Final Report Summary - MYCORED (Novel integrated strategies for worldwide mycotoxin reduction in the food and feed chains)

Executive Summary:
MycoRed developed novel solution driven methodologies, handling procedures and practically useful tools to reduce significantly both pre- and post-harvest toxin contamination of selected and economically important commodities in food and feed chains.

MycoRed applied integration of specific technologies to a set of food/feed chain targets with respect to wheat, maize, grape, nuts and dried fruits, by new advanced integrated approach based on multidisciplinar know-how and technology. A significant reduction of mycotoxins in pre-harvest was obtained by optimization of plant resistance, fungicide use, biocontrol, modelling and developing a decisional support system (DSS). Wheat genotypes and maize hybrids were identified to be resistant to Fusarium Head Blight and Fusarium Ear Roat respectively. A significant reduction in DON contamination (80-90%) was reached by using fungicide optimization technology and additional 50% reduction compared to the best technology found was reached by developing a better nozzle composition.

Field trials also on large scale in Nigeria, Argentina, The Netherlands and Italy, were done with selected biological control agents in maize, peanuts and wheat; so far up to 80% aflatoxin control reduction in maize and in peanuts was achieved with non-toxigenic strains while F. graminearum inoculum was decreased about 70% in wheat stubble by using antagonists. Predictive models for DON/ZEa contamination in wheat, FUM and AFLA in maize, OTA in grapes, were developed and validated supporting DSS. In post-harvest and processing, relationships between environmental factors and dry matter loss relevant to EU legislative limits (DON, FUM and AFLA) were identified in wheat, maize and hazelnuts. Novel solution driven methodologies and handling procedures based on gases O3 in wheat and maize, reduced the fungal growth and FUM production (100%) in maize. In addition, novel anti-fungal natural compounds were identified and economically evaluated to be potentially used for post-harvest control of DON, FUM and AFLA. An intelligent system based on wireless sensor network devices was developed to monitoring temperature, humidity and CO2 into grain silos. Some agricultural by-products can find technological applications as low cost feed/food additives for mycotoxin reduction mycotoxins (up to 95% AFB1, 83% ZEA, 83% OTA and 47% FB1), and represent a potentially valuable source of phenolic antioxidants and undegradable fibre, which could promote health also through their ability to “trap” in the digestive tract. Dried fruits processing by sulphuration, dehulling/peeling, sorting and roasting reduced AFLA B1 in apricot seeds (up to 99.5%), in pistachio (83%), and almonds (50%). Advanced molecular technologies were developed for identification of toxigenic fungi from several host plants, and novel approaches to control mycotoxigenic fungi by application of light at different wave length, permitting a better control of fungal growth and toxin production/reduction. Advanced analytical methods were developed and validated for multi-mycotoxin analysis in a range of food matrices from the chosen food chains and for simultaneous determination of multi-biomarkers for main mycotoxins in human and animal urine. Rapid test kits (strip tests) for the detection of DON, AFLAs and FUMs were thoroughly validated and checked for cross-reactivity against conjugated and other altered forms of mycotoxins.

The project had a significant impact in the scientific international community as well as in the stakeholders, policy makers and industry. In addition the project improved awareness and advanced knowledge on mycotoxins concerns in the world, by dissemination and training with unexpected interest expressed by different communities. International events, in cooperation with ISM and/or other organizations, were organized in the world (Austria 2009, Malaysia 2010, Africa 2011, Argentina 2011,
Canada 2012, Italy 2013) with participation of about 2000 experts. Workshops on specific topics (Hungary, 2010, Turkey 2010, Russia 2011, Egypt 2011, The Netherlands 2012); 4 training courses on Detection techniques, on line too (Italy, Malaysia, Indonesia). More than 150 Young researchers/students were involved in this learning process, participating at Short Term Visits mostly from ICPC countries (24) in EU/Africa labs and Home Education sessions in China/Argentina/Indonesia/USA. Twinnings (EU-Canada; EU-Argentina) and scientific networks were activated by signing 20 alliances/agreements worldwide.

Project Context and Objectives:
Reducing mycotoxin contamination in the worldwide food and feed chains is a major challenge to improve human and animal health. The presence of mycotoxins in food and feed may affect human and animal health as they may cause several effects such as induction of cancer and mutagenicity, estrogenic, gastrointestinal and digestive, blood, kidney and nerve problems. These toxins are health hazards that could contaminate a wide variety of crops and are also primary sources of both yield losses and increase of management costs worldwide. The mycotoxic risk is difficult to evaluate, as mycotoxins are natural contaminants and the regulation way is one of the main system to limit exposure to mycotoxins. One quarter of the world’s food crops, including many basic foods, are potentially contaminated by mycotoxin-producing fungi. The mycotoxic problem is particularly important for human health in tropical areas, e.g. Sub-Saharan Africa, where crops are particularly susceptible to contamination with the carcinogenic aflatoxins and fumonisins. Globalization of trade has complicated the way we deal with mycotoxins in that regulatory standards often become bargaining chips in world trade negotiations. Many countries worldwide have adopted laws and regulations that set maximum allowed levels of mycotoxins for the affected commodities, in order to decrease the risk to human health or safety. These regulations include the detention of contaminated goods at the borders to avoid their entrance in the market. Growers, processors and exporters are being directly affected, as they lose part of the goods that are rejected on borders and markets, which sometimes have to be destroyed. While developed countries have numerous mycotoxin regulations and a well-developed infrastructure for enforcing food quality standards, people in developing countries are not protected by food quality monitoring or by the enforcement of safe standards within their countries. Food commodities that enter international commerce are expected to comply with CODEX Alimentarius standards. This requirement may indirectly increase the risk of mycotoxin exposure in developing countries because the best quality foods leave the country, while the lower quality food is consumed, often by humans, and not discarded. The consequences of this requirement to comply with the needs of the European Union and its consequences for global strategies for mycotoxin reduction need further consideration.

