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Safety Aspect in Soybean Food and Feed Chains: Fungal and Mycotoxins Contamination

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1. Introduction

Soybean (*Glycine max* L. Merr.) is an Asiatic leguminous plant cultivated in many parts of the world for its oil and proteins, which are extensively used in the manufacture of animal and human foodstuffs (FAO, 2004a; Hepperly, 1985). The production reached 47.5 million tons during the 2006/2007 harvest season ranking Argentina third as soybean producer in the world. In Argentina, during the last quarter of the century, soybean production has increased at an unprecedented rate from a cultivated area of 38.000 hectares in 1970 to 16 million hectares today. Around 70% of the soybean harvested is processed, providing 81% and 36% of the world's exported soybean oil and meal, respectively (SAGPyA, 2010). Soybean is often attacked by fungal infections during cultivation, or post-harvest (in transit or in storage), significantly affecting its productivity. Seeds and infected harvest debris are the main sources of primary infections, and the level of seed damage depends on environmental conditions such as high relative humidity, dew, and temperatures above 25 ^{o}C. These species can be potential mycotoxin producers. Mycotoxins (from "myco" fungus and toxin) are relatively low-molecular weight, fungal secondary metabolic products that may affect exposed vertebrates such as animals in a variety of ways. Mycotoxins are considered secondary metabolites because they are not necessary for fungal growth and are simply a product of primary metabolic processes. The functions of mycotoxins have not been clearly established, but they are believed to play a role in eliminating other microorganisms competing in the same environment. They are also believed to help parasitic fungi invade host tissues. The amount of toxins needed to produce adverse health effects varies widely among toxins, as well as within each person's immune system (Brase et al., 2009).

Some mycotoxins are carcinogenic, some are vasoactive, and some cause central nervous system damage. The mycotoxins can be acutely or chronically toxic, or both, depending on the kind of toxin, the dose, the health, the age and nutritional status of the exposed individual or animal, and the possible synergistic effects between mycotoxins. The most frequently studied mycotoxins are produced by species of *Aspergillus*, *Penicillium, Fusarium* and *Alternaria.*

There is an increasing world consumer demand for high quality and inocuous food and drink products with the lowest possible level of contaminants such as mycotoxins. As a result, the food industry in the developed world demands raw ingredients of the best

quality and that conform to statutory limits where these have been set for mycotoxins. Because the mycotoxins are unavoidable, it is important to know how the concentrations of mycotoxins present in raw materials change through the food and feed chains. The development of prevention strategies today has been predominantly based on using the HACCP approach and to identify the critical control points in the pre- and post-harvest food chain. This approach enables strategies for minimizing consumer exposure to be developed through appropriated management of the products (Sanchis & Magan, 2004; Scudamore 2004).

2. Fungal and mycotoxin contamination in soybean at preharvest stages

2.1 *Fusarium* species dynamic and their mycotoxins at different soybean reproductive growth stages

The occurrence of fungi in soybean seeds has received far more attention than the occurrence of fungi in pods and flowers. This is understandable from a practical standpoint: infected seeds and infected seedlings developing from them represent greater economic risks in soybean production, and seed contamination with mycotoxins represents a health risk to human and animals (Roy et al., 2001).

