolated from stored peanuts (Arevabini et al., 2014). A number of studies have determined the effect of synthetic and natural food additives on AF reduction in food products including nuts.

The use of food-grade antioxidants (butylated hydroxyanisole [BHA], butylated hydroxytoluene [BHT] and propyl paraben [PP]) alone and in mixtures was found to be significantly influenced on the growth of fungal populations in stored peanuts. BHA and BHT are synthetic antioxidant which are authorised as a food additive in Europe. EFSA lately delivered two scientific opinions re-evaluating the safety of these two food additives (EFSA, 2011, 2012). A recent study investigated the antifungal and insecticidal activity of both BHA and BHT against *Aspergillus section Flavi* and *Oryzaephilus surinamensis* (L.), a vector carrier of aflatoxigenic fungi on stored peanuts. Results have shown that the BHA formulation completely inhibited *Aspergillus* section *Flavi* development regardless of *aw* and doses assayed and no aflatoxin accumulation was detected in treated samples. Antifungal effect was also demonstrated in samples treated with microencapsulated BHT but it was highly dependent on *aw* (Garcia et al., 2016).

* + - 1. Decontamination

Several methods are used to detoxify hazelnuts from AFs (Basaran and Akhan, 2010; Dasan et al., 2016). Cold atmospheric plasma using different gases is quite well established in various industrial processes, and its use is also potentially promising in the food sector, in particular for microbial and mycotoxin inactivation. Siciliano (2016) conducted a study to investigate the efficacy of low temperature atmospheric pressure plasma, generated by ionization of nitrogen and different mixtures of nitrogen/oxygen, on the reduction of four aflatoxins (AFB1, AFG1, AFB2 and AFG2) both *in vitro* and on hazelnuts. The highest detoxification efficacy was obtained with nitrogen or nitrogen/oxygen mixtures (0.1% O2) for plasma generation and the longest exposure times (12 min) (Siciliano et al., 2016).

Food processes, including roasting, may have different effects on mycotoxins, and high temperatures have proven to be very effective in the reduction of AFs. Two different roasting methods, traditional static hot air roasting and infra-red rays roasting, were tested for detoxification of hazelnuts from Italy and Turkey at a temperature of 140°C for 40 min of exposure. The detoxification resulted effective for both roasting techniques (Siciliano et al., 2017). Gamma-irradiation of peanuts at 5.2 kGy has been found to be an effective treatment to prevent the growth of potentially aflatoxigenic fungi during storage (Costa de Camargo et al., 2012). The inhibitory effects of both biotic and abiotic agents on presence of mycotoxin producing fungi in stored peanuts have been previously tested. El-Saidy and El-Hai (2011), reported that the treatment of seed with yeast extract (*Saccharomyces cervisiae*) followed by salicylic acid result in relevant reduction of AFB1 content in peanut seeds after 6 months of storage (El-Saidy and El-Hai, 2011; Al-Othman et al., 2013).

Zhang (2012) reported another novel technology that has been applied to inhibit AF contamination called acidic electrolyzed oxidizing water, which is an electrolyte solution prepared using an electrolysis apparatus with an ion-exchange membrane, used to decontaminate AFB1 from naturally contaminated peanut samples. The content of AFB1 in peanuts decreased about 85% after soaking in the solution. Remarkably, the nutritional content and colour of the peanuts did not significantly change after treatment (Zhang et al., 2012).

**A number of nut crops are affected by contamination with AFs. The principal nuts of concern include peanuts and some of the most popular tree nuts in the world, such as pistachio and almond. The most frequent mycotoxins detected in nuts are AFs, whereas poor data is available on co-occurrence with other mycotoxins.**

Abbreviations

|  |  |
| --- | --- |
| ABC | Almond Board of California |
| AFB1 | Aflatoxin B1 |
| AFB2 | aflatoxin B2 |
| AFG1 | aflatoxin G1 |
| AFG2 | aflatoxin G2 |
| aw | Activity water |
| BCAs | Biological control agents |
| BEA | beauvericin |
| BHA | Butylated hydroxyanisole |
| BHT | Butylated hydroxytoluene |
| ECS | Electronic colour sorting |
| ENNs | enniatins |
| EOs | Essential oils |
| GAPs | Good agricultural practices |
| GLCs | Glucosinolates |
| HPP | High pressure treatment |
| ICRISAT | Crops Research Institute for the Semi-Arid Tropics |
| ITCs | Isothiocyanates |
| LCB | Lesser cornstalk borer |
| LSK | Loose shelled kernels |
| MFC | Minimal Fungicide Concentration |
| NOW | Navel orangeworm |
| OTA | Ochratoxin A |
| PP | Propyl paraben |
| USDA | U.S. Department of Agriculture |

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