Table 1. List of major plants exposed to mycotoxin risks and addressed by MycoRed

<table>
<thead>
<tr>
<th>Plant</th>
<th>Chain</th>
<th>Toxin</th>
<th>Fungal genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>Food/feed</td>
<td>Fumonisins/Aflatoxins</td>
<td>Fusarium/Aspergillus</td>
</tr>
<tr>
<td>Wheat</td>
<td>Food/feed</td>
<td>Trichothecenes/Zearalenone/Ochratoxin A</td>
<td>Fusarium/Fusarium Penicillium</td>
</tr>
<tr>
<td>Grape*</td>
<td>Food**</td>
<td>Ochratoxin A/Fumonisins</td>
<td>Aspergillus</td>
</tr>
<tr>
<td>Nuts</td>
<td>Food</td>
<td>Aflatoxins</td>
<td>Aspergillus</td>
</tr>
<tr>
<td>and dried fruits</td>
<td></td>
<td></td>
<td>(Peanuts, figs, pistachios, almonds, apricot seeds)</td>
</tr>
<tr>
<td>*including raisins and sultans ** wine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wheat and maize have major mycotoxin safety concerns worldwide, and they were studied throughout their food and feed chains. The mycotoxins minimization may have important impact at economic level, by prevention and optimization of food and feed processing. Maize is considered to be one of the commodity most susceptible to mycotoxins worldwide. The dominant mycotoxigenic species is strictly related to meteorological conditions in the regions of cultivation. The impact of climate change has been identified as an emerging issue for food and feed safety. In particular, in dry and hot conditions maize grains resulted highly contaminated by AFLA B1 while in wet and mild conditions FUM B1 is the most frequently reported mycotoxin in maize.

Specific strategies for raisin and sultanas also were considered for ochratoxin A reduction in addition to the grape-wine chain, for which promising pre-harvest strategies were developed in previous FP project. Grapes and their processed products are very important in Europe where about 47% of worlds vineyards are found and 75% of the world’s wine. Finally, dried fruits and
nuts also were considered because of the high risk for aflatoxin contamination and the high social and economic impacts resulting from stringent EU limits.

In this context, MycoRed was based on the multidisciplinary integration of specific technologies in the whole food/feed chain with respect to wheat, maize, grape, nuts and dried fruits. Multidisciplinary integration of know-how and technology has been applied in order to address the broad requirements for reducing mycotoxins in agro-food chains.

The overall objectives of the project were:

a. to develop novel solution driven methodologies and handling procedures to reduce both pre- and post-harvest contamination in selected and economically important commodities in feed and food chains;

b. to generate and disseminate information and education strategies to reduce mycotoxin risks at a global level. High risk areas received major attention by cooperation with international agriculture and food organizations and by applying the results of all technical workpackages of the project.

The project was addressed also to support and spread innovation at different levels in the food/feed chain and in the SME applications based on:

- development of new pre- and post-harvest strategies for achieving the required specifications addressed due to the selected applications;
- combination of advanced technological solutions for improving specifications;
- cost reduction for novel analytical/molecular solutions at the laboratory level, allowing the massive use and thus, the improvement of monitoring of safety and quality;
- application of very high-tech multidisciplinary solutions (i.e. ambient intelligence, webinar, DNAarrays, biomarkers) to traditional activities (i.e. conferences, training courses) in Europe;
- first validation of solutions developed under the project referred to the ambient intelligence.

Specific objectives were:

- improving prevention to minimize mycotoxins in products at different critical steps of the food chain;
- developing a set of systems with clear breakthrough solutions to specific mycotoxicological problems;
- disseminating information and best practice education strategies to enhance the involvement of operators at all levels along food and feed chains. To reduce mycotoxin risks at a global level, high risk areas should receive major attention by cooperation with international agriculture and food organizations and by applying the results of all technical workpackages of the project;
- strengthening the international participation and cooperation at European and global level.

All these general objectives have been reached at the end of the project. The project addressed the prevention problem to minimize mycotoxins in products at different critical steps of the food chain (raw materials, storage, feed supply, food processing, final products) by vertical (across food and feed chains) and horizontal (among methodologies and procedures) integration of experiences to develop a set of systems with clear breakthrough solutions to specific mycotoxicological problems. MYCORED enforced its efforts by a sound dissemination strategy, acting on several levels of information and best practice education to enhance the involvement of players along food and feed chains, facilitating the participation and cooperation at global level. The web-communication was based on a massive action to reach the maximum audience and large public, by using website, newsletters, links, web-seminars, webconferences. The project dealt with the themes of knowledge and reduction of aflatoxins, trichothecenes (deoxynivalenol, nivalenol, T-2 and HT-2 toxins, etc.), zearalenone, fumonisins and ochratoxin A, taking into consideration indications given by several international food organizations (IARC, FAO, CIMMYT, EFSA, IITA, SAFE consortium), EU reports and relevant food industry representatives. The project, based on integrated approach of a reduction system as a horizontal task, was planned to create a synergetic action by disseminating technological solutions developed by the research activities and to address geographic target areas affected by mycotoxin problems. The most effective strategies were disseminated at European and global level.

The MYCORED approach complied with the needs of the EU vision by using global, multidisciplinary and integrated strategies, that can be effectively applied along the food and feed chains and linked to decision-making bodies and consumers through
effective mycotoxin risk assessment and information and education programmes. Five work-packages developed novel-solution driven strategies and handling procedures to reduce both pre- and post-harvest contamination in feed and food chains. They involved: - optimization of plant resistance and fungicide use (WP1); - biocontrol to reduce toxigenic fungi in cropping systems (WP2); - modelling and development of a Decision Support System (WP3); - novel post-harvest and storage practices (WP4); and application of new food processing technologies (WP5). Two additional horizontal work-packages developed methodologies for advanced diagnostics and quantitative detection of toxigenic fungi (WP6) and rapid and multi-analyte detection and quantification of mycotoxins (and relevant biomarkers) (WP7). A horizontal work-package (WP8) covered all information, education, dissemination and demonstration activities to reduce mycotoxin risks worldwide based on the outcomes of the above WPs and other knowledge of methodologies and handling procedures actually in use for particular crops and geographical areas. Finally WP10 demonstrated the creation of an “ambient intelligence” for real time and for periodic monitoring of mycotoxicogenic fungal contamination during storage of cereals. A set of food/feed targets (which include the most important crops for human and animal consumption) and relative mycotoxins was identified in MYCORED.