The mycoflora of soybean seed may be affected by environmental factors, geographic location, cultural practice and degree of host susceptibility to pathogens (Villaroel et al., 2004) Previous studies carried out in Argentina evaluated the fungal contamination on freshly harvested soybean or soybean seeds under storage (Boca et al., 2003; Broggi et al., 2007). However, other studies have demonstrated that the isolation frequency of fungi from living plant tissues differs from those of senescing or dead tissues of soybean plants. For this reason, three reproductive stages of soybean development and samples of flowers, pods and seeds were examined. The flowers were obtained during R2 growth stage (full bloom), while pods and seeds were recovered during R6 (full seed) and R8 (full maturity) growth stages. The mycoflora isolated from flowers, pods and seeds were dominated by two genera: *Alternaria* and *Fusarium*, at similar levels across all the stages. Among *Fusarium* isolates, 45% were isolated from pods, 38% from seeds and 17% from flowers. *Fusarium* contamination across different stages showed that the high isolation frequency was found in pods and seeds at stage R6 (full seed), being the a_w of immature seeds 0.992. At stage R8 (full maturity), the water content of the seeds dropped dramatically to 0.70 and the percentage of *Fusarium* spp. also diminished compared to stage R6. Villarroel et al. (2004) showed that *Fusarium* spp. isolated at R6 and R8 growth stages from pods and seed tissues were significantly greater on conventional than on transgenic cultivars. In our study, a transgenic cultivar with tolerance to glyphosate was used since more than 90% of the planted area in Argentina belonged to this category (Lopez et al., 2008). The *Fusarium* species identified are showed in the Fig. 1, being *F. equiseti* the most frequently recovered from flowers, pods and seeds (40% of isolates), followed by *F. semitectum* (27%) and *F. graminearum* (11%). The distribution of *Fusarium* species was similar in the three parts of the plants and the reproductive stages evaluated.

Members of *Fusarium* genus are known to produce a broad spectrum of toxins including trichothecenes of A- and B-types. Among B-type trichothecenes, deoxynivalenol (DON) and nivalenol (NIV) are important mycotoxins produced by members of the *F. graminearum* species complex (*Fg* complex). DON is the most distributed *Fusarium* mycotoxin and occurs world-wide in crops from temperate regions. NIV also occurs in cereals and has been

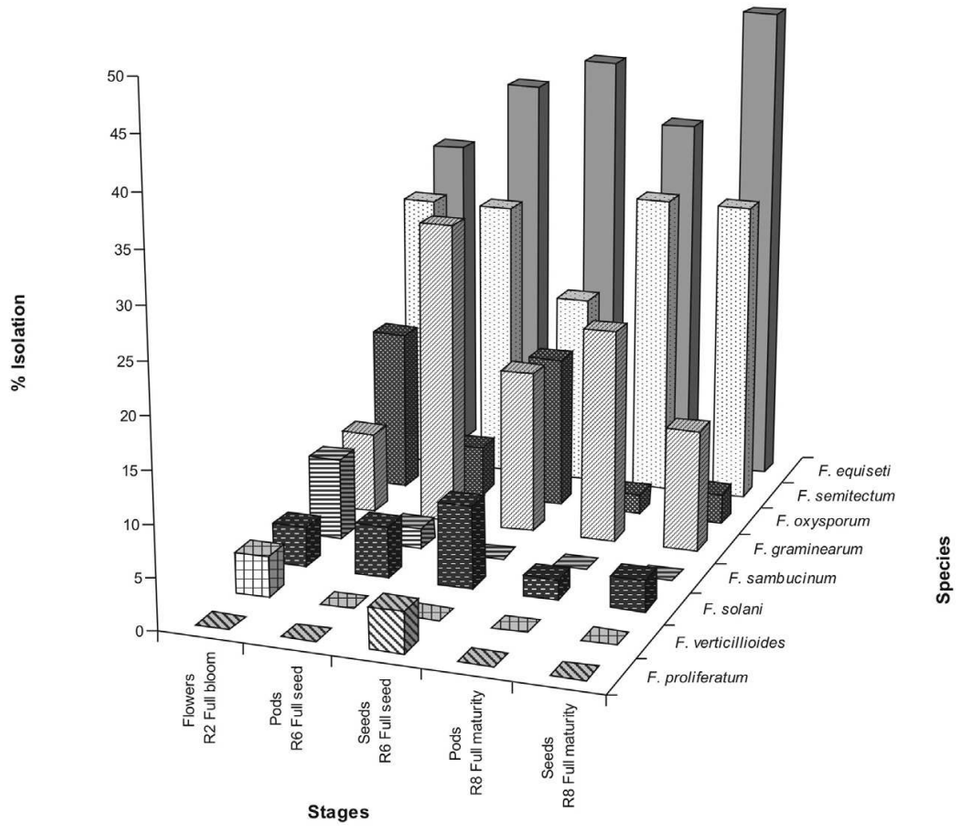

Fig. 1. *Fusarium* species distribution at different soybean reproductive stages of development.