Project Results:
The research activities considered mainly toxins and commodities in economically important food and feed chains by a multidisciplinary integration of know-how and technology.
Four important food/feed chains and relative main mycotoxin risk were investigated in pre-and post-harvest contamination processes: wheat (Trichothecenes, Zearalenone, Ochratoxin A), maize (Fumonisins, Aflatoxins), grape-wine (Ochratoxin A), nuts and dried fruits (Aflatoxins).

WHEAT CHAIN

Introduction
Food safety and security are important issues worldwide due to both growing request of food at global level and the need to ensure basic levels of safety. Wheat is a key crop in this perspective, being the first more cultivated crop worldwide with around 216 million ha. However, wheat is a crop that can be commonly contaminated by mycotoxins, considered among the highest reason of concern for their chronic health effect on consumers (Kuiper-Goodman, 2004). Indeed, wheat is a crop contaminated, at a different incidence, by three of the most important mycotoxin-producing fungi: Aspergillus ochraceus, Penicillium verrucosum and Fusarium graminearum (syn. G. zeae; Marasas et al., 2008). Therefore, three of the most important mycotoxins occurring in agricultural products, ochratoxin A (OTA; produced on wheat mainly by A. ochraceus and P. verrucosum) and deoxynivalenol (DON) and zearalanone (ZEA), both produced on wheat mainly by F. graminearum, can be common contaminants of wheat (Miller et al., 1995).

Fusarium head blight of wheat (FHB) is a significant threat to wheat production worldwide. The main causal agent of FHB worldwide is F. graminearum (Logrieco and Moretti, 2008). However, many other Fusarium species have been associated with the disease. FHB of wheat is associated with mainly F. avenaceum, F. culmorum, and F. poae, and at a lesser extent, F. acuminatum, F. cerealis (syn. F. crookwellense), F. equisetii, F. langsethiae, F. sporotrichioides and F. tricinctum. The toxigenic potential of FHB pathogens can greatly vary between species. Indeed, F. culmorum, F. graminearum, F. langsethiae, F. poae and F. sporotrichioides, F. cerealis, F. equisetii, can each produce a specific range of trichothecenes, while F. avenaceum and F. tricinctum are known to produce moniliformin, beauvericin and enniatins (Desjardins, 2006). Predominance of FHB species is determined, to a large extent, by climatic factors, particularly temperature and moisture (Xu et al., 2008). Agricultural practices also play an important role in the prevalence of FHB pathogens since, for example, overwintered plant debris favours infection by F. graminearum (Bai and Shaner, 2004). Moreover, combinations of these pathogens can occur on wheat heads leading to multiple mycotoxins in the harvested grain. Wheat plants are most vulnerable to infection during anthesis, and FHB is most severe when warm and wet conditions prevail during anthesis. In addition, each FHB species may differ in response to different fungicides used to control FHB (Bai and Shaner, 2004). Therefore, it is vital to determine the exact nature of the FHB complex for more reliable disease prediction and management, and for managing the food risks associated with mycotoxins.

Ochratoxin A, that is produced by P. verrucosum and A. ochraceus, is considered mainly a post-harvest issue and not signaled
as a relevant problem in the field. However, due to climate changing and other variations in the wheat cropping system, the attention addressed to this mycotoxin on wheat is always high. Recent surveys of wheat in Europe have shown that P. verrucosum is commonly isolated from wheat, while A. ochraceus is present only occasionally.

Specific actions developed in MYCORED to support the wheat chain management were considered. Above all: hybrids resistant to mycotoxin producing fungi, Fusarium spp in particular, biocontrol agents to reduce fungi inoculum or to compete with toxigenic strains, novel post-harvest and storage handling practices, novel applications of food processing technologies, advanced technologies for diagnostics, so as advanced and rapid analytical tools.

European Commission regulations (2007, 2010) were considered as reference for the contamination threshold to be attended at harvest in raw wheat or in processed products.

Optimization of plant resistance & fungicide use (WP1)
Wheat resistance to FHB. Identification of the effect of QTLs on trichothecene contamination

To identify genetic traits (QTLs) responsible for FHB and DON resistance in wheat is a major issue for reducing the risk of DON accumulation in the wheat plants. Two populations were tested in order to confirm the effect of several QTLs for FHB resistance. Two QTLs from CM 82036/Remus population on chromosomes 3B and 5A conferred high level of FHB resistance. As visual symptoms and grain infection (FDK) gave larger differences, the DON contamination of the plant lines belonging to the two QTLs was nearly the same. The combined effect was very high, only the presence of the two QTLs secured enough resistance. We had similar experience with the Sgv/Nobeoka Bozu/MM/Sumai 3 population, where the most resistant genotypes had the highest number of QTLs. In addition, according to the JIC tests the QTLs on chromosome 4A and 5A gave significant resistance to initial infection (type 1 resistance). The combination 1B and 3B QTLs conferred a significant degree of FHB resistance to fungal spread (type 2 resistance). The DON relations were also better expressed than symptom severity data, an agreement with the Szeged data.

Cultivar registration, Resistance testing of winter wheat cultivars and maize hybrids
In Mycored, cultivar registration and assessment to disease and mycotoxin resistance in wheat varieties and corn hybrids was carried out. Variety registration is an important task in order to identify susceptible genotypes. Wheat cultivars were tested using three inoculation methods and isolates of F. graminearum and F. culmorum. Visual symptoms, kernel infection and toxin contamination were evaluated and significant differences between genotypes were detected for FHB (Fusarium Head Blight), FDK (Fusarium Damaged Kernels) and DON content. The results obtained with the different spraying methods were highly correlated with the disease incidence, severity and DON contamination.

Optimization of fungicide use in wheat and maize
To improve fungicide distribution technology and to evaluate different fungicides to provide better protection for wheat crop has been an important object developed in MycoRed. In wheat, fungicides reduced FHB and FDK (Fusarium Damaged Kernels) severity consistently (from 40% to more than 90%). One important clue is to optimize the spikelet coverage by the fungicides using efficient spraying nozzles. New nozzle combinations proved to be superior in reducing FHB and DON contaminations. DON reduction was highly significant; the best nozzle gave about 50 % less visual symptom severity than the traditional ones, in FDK the difference was smaller as the light infected grains were blown out by combine. The least effective fungicide gave 20 % yield increase, the most effective Prosaro gave 49 % across three cultivars and nozzles. So with improved technology and better fungicides the reduction of DON may reach 70-80 %. However, this reduction at highly sensitive cultivars is not enough to reach the 1.25 mg/kg DON, at more resistant cultivars this was also achieved where DON contamination of the naturally infected controls was 5-11 mg/kg depending on cultivars.

Biocontrol strategies to reduce Fusarium head blight and effect on deoxynivalenol accumulation in wheat (WP2)
Biological control has been demonstrated as an alternative strategy both in greenhouse trials and under field conditions for reducing FHB (Jochum et al., 2006; Schisler et al., 2006; Khan & Doohan, 2009; Palazzini et al., 2009). Since the 1980s, conservative tillage practices, including no tillage, are increasingly followed in Argentina, so that crop stubble and straw residues are left on field soils. F. graminearum and other Fusarium species including F. verticillioides, F. avenaceum and F. poae have a saprophytic stage and can survive in residues of crops such as maize, soybean and wheat (Leslie & Summerell, 2006). Such colonized residues can be the inoculums sources within and outside wheat fields. In many studies, residues of
previously infected crops have been found to be the main sources of spores causing head blight of wheat and maize (Dill-Macky & Jones, 2000; Shaner, 2003; Vogelsang et al., 2011). Antagonistic microorganisms applied to crop stubbles can reduce the survival and multiplication of pathogens present in the residues and thus prevent or delay disease epidemics. Reduction of pathogen populations through biocontrol applied to crop stubble has been evaluated by Luongo et al. (2005) by using strains 016 and 1457 of the fungus Clonostachys rosea (teleomorph Bionectria ochroleuca; Schroers et al., 1999). In this study, the authors demonstrated biocontrol against various Fusarium spp. on wheat and maize under controlled conditions and on stalks and maize ears under field conditions by traditional methodologies. In a further study, the biocontrol effect of C. rosea (strains 016 and 1457) on F. graminearum, F. avenaceum, F. verticillioides, F. langsethiae, F. poae, F. sporotrichioides, F. culmorum and M. nivale was evaluated on naturally infected wheat stalks exposed to field conditions in Argentina. The biocontrol strains showed potential to reduce Fusarium spp. inoculum on wheat stalks and the study demonstrated that wheat stalks were important reservoirs for F. avenaceum and F. verticillioides populations under field conditions in Argentina while stalks were less favorable for F. graminearum survival (Palazzini et al., 2013).

Two potential biocontrol agents selected in in vitro experiments and physiologically improved through osmotic treatments favouring betaine accumulation, were evaluated under field trials with artificial inoculation of F. graminearum for their potential to reduce Fusarium head blight and DON accumulation. Disease severity was diminished by all treatments evaluated during both 2010 and 2011 field trials. After biocontrol treatments no DON accumulation was observed in wheat heads; meanwhile in control plots an average of 1372 µg/kg DON was detected during the two trials. The results showed the effectiveness of the two formulated biological control agents in reducing both FHB disease severity (about 50 %) and DON accumulation by F. graminearum under field conditions.

Predictive modelling (WP3)
As contamination occurs mainly during the cropping period, predicting DON contamination in wheat kernels at harvest can support decision making for tactic and strategic actions (Van der Fels-Klerx and Booij, 2010) and can be useful tools for control authorities and the industry to avoid or limit potential food/feed safety problems. For this reason, mathematical models have been developed to predict FHB and/or DON in small grain cereals. During MycoRed project, WP3 led the validation in different countries (Russia, Mexico and Italy) of the mechanistic model FHB-wheat (Rossi et al, 2003) able to predict the risk of DON contamination from flowering to grain harvest. During the project also the predictive performance of an existing Decision Support Systems (Rossi et al., 2007) was evaluated. DSS are developed using predictive models as core and they are provided with data bases that help in supporting decisions concerning the cropping system choices and the monitoring needs of grain lots for mycotoxins. Moreover, the mechanistic model was cross-validated with an empirical model recently developed in the Netherlands (Van der Fels-Klerx et al., 2010), recalibrated with Italian data (Camardo Leggieri et al., 2013); an Italian data-set and one from The Netherlands were used to perform a cross-validation of the 2 models. The prediction of DON contamination in wheat at harvest using FHB-wheat model gave good results in almost all the geographic areas monitored; correct predictions were around 77%. As expected, the relevance of meteorological data was confirmed, but the application of the DSS including cropping system and geographic data slightly improved the predictions (81%). Wheat contamination was limited in the considered years and it is expected that in high risk years the cropping system should play a more relevant role. From the activity performed for models cross-validation, where the mechanistic model was compared with an empiric one, it was shown that both modelling approaches can give good results if they are accurately developed. The combined use of the two models reduce the uncertainty if models results are in agreement and move to more reliable and confident predictions.

FHB-wheat model cross-validation
Results showed that predictions of both modelling approaches for independent wheat fields (sampled in Italy and The Netherlands) were good. Both models predicted correctly around 90% of the samples, to contain DON in kernels below the legal limit. Using the common data-set (10% of Italian data from 2001 to 2011) for cross validation of the two models, all samples predicted as not contaminated above the EU legal limit by FHB-model were confirmed by the empirical model; 6% of samples were underestimated by the empirical model, whereas with the mechanistic approach no “false negatives” were predicted. However, with the latter model, a remarkable amount of samples (38%) were overestimated (“false positives”). Checking the recorded cropping system data, around 50% of the samples were chemically sprayed against FHB at flowering.
This could explain the overestimates, due to the relevant role of chemical control of Fusaria. This study was recently published (Camardo Leggieri et al., 2013).

Novel post-harvest and storage handling practices to reduce trichothecene and ochratoxin A contamination (WP4)

Three aspects were examined for the reduction of trichothecene and ochratoxin A contamination in post-harvest: (i) relationship between dry matter losses and mycotoxin contamination of wheat under different storage regimes; (ii) efficacy of gaseous Ozone (O3); and (iii) use of natural compounds to replace existing preservatives for feed wheat.