extensively found in Japan and Korea, and at relatively low levels in samples from Europe, Southern Africa and South America (Placinta et al., 1999). DON is associated with feed refusal, vomiting and suppressed immune functions (WHO, 2001), and NIV is more toxic to humans and domestic animals than DON (Ryu et al., 1988). Due to their toxicity, international regulations limit the content of DON in the food chains (FAO, 2004a; Verstraete, 2008). Trichothecenes also are potent phytotoxins (Eudes et al., 2000), with DON being more phytotoxic than NIV (Desjardins et al., 2006).

In our study, soybean seed samples from each reproductive stage (R6 and R8) were evaluated for DON and NIV contamination. Out of 40 samples, two were contaminated with DON at levels of 1.6 μg/g (R6 stage) and 0.9 μg/g (R8 stage). Only one sample at stage R8 showed T-2 toxin contamination at level of 280 μg/kg. Neither NIV nor HT-2 were detected (Barros et al, 2008b). These results demonstrate that the soybean could be contaminated with mycotoxins at low levels and the presence of *Fusarium* species potentially toxigenic might be considered for further studies on natural contamination. Generally, grains with a moisture content equivalent to less than 0.70 a_w (<14.5% moisture by weight) will not be subject to fungal spoilage and mycotoxin production (Aldred and Magan, 2004). In the present study, similar a_w was observed in seeds at stage R8, this fact suggest that the mycotoxin contamination occurred between stages R6 and R7 were the level of a_w was around 0.99. Other studies have analyzed the importance of soybean pathogens such as *Diaporthe/Phomopsis* complex and *Cercospora spp.* in tissues prior to harvest maturity (Baird et al., 2001; Ploper et al., 1992; Roy et al., 2001; Villarroel et al., 2004). However, this is the first

report to analyze the *Fusarium* species diversity and densities and mycotoxin contamination during the soybean grain ripening.

2.2 Chemotype and genotype within *Fusarium graminearum* species complex

Based on the DON contamination on soybean seeds at stages R6 and R8, we considered interesting to investigate the toxigenic ability of isolates belonging to *F. graminearum* species complex. Strains of *F. graminearum* usually express one of three sets of trichothecene metabolites either: (i) nivalenol and acetylated derivatives (NIV chemotype), (ii) deoxynivalenol and 3-acetyldeoxynivalenol (3-ADON chemotype), or deoxynivalenol and 15- acetyldeoxynivalenol (15-ADON chemotype) (Ward et al. 2002). Surprisingly, *Fusarium* isolates that produce both DON and NIV (NIV/DON chemotype) have been reported and described as "unknown" chemotypes (Quarta et al., 2006; Ward et al., 2002).

The 15-ADON chemotype is predominant in North America and the 3-ADON chemotype is predominant in same areas in Asia, including China, Australia, and New Zealand (Guo et al., 2008). Due to the toxicological differences between NIV and DON (Desjardins and Proctor, 2007), it is important to determine the chemotypes of strains present in a given region on different crops. With the identification of the genes responsible for trichothecene biosynthesis, PCR assays have been developed to distinguish the toxin producing genotypes (Chandler et al., 2003; Jennings et al., 2004; Lee et al., 2001; Waalwijk et al., 2003). Primers based on the sequences of alleles at *Tri3*, *Tri5* and *Tri7* have been designed to differentiate the three toxin genotypes (Quarta et al., 2005, 2006).