The relationship between storage environmental factors (water activity (aw) (0.70-0.97) and temperature (15-30°C), colonisation of wheat by F. graminearum and the dry matter losses (DMLs), and contamination with DON and zearalenone (ZEA) during storage were determined for the first time. Fungal growth was assessed by the amount of CO2 produced under different interacting conditions of aw and temperature. DMLs were quantified using the cumulative CO2 data and these were shown to increase as temperature and aw increased. The amount of DON, ZEA (wheat for human consumption) produced was significantly affected by the storage conditions. The two toxins however showed different patterns of production. Optimum for DON was at the wettest conditions (0.97 aw) and the higher temperature assessed (30°C), while for ZEA this shifted to 25°C. Polynomial models were developed for the effect of the storage factors on DMLs and toxin production. DMLs under different environmental conditions were significantly correlated with DON. DON contamination was above the EU limits in at least 80% of the wheat samples with DMLs>1%, while at least 70% of the same samples contained ZEA above the respective EU legislative limits. These results show that it may be possible to use temporal CO2 production during storage of grains as an indicator of the level of contamination of the grain with mycotoxins.

The use of O3 as a gaseous treatment showed that for up to 300 ppm O3 (3-6L/hr) for a period of 30-60 mins could completely inhibit spores of F. graminearum and P. verrucosum and also other mycotoxigenic species in vitro. However, mycelial growth was relatively unaffected by exposure to 100-300 ppm O3 over these time periods. Treatment of naturally contaminated wheat showed a significant reduction in total fungal populations with 200-300 ppm O3 (6 L/hr) for 1 hr during storage at different aw levels for up to 30 days. In situ treatment of wheat stored at intermediate moisture contents resulted in about 60% control of DON, ZEA (F. graminearum) and ochratoxin A (OTA) by P. verrucosum. However, under very wet conditions the gaseous treatments were ineffective in controlling mycotoxin contamination. This suggested that overall, at intermediate and drier storage conditions these fungi can be inhibited and mycotoxin contamination minimised.

The use of natural compounds from natural plants, tubers and basidiomycetes were screened for efficacy against F. graminearum and F. langsethiae (type B and type A trichothecenes, respectively) in vitro and in wheat and oats. This showed that the efficacy to control contamination below the EU legislative limits was influenced by storage temperature and water availability. The extracts from basidiomycetes (Trametes versicolor) were found to be most effective for control of both DON and T2+HT-2 toxins in stored wheat and oats respectively. Extracts of Allium species were also very effective, but control to below the EU legislative limits depended on wheat/oats storage moisture content and temperature. Under intermediate aw conditions they were more effective. Based on the relative cost-benefit analyses the extracts from the basidiomycetes were considered to be the most practically and economical to use.

Wireless sensors in grains, a tool for monitoring toxigenic fungi growth during storage (WP4).

All kind of grains are constantly subjected to a risk of fungal contamination ever since when they are still present in the field, both during the harvest that even during transportation or storage. Even when they are harvested and stocked, the grains are not lifeless, because they form a real living ecosystem inside that, under certain conditions, can be attacked by toxigenic fungi and contaminated by mycotoxins. Several solutions already exists on the market for monitoring the conservation of grains, mostly based on cables with measurements of temperature and rarely even humidity: however one cannot analyze such a complex phenomenon without considering the reactions that occur at the base. In the WP4 we have analyzed the breakdown of glucose by microorganisms in order to follow their growth: so we simultaneously monitored the trend of temperature, relative humidity and carbon dioxide with wireless sensors dispersed in the grains, which have measured precisely their variations in time. All these measures are immediately made available to a user, accessing to an online platform in which the user himself is able to follow trends inside silos or container, and be advised if any risk thresholds are exceeded. With a predictive model running in background, the user can also be advised well in advance compared to traditional methods now on the market, if their grains are well stored or have a risk of contamination such as to require user intervention. Thanks to low-power sensors developed during Mycored, the electronic devices are battery powered and can run for several years also
Diversity of Fusarium species colonization of wheat worldwide (WP6)

Differences in environmental conditions could influence the distribution of various toxigenic fungi and related mycotoxins worldwide. Therefore, they can have consequence on the diversity of toxigenic fungi colonizing food commodities, among which wheat can be heavily contaminated, mostly by species of Fusarium genus. Moreover, such variability of environmental conditions can deeply influence also the distribution of toxigenic fungi on the wheat cultivated in the different geographical areas. The importance of a correct knowledge of the distribution of toxigenic fungi is mainly due to capability of each species belonging to Fusarium, to produce specific profiles of mycotoxins and the predominance of a given species can be related to the a specific risk for the food commodity colonized. Emerging problems due to climate change and new mycotoxin/commodity combinations add further concern and seem confirming the importance of keeping monitoring the diversity of toxigenic fungi. In addition, trans-global transposition and trade exchanges of plant products significantly contribute to the spreading of toxigenic fungi worldwide. Therefore, the evaluation of the distribution of the main toxigenic species belonging to Fusarium genera on wheat has been carried out in WP6 in order to add detailed information on the current knowledge on the predominance of specific species at global level.

Geographical distribution of toxigenic fungi on wheat (WP6)

The worldwide collection of wheat and, as comparison, from oat, has led to the morphological identification of 342 strains belonging to Fusarium species. The isolation of strains was obtained from 139 samples of wheat (265 strains) and 70 of oat (77 strains) collected in Argentina, Canada, Croatia, Greece, Italy, Mexico, and Russia. All strains were firstly identified morphologically and then were studied by a multilocus sequence analysis of four independent gene regions (β-tubulin, calmodulin, RNA polymerase II and elongation factor 1α). By the identification analyses, the strains isolated from oat and wheat belonged mostly to species that produce type A and type B trichothecenes, which are potent inhibitors of proteinous synthesis. Interestingly, a wide population of the F. incarnatum/semitectum complex (former Gibbosum and Arthrosporiella sections), where a wide genetic and mycotoxin profile variability does exist, was identified occurring in several countries on both oat and wheat. Moreover, strains belonging to the forme Sporotrichiella section and to F. graminearum species complex were identified. It is important to notice that the results from the strain collection evidenced a higher and wide presence of the species belonging to Sporotrichiella section (F. langsethiae, F. poae and F. sporotrichioides) than can be producers of the type A trichothecenes T-2 and HT-2 toxins, which are the most harmful known Fusarium toxins. The knowledge of the distribution of these toxigenic species according with the different environmental conditions provides a useful source of information on the toxigenic risk related to the consumption of maize products related to specific geographical areas. Finally the high number of new fungal strains deposited in ITEM collection is an important source of biodiversity of toxigenic fungi useful for further investigations aimed to elucidate genetic, biochemical and pathogenic traits of each single species identified.

Early detection of toxigenic fungi in the wheat chain: development and use of primers and quantitative PCR assays for FHB species (WP6)

Primers designed for F. langsethiae and F. sporotrichioides proved to be robust on a set of 30 strains from different European countries. These sequences were used to develop quantitative PCR assays, qPCRs, that allowed quantification between 1 pg and 10 ng of fungal DNA. Because these qPCRs did not react with DNA from other Fusarium species causing FHB in wheat (e.g. F. avenaceum, F. culmorum, F. equiseti, F. graminearum, F. poae or F. tricinctum) they can be applied in the quantification of T2 and/or HT2 producing fungi and risk assessment of these mycotoxins in cereals.

Variable Number of Tandem Repeats, or VNTRs, are suitable for population studies and their usefulness was shown in the geographic substructuring of the F. asiaticum in China (Zhang et al., 2010b) and migration of certain genotypes (Zhang et al., 2012).

Previously, PCRs were mostly based on unidentified regions, also known as SCARs (e.g. Waalwijk et al., 2003; 2004). These targets are prone to sequence variations, making them less robust. During the MycoRed project, novel PCRs were developed based on functional sequences, like the tri11 gene from the gene cluster involved in the production of trichothecene type B mycotoxins 3ADON, 15ADON and NIV. Based on sequence variation within that gene, discrimination between producers of either NIV, 3AcDON or 15AcDON was accomplished (Zhang et al., 2010a).
Sequences from the tri3 and tri12 gene as well as from the cyp51 gene, that is involved in sterol biosynthesis and target for several fungicides (Fernandez-Ortuno et al., 2013) were used to design TaqMan based qPCRs. These assays were applied on field samples collected in The Netherlands, UK and China, respectively. It could be concluded that there is a positive correlation between total amounts of deoxynivalenol and F. graminearum in The Netherlands. In addition, F. graminearum is the predominant FHB representative in Europe, while in China the disease is mainly caused by F. asiaticum (van der Fels-Klerx et al., 2012; Fernandez-Ortuno et al., 2013). To improve the robustness of the assays, qPCRs based on different loci were compared. The reproducibility of the amplification depended on the fineness of the grinded sample.

Seedlots were artificially inoculated with either a 15ADON producing F. graminearum isolate, a 3ADON producing F. culmorum isolate or a NIV producing isolate of F. asiaticum and blended in various ratios. The correlation between quantification of different targets in a mixture composed of equal amounts of the three different Fusarium spp. (e.g.1:1:1) was tested. Side-by-side comparisons were performed on all combinations of qPCRs so for each target (tri3, tri12, and species specific PCR) as well as for each chemotype (3ADON, 15ADON or NIV) and all correlations were highly significant (R2 > 0.9).

Advanced technologies for diagnostics, quantitative detection and novel approaches to control toxigenic fungi (WP6)

In recent years enniatins (ENs) are frequently reported as small grains contaminants in Europe, especially wheat, rye, oats, barley and sorghum, (Logrieco et al. 2002; Jestoi et al. 2004; Uhlig et al. 2007), and F. avenaceum is the dominant species recorded in ENs-contaminated cereal grains (Kosiak et al. 2003). Within the Mycored project we analyze the biosynthetic pathway of enniatin production in F. avenaceum, demonstrating for the first time a transcriptional regulation of esyn1 gene, responsible for the modulation of ENs biosynthesis (Fanelli et al., 2013). These data improved the knowledge on the molecular mechanism underlying their production in toxigenic fungal species, and provided information needed to face the emerging concern of ENs in cereals contamination, their toxic effect on human and animal health, together with the possibility to use these compounds as pharmaceutical products.

Mutual regulation of ochratoxin and citrinin biosynthesis by Penicillium verrucosum and its relation to the wheat environment (WP6)

Penicillium verrucosum is the main responsible for the occurrence of ochratoxin A in wheat and wheat products (Lund and Frisvad, 2003). However because it has no known plant pathogenic activity it is regarded as a storage fungus, which means that it mainly produces ochratoxin A in a non-natural, controllable environment. Citrinin is a mycotoxin with a very similar polyketide structure like ochratoxin A. It is also a nephrotoxin and its toxicity acts synergistically to the toxicity of ochratoxin (Braunberg et al., 1994). Nevertheless not so much attention is laid on the presence of citrinin, albeit it may be present in high amounts and may be found frequently in wheat (Vrabcheva et al., 2000). Until now no regulatory limits have been set although recently the EFSA stated the importance about further research on citrinin (2012).

During the work done at MycoRed a direct correlation of the amounts of citrinin and ochratoxin could be detected and the regulatory mechanism behind this regulation could be completely unraveled. It was shown that the two mycotoxins citrinin and ochratoxin are mutually regulated, meaning that, dependent on the conditions, generally either high amounts of citrinin or high amounts of ochratoxin were being produced. It could be demonstrated that this capability to shift between both secondary metabolites has ecological reasons and plays a role in the adaptation towards different stressful environments. P. verrucosum is generally adapted to the cereal environment and is regarded as a storage fungus producing ochratoxin A under favourable conditions during the storage of cereals, e. g. in the dark. On the other hand P. verrucosum is a ubiquitously occurring saprophyte and it can be expected, that spores of this fungus contaminate cereals already on the field, which means that these spores are exposed to natural conditions like sunlight.

Based on these results obtained under Mycored project, a model can be established which describes the production of citrinin and ochratoxin under certain conditions. According to the results about the mutual regulation of citrinin and ochratoxin citrinin is more produced under oxidative/light conditions, e. g. when the fungus is exposed to sunlight. As already mentioned above P. verrucosum is a ubiquitous saprophytic fungi, so spores of this fungus may already contaminate cereals on the field before harvest. This should result in a higher citrinin production under these conditions. On the other hand during storage of wheat, e. g. mostly under dark conditions, the biosynthesis of ochratoxin A is apparently be preferred and lead to the contamination of wheat with ochratoxin A. The ecological reason for the production of ochratoxin A under these conditions is not clear.