Examination of trichothecene profile among the strains isolated from the soybean agroecosystem revealed that the 15-ADON was the dominant chemotype identified by chemical and PCR analysis (Barros, personal communication). Similar results were obtained in previous studies that evaluated the toxigenic potential of *F. graminearum* isolates from wheat in Argentina (Alvarez et al., 2009; Reynoso et al., 2011) and South of Brazil (Scoz et al., 2009). However, a 12% of the isolates showed an unusual pattern of trichothecenes with a simultaneous DON and NIV production. The finding of isolates with an unusual pattern of trichothecenes production (DON and NIV producers) in this study agree with previous reports by Reynoso et al. (2011) and Fernandez Pinto et al. (2008) who identified this type of strains from wheat in Argentina. This fact is not surprising since e regular crop rotation is soybean/wheat and the pool of strains could move through one to another agroecosystem.

Several reports examined the trichothecene production by strains of *F. graminearum* isolated from cereals in Argentina based on chemical analyses (Alvarez et al., 2009; Faifer et al., 1990; Fernandez Pinto et al., 2008; Lori et al., 1992; Molto et al., 1997). However, this is the first report on determine trichothecene chemotype and genotype among the *Fg* complex isolated from soybean in Argentina. Although a good relation between multiplex PCR (genotype) and chemical analysis (chemotype) was observed, more studies are necessary to evaluate variations within the field populations on soybean. For example, in North America the 15-ADON chemotype is predominant, but recent molecular surveillance has shown that 3-ADON chemotype is replacing 15-ADON from eastern to western Canada (Ward et al., 2008).

The *Fg* complex is composed of at least twelve lineages (O'Donnell et al., 2008). *F. graminearum* populations from wheat in Argentina are genotypically diverse, and belong to *F. graminearum* lineage 7 (Ramirez et al., 2006, 2007) also termed *F. graminearum sensu stricto* (O'Donnell et al., 2000, 2004). Further studies using molecular markers and sequence analysis on the *F. graminearum* species complex population isolated from soybean

agroecosystem are in progress, these results will allow us to know the isolates characteristics such as genotype, genetic diversity, lineage and pathogenicity.

3. *Alternaria* species and their mycotoxins on soybean at harvest time

A diverse group of saprophytic and parasitic fungi can colonize and infect soybean pods and seeds prior harvesting (Villarroel et al., 2004). *Alternaria* and *Fusarium* species are the most commonly isolated fungi from soybean in Argentina and in others regions of the world (Boca et al., 2003; Broggi et al., 2007; Gally et al., 2006; Roy et al., 2001). The most common *Alternaria* specie found on soybean seeds is *A. alternata*.

From a survey done on *Alternaria* species contamination on soybean samples (n=50) harvested in two provinces of Argentina during two harvests seasons (2006-2007 and 2007-2008) years. We observed that around 80% of the samples presented *Alternaria* spp. contamination at levels ranging from 2 to 84%. One hundred and forty strains were morphologically indentified at species level according to Simmons (1992, 2007) based mostly on the three-dimensional sporulation patterns. Seventy five strains were identified as *A. alternata*, 65 as *A. infectoria*, 8 as *A. oregonensis*, 5 as *A. graminicola* and 1 as *A. tritimaculans*, respectively. These results were noticeable, considering that except *A. alternaria*, all the other strains were members of the *A. infectoria* (morphological) group and also all belonged to the infectoria species-group, which is genetically distinct and phylogenetically distant from the other species-group (brassicola species-group and the alternata species-group). The *A. infectoria* group comprises at least 30 known species (Andersen et al., 2009). Morphologically the *A. infectoria* group differs from others *Alternaria* species-groups in the three dimensional sporulation pattern (Simmons and Roberts, 1993). The mains characteristic for the *A. infectoria* group is the production of small conidia in branched chains with long, geniculate multilocus secondary conidiophores between conidia (Simmons, 2007). This data were the first report on the presence of member of species belonging to the *A. infectoria* group on soybean. Recently, *A. infectoria* have also been isolated in high frequency on wheat probably due to changes in cropping systems in most of the different agroclimatic zones in Argentina (Ramirez et al., 2005). Also, Perello et al. (2008) have associated *A. infectoria* as the ethiological agent of black point in wheat grains in Argentina. We explain the presence of *A. infectoria* and *A. infectoria* group on soybean as a consequence of the practice of double cropped soybean cultivation widely used in our country, which consist of sowing the soybean immediately after harvesting a wheat crop.