However induction of ochratoxin A biosynthesis under high NaCl conditions with the aid of a MAP kinase signal cascade
establishes a new habitat for P. verrucosum. Under these conditions the fungus adapts to this environment by this shift in secondary metabolite biosynthesis and can indeed occasionally be found on NaCl rich dry cured meat products (Comi et al., 2004) or on olives (Heperkan et al., 2009). The work is presented has been published Schmidt-Heydt et al (2010; 2011; 2012).

MAIZE GRAIN CHAIN

Introduction

Sustainable agriculture and safe food and feed can be considered the key words for agriculture in the XXI century. Mycotoxins, rated as the highest chronic health risk from food (Kuiper-Goodman, 2004), have been put on the top of concern list and the efforts devoted to mitigate their presence are totally justified. 

Maize is a crucial crop in this context, being the second most cultivated crop worldwide with around 161 million ha. Maize is susceptible to mycotoxin producing fungi belonging to the main genera, Fusarium, Aspergillus and Penicillium spp., therefore potentially contaminated by fumonisins, trichothecenes, aflatoxins and ochratoxins, limiting the list to the regulated compounds (Logrieco and Moretti, 2008; Battilani and Logrieco, in press; EC, 2006, 2007, 2010).

Fusarium verticillioides prevails as the causal agent of ear rot in most maize-growing areas of southern Europe when hot and dry weather occurs (Logrieco et al., 2003, Battilani et al., 2008b), while F. graminearum causes more worries in northern areas (Logrieco et al., 2003). Aspergillus flavus was reported mainly in tropical and subtropical areas till to recent years, but the outbreak of 2003 in Italy (Battilani et al., 2008a; Piva et al., 2006) and the severe aflatoxin contamination spread in many European maize growing areas in 2012 suggested to increase the attention also for this toxin. Ochratoxin A, produced by P. verrucosum and A. ochraceus, is considered mainly a post-harvest issue; it is not signaled as a relevant problem, but in this changing period, due to climate and other variations in the maize cropping system, it is good to keep the toxin monitored.

High mycotoxin contamination is commonly associated to high stress for plant and fungi; therefore, guidelines to optimize maize management have been prepared (see www.ermesagricotura.it as an example) where all the steps of cropping system are discussed in relation to plant wellness and mycotoxin risk. Nevertheless, the support of predictive models is acquiring importance due to the difficulty to take into account the effect of meteorological conditions without a mathematic/statistic computerized support. Besides, the high number of factors to be considered in strategic and tactic decisions for maize management could take advantage from Decision Support Systems (DSS; Rossi et al., 2012), perfectly fitting the sustainable agriculture approach. Strong efforts were finalized in modelling and DSS development to obtain reliable tools.

Good crop management is frequently not sufficient to guarantee maize production with mycotoxin contamination below the legal limits. Therefore, in a “chain vision”, MYCORED research tried to contribute with additional tools to prevent mycotoxin contamination, improve food processing and make easier the diagnosis and mycotoxin analysis.

Specific actions developed in MYCORED to support the maize chain management were considered. Above all: hybrids resistant to mycotoxin producing fungi, Fusarium spp in particular, biocontrol agents to reduce fungi inoculum or to compete with toxigenic strains, novel post-harvest and storage handling practices, novel applications of food processing technologies, advanced technologies for diagnostics, so as advanced and rapid analytical tools.

European Commission regulations (2007, 2010) were considered as reference for the contamination threshold to be attended at harvest in raw maize or in processed products.

Pre-harvest

Maize resistance: reduction of fumonisins in maize (WP1)

Fusarium verticillioides is the causal agent of Fusarium ear rot (FER) in maize and contaminates the grain with fumonisins, a family of mycotoxins that affects feed and food. Genomic regions and candidate genes for kernel resistance to F. verticillioides infection were detected through the comparison of resistant and susceptible maize inbred lines (Lanubile et al., 2011), by adopting three different approaches: Quantitative Trait Locus (QTL) mapping, transcriptomic (microarrays) and metabolomic analyses. We observed more than 2,000 differentially expressed genes 72 hours after infection for the resistant and susceptible maize genotypes, of which about 900 were in common and showed more than 5,000 single nucleotide polymorphism (SNP) variants (Lanubile et al., 2010).

The same kernel samples were subjected to a metabolomic analyses. 433 putative maize metabolites were detected through high resolution liquid chromatography-mass spectrometry (LC-MS). A large part of these metabolites were involved in defense
mechanism, fitting with transcriptomic results. Some pathways resulted particularly interesting for the number of involved metabolites and differentially expressed genes: shikimate pathway, biosynthesis of aromatic amino acids, phenylpropanoid and flavonoid biosynthesis, linoleic and α-linolenic acid metabolism. Abundant transcripts derived from terpenoid and diterpenoid biosynthesis. The integration of transcriptomic and metabolomic data allows the detection of new differentially expressed genes involved in defense pathways that can be used as candidate genes for FER resistance (Lanubile et al., 2012a; Lanubile et al., 2012b; Lanubile et al., 2013a).

A segregating population of 192 F2:3 families was phenotypically evaluated in two different sowing periods in 2011 and 2012 after artificial infection by two side-needle inoculation methods. Significant genotypic variations in response to Fusarium infection and fumonisins accumulation were detected. A genetic linkage map was constructed based on polymorphic simple sequence repeats (SSRs) and SNPs detected in differentially expressed genes between resistant and susceptible inbreds. Sixteen QTLs for fumonisin B1 reduction, partially overlapping with nine QTLs for Fusarium ear rot resistance, were revealed. All identified QTLs for both traits have small effects, with the majority of them explaining about 10% of phenotypic variation (Maschietto et al., 2011; Lanubile et al., 2013b).