Most *Alternaria* species are saprophytes that are commonly found in soil or on decaying plant tissues. Some species are opportunistic plant pathogens that, collectively, cause a range of diseases with economic impact on a large variety of important agronomic host plants including cereals, ornamentals, oil crops and vegetables. *Alternaria* species are also well known as post-harvest pathogens. Some *Alternaria* species are well known for the production of toxic secondary metabolites, some of which are powerful mycotoxins that have been implicated in the development of cancer in mammals (Thomma, 2003). Among these metabolites with mammalian toxicity are alternariol (AOH), alternariol monomethyl ether (AME) (Logrieco et al., 2003; Ostry, 2008). Recently have been reported that AOH and AME posses cytotoxic, genotoxic and mutagenic properties *in vitro* (Brugger et al., 2006; Fehr et al., 2009; Lehmann et al., 2006; Wollenhaupt et al., 2008), and there is also some evidence of carcinogenic properties (Yekeler et al, 2001). Tenuazonic acid (TA) is a mycotoxin and phytotoxin, produced primarily by *A. alternata*, but also by other phytopathogenic *Alternaria* species (Logrieco et al., 2003). This toxin is considered as a

possible causal factor of Onyalai, a human hematological disorder (Ostry, 2008). TA also exhibits phytotoxic, insecticidal, zootoxic, cytotoxic, antibacterial and antiviral activity. TA has been shown to be more toxic than other mycotoxins produced by *Alternaria* species such as altenuene (AE), alternariol (AOH) and alternariol monomethyl ether (AME) (Ostry, 2008). These mycotoxins have been demonstrated to be produced by *Alternaria* species on wheat, tomato, sorghum, pecans, sunflower and on cotton (Scott, 2001; Ostry, 2008).

3.1 Ecophysiology of *Alternaria alternata* on soybean based media

Fungal growth and mycotoxin production result from the complex interaction of several factors and, therefore, an understanding of each factor involved is essential to understanding the overall process and to predict and prevent mycotoxin development (Chamley et al., 1994). Temperature and water activity (a_W) are the primary environmental factors that influence growth and mycotoxin production by several *Alternaria* species in cereals and oil seeds (Etcheverry et al., 1994; Magan & Baxter, 1994; Magan & Lacey 1984; Torres et al., 1992; Young et al., 1980).

Prevention of mycotoxin contamination of food raw materials is now considered more effective than subsequent control. Thus, hazard analysis critical control point (HACCP) approaches are being developed to examine the critical control points (CCPs) at which mycotoxigenic fungi and mycotoxins may enter a range of food and feed chains (Aldred & Magan, 2004). Therefore, accurate information is needed on the impact of key environmental factors such as a_W, temperature and their interactions, and on identifying marginal and optimum conditions for growth and toxin production. Few studies have attempted to build two-dimensional profiles for growth and mycotoxin production by *Alternaria* species (Sanchis & Magan, 2004). Due to the lack of information on *Alternaria* growth and toxin production on soybean or culture medium based on soybean, we decided to evaluate the effect of water activity (a_W; 0.995, 0.98, 0.96, 0.94, 0.92 and 0.90), temperature (5, 18, 25 and 30°C), incubation time (7-35 days) and their interactions on mycelial growth and AOH, AME and TA on soybean extract agar by two *A. alternata* strains isolated from soybeans in Argentina. Maximum growth rates were obtained at the highest a_W (0.995) and 25°C with grow decreasing as the water availability of the medium was reduced. Maximum amount of AOH was produced at 0.98 a_W and 25 °C for both strains. Maximum AME production was obtained for both strains at 30 ºC, but a different a_W 0.92 and 0.94 for the strains RC 21 and RC 39 respectively. Maximum TA production was obtained for both strains at 0.98 a_W, but at 30 and 25 ºC for the strains for RC 21 and RC 39 respectively. The concentrations range of three toxins varied considerably depending on a_W and temperature interactions assayed. Further, the three metabolites were produced over the temperature range from 5 to 30 ºC and a_W range from 0.92 to 0.995. Although at 5 and 18 ºC little of any mycotoxin was produced at a_W lower than 0.94. Two-dimensional profiles of a_W x temperature were developed from these data to identify areas where conditions indicate a significant risk from *A. alternata* growth and mycotoxin accumulation on soybean (Fig. 2) (Oviedo et al., 2009a, 2010). All the conditions of a_W and temperature were maximum production of the three toxins are those found during soybean development in the field. Thus, field conditions are likely to be conducive to optimum grow and toxin production of this specie.

Taking in to account these results, it appears that different combinations of a_W and temperature are necessary for optimal production of these 3 toxins by *A. alternata* and that the limiting a_W for detectable mycotoxin production is slightly greater than that for growth.

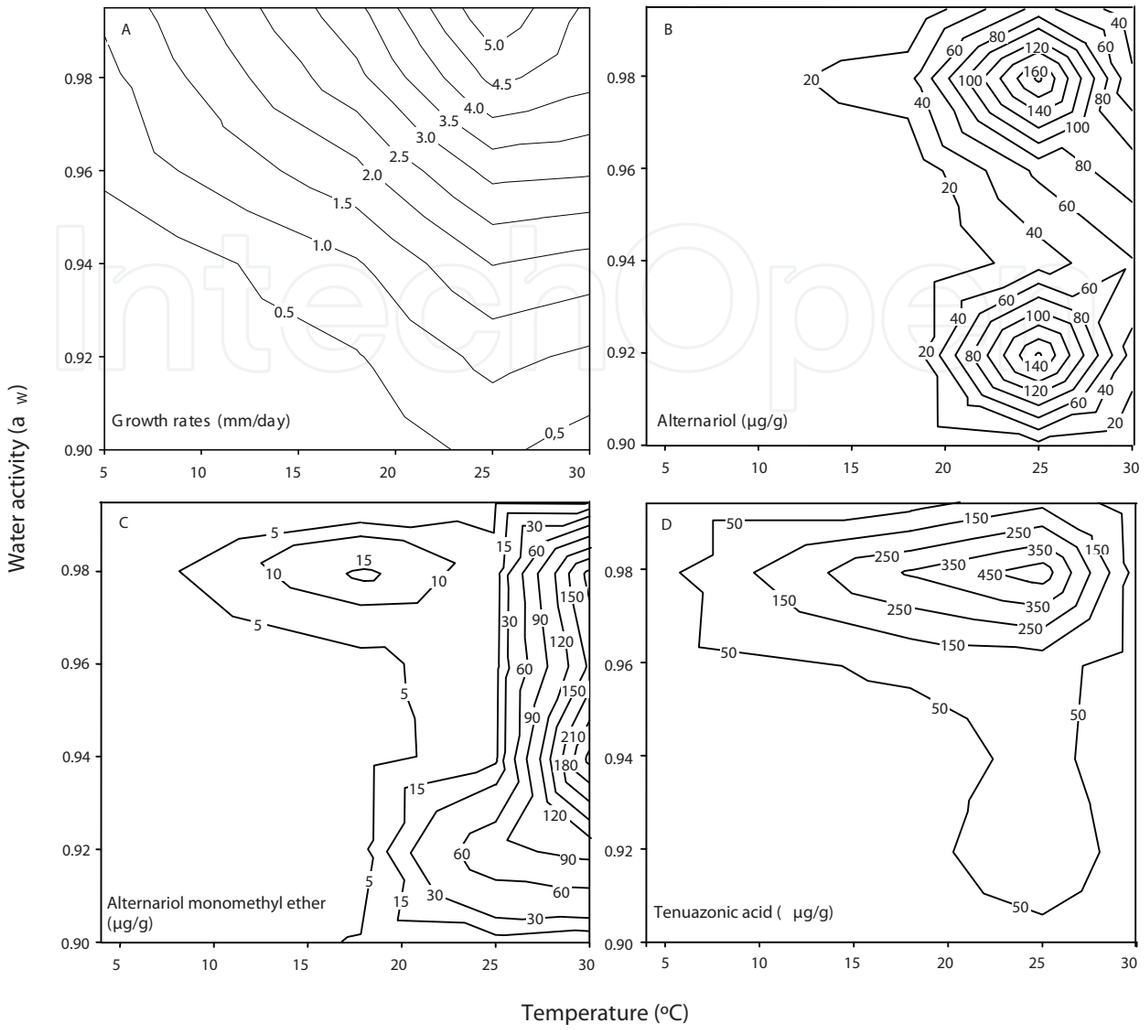

Fig. 2. Comparison of profiles for growth and different mycotoxins by *Alternaria alternata* on soybean based media.

In the present study, the knowledge of interacting environmental conditions provides very useful information for predicting the possible risk factors for AOH, AME and TA contamination of soybean. The a_W and temperature range used in this study simulate those occurring during grain ripening. Also, the data demonstrated the contrasting impact of a_W, temperature and incubation time on growth and AOH and AME production by the two strains examined. The knowledge of AOH, AME and TA production under marginal or sub-optimal temperature and a_W conditions for growth can be important since improper storage conditions accompanied by elevated temperature and moisture content in the grain can favour further mycotoxin production and lead to reduction in grain quality.

3.2 Natural contamination of *Alternaria* mycotoxins on soybean seeds

Alternaria toxins have recently received much attention, both in research programs and in risk assessment studies. At present, no statutory or guideline limits set for *Alternaria* mycotoxins have been set by regulatory authorities (FAO, 2004b). Current data on the

natural occurrence of *Alternaria* toxins point to low human dietary exposure. Further studies are necessary to develop strategies for safe food and feed supplies by developing detection methods, identifying *Alternaria* mycotoxins risk in the production chain, determining the critical control points, and developing preventive measures.

Numerous methods have been developed for AOH and AME determination in different agricultural commodities (Logrieco et al., 2009; Ostry, 2008; Scott, 2001); however there was no an available technique for determining these mycotoxins in soybean. Solid-phase extraction columns have been used for extraction and clean-up of AOH and AME in apple juice and wheat. The natural occurrence AOH and AME on soybean seeds harvested in Argentina was evaluated. Both toxins were simultaneous detected by using HPLC analysis coupled with a solid phase extraction column clean-up. Characteristics of this in-house method such as accuracy, precision and detection and quantification limits were defined by means of recovery test with spiked soybean samples. From a survey of 50 soybean seed samples evaluated for AOH and AME contamination, it was found that 44% of them were contaminated with AME. AME was found in levels ranging from 62 to 1,153 ng/g. Although a limited number of samples were evaluated, this data were the first report on the natural occurrence of *Alternaria* toxins in soybean seeds and is relevant from the point of view of animal public health (Oviedo et al., 2009b). Also the results showed that AOH and AME are produced on soybean seeds at harvest time. This data agree with previous studies (Oviedo et al., 2009a, 2010) in which we have demonstrated that the environmental conditions (a_W and temperature) optimum for growth and mycotoxin production by *A. alternata* on soybean-base media were similar to those occurring during soybean development in the field until harvest.

During the last five years numerous studies dealing with AOH and AME toxicity have been published. Both mycotoxins have been reported to have genotoxic, mutagenic and carcinogenic effects (Ostry, 2008; Logrieco et al., 2009). Also, have been suggested that, the mutagenicity of AOH may have bearing on the carcinogenicity of this mycotoxin. Recently Tiemann et al. (2009) have demonstrated that AOH and AME, at similar concentration levels found in the present study, negatively affected progesterone synthesis in porcine granulosa cells *in vitro*. In view of the fact that granulosa cells directly influence the metabolic and structural growth of the oocyte (Albertini et al., 2001), exposure to AOH or AME may eventually affect reproductive performance by interfering with follicular development in swine and possibly other mammalian species. Feedstuff should therefore be carefully controlled for *Alternaria* toxins content.

4. Fate of fungal and mycotoxin contamination during soybean meal production process

Soybean production and its by-products (oil and meal) form one of the most important economic activities in Argentina. At present, Argentina is the world´s first exporter of soybean meal and oil and the third producer of soybean, behind USA and Brazil (Lopez et al., 2008). Hygienic safety of soybean and by-products depends on fungal contamination among other microorganisms. However, with European Union legislation imminent, the consideration of mycotoxins is becoming increasingly important.

The fungal and mycotoxin contamination on soybean used in the soy meal production was examined, in order to identify critical control points (CCP_s) in the process. Respect to fungal contamination, the levels of fungal propagules in all points of the process were no higher

than 10^4 cfu/g, value considered safe by GMP14 normative. However several potential toxigenic fungi were detected, especially species belonging to the genera *Fusarium* and *Aspergillus*. Among *Fusarium* species, *F. verticillioides* was most frequently recovered (60% of isolates), followed by *F. oxysporum, F. subglutinans, F. proliferatum, F. semitectum* and *F. graminearum*. The genus *Aspergillus* was the second most frequent genus isolated and the dominant *Aspergillus* species identified belong to the section *Flavi* (*A. flavus*) and section *Nigri* (*A. niger* aggregate). According to the species identified, the natural occurrence of aflatoxins (produced mainly by *A. flavus*), fumonisins (produced by *F. verticillioides* and *F. proliferatum*) and deoxynivalenol (DON) and zearalenone (produced mainly by *F. graminearum*) were analyzed at six points in soybean meal production process. Previously, in order to evaluate the natural occurrence of these mycotoxins, adequate methodologies for their determination on soybean and soy meal by using HPLC analysis were optimized (Barros et al., 2008a, Barros personal communication).

Aflatoxin B_1 and fumonisin B_1 were detected in a few samples at low levels and no zearalenone contamination was observed. DON showed higher incidence than aflatoxins and fumonisins and was detected in different points of the process at ppm levels. However, *F. graminearum*, the main responsible for cereal contamination with DON in Argentina, was recovered at very low frequency. This result showed that DON contamination of soybean seed occurred at field stage previous to harvest. For this reason, the dynamics of *Fusarium* populations at field stage and specifically in the reproductive growth stages where the soybean seed is developed was evaluated.

5. Conclusion

This chapter summarizes new information on toxigenic fungi and mycotoxin contamination on soybean at preharvest, harvest and processing stages. Also data on ecophysiology of the most important genus and species isolated are provided. Although, *Alternaria* mycotoxins are not yet regulated, their toxicity is at present under revision. *Fusarium* mycotoxins are regulated both in food and feedstuff. All the presented information are relevant from the point of view of food safety, since mycotoxins are natural contaminants and their presence is unavoidable. It is important to reduce their presence and optimized prevention strategies at all stages of food and feed chains.

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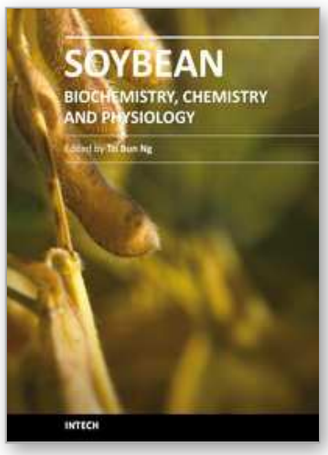

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