Biological control of toxigenic Fusarium spp. in maize stalks (WP2)
Crop residues are an important niche for saprotrophic plant pathogens. Maize stalks from preceding crops colonized by toxigenic Fusarium spp. are the main source of inoculum for ear rot epidemics in maize. Antagonistic isolates belonging to Clonostachys rosea had been selected earlier for their potential to reduce Fusarium spp. in overwintering stalks of wheat and maize (Luongo et al., 2005). In the Mycored project, maize stalks originating from various fields in The Netherlands and Italy were treated with conidial suspensions of the C. rosea isolates 016 and 1457. Treated stalks were placed on field soil under Dutch and Italian conditions. The colonization of stalks by toxigenic Fusarium spp. was quantified after field exposure of 3 and 6 months using species-specific qPCR. The overall results showed that the antagonists reduced the colonization by Fusarium spp. only in a few cases. Additional measurements of individual maize stalks showed that there was a significant variation in colonization by Fusarium spp. from all sampled fields. Possibly, the antagonistic fungi were not successful in competitive exclusion of Fusarium spp. from stalks which were already heavily colonized by Fusarium spp. highly colonized by the pathogens at harvest.

In a second research line, the colonization of individual maize stalks collected from various fields in The Netherlands, Italy and Nigeria by toxigenic Fusarium spp. was quantified by species-specific qPCR directly after harvest and after 6-month field exposure. It was demonstrated that the increase in colonization by Fusarium spp. during field exposure varied considerably between individual maize stalks. Bacterial and fungal communities in stalks with high versus low increase in pathogen colonization were analyzed using Denaturing Gradient Gel Electrophoresis DGGE (Köhl et al., in preparation). In line with research on suppressive soils, such ‘suppressive stalks’ can be a valuable source of new antagonists. Several bacterial groups and fungal species were identified which were associated with a slower increase in pathogen colonization. The MycoRed study focused on the primary colonizers of stalks present already at harvest because pathogenic Fusarium spp. invades stalks early during or after senescence. In this situation, competitive exclusion by the pathogens by applied antagonists will only be successful if antagonists invade the stalk tissue early and vastly during senescence before niches have been occupied by Fusarium spp. The hypothesized antagonistic potential of microbial groups identified in this study has to be tested in a biocontrol screening program.

Biological control of aflatoxins in Africa (WP2)
Aflatoxin is a genotoxic, immunosuppressive carcinogen that frequently contaminates maize and groundnuts. The toxicity of aflatoxins to humans and animals has led to regulatory controls. Aspergillus species survives in the soil and plant debris and infect the grains in the field. Before harvest, the crop is often contaminated with aflatoxins. These fungi are carried from the field to the stores where the aflatoxin contamination further increases. An innovative scientific solution to the aflatoxin problem has been developed by the United States Department of Agriculture’s Agricultural Research Service (USDA-ARS). The International Institute of Tropical Agriculture (IITA) in collaboration with USDA-ARS, the University of Ibadan, the University of Bonn and other partners have developed aflasafe™, an indigenous biological control product to mitigate aflatoxin contamination in Nigeria. Aflasafe™ contains a mixture of four atoxigenic (lack of aflatoxin producing capacity) A. flavus L-
strain isolates that occur naturally in maize in Lafia, Kaduna and Ogbomosho states in Nigeria. These strains were carefully selected to out-compete resident toxigenic Aspergillus strains after Aflasafe™ is applied on the soil. The National Agency for Food and Drugs Administration and Control (NAFDAC) granted approval of provisional registration (i.e. listing status) of aflasafe™ for five years in 2009.

Product efficacy trials were carried out in 51 farmers’ fields in 2009, and 14 farmers’ fields in 2010 in collaboration with the Commercial Agriculture Development Project (CADP) of Kaduna State and Pampaida Millennium Villages Project of the United Nations Development Program (UNDP). In addition, carry-over effect of aflasafe™ was evaluated in farmers’ fields. Aflasafe™ was applied by farmers in their maize fields at a rate of 10 kg/ha, 2 to 3 weeks before crop flowering. A neighboring untreated field near each treated field served as control. Aflatoxin concentration in maize and presence of various species and strains of Aspergillus were measured. A carry-over trial of aflasafe™ strains in maize fields was also conducted in 2009 and 2010. Our results showed that aflasafe™ strains are native in the Nigerian environment, and occur in crops and soil at low frequencies.

Farmers who applied aflasafe™ reduced aflatoxin contamination by 82% to 94% at harvest and 93% reduction after poor storage compared to fields where aflasafe™ was not applied. Reduction of aflatoxin in treated fields resulted from displacement of toxin-producing strains and species of Aspergillus by the atoxigenic aflasafe™ strains. Aflatoxin contamination in maize grains was significantly less in maize planted in fields treated with aflasafeTM during the previous year compared to control fields not receiving any treatment during either year. However, the effectiveness of aflasafe™ increased with its repeated application every year. AflasafeTM strains persisted in high frequency in soil for at least two years and thus had significant carry-over effect from one year to the next. Repeated applications of aflasafeTM continually increased the population of aflasafe™ strains in soil and grain. CADP is now promoting use of aflasafe™ by farmers in Kaduna state by advising farmers to use the product as one of the essential input in maize production.

Predictive modelling (WP3)

Two predictive models were developed, AFLA-maize (Battilani et al., 2013) and FER-maize (Battilani et al., 2003; Battilani et al., in preparation), served by hourly data on air temperature and humidity and rain as input. Cropping system information were also integrated to improve models performance following the Decision Support System (DSS) approach. The models were developed following the principles of “system analysis” (Leffelaar & Ferrari, 1989). The models define, by mathematical equations, all variables influencing the infection cycle, their sequence from one state to another and their interaction with meteorological conditions and host growth stages. The model output is a cumulative index, resulting from the summation of the daily output. The logistic regression was used to relate model index and field data on mycotoxin contamination in maize and compute, on a daily base from silk emergence to crop harvest, the probability to overcome the legal limit for the specific toxin, aflatoxin B1 or fumonisin B1+B2, in this case (respectively AFB1 ≥ 5 μg/kg and FB1+FB2 ≥ 4000 μg/kg).

More than 500 maize samples were collected worldwide and used to develop and validate the model. Correct prediction obtained by using only meteorological data were 72% and 69% respectively for AFLA-maize and FER-maize; they improved to 77% and 75% when the DSS approach was followed. The results obtained with the DSS suggest that further research is needed to acquire quantitative data on the role of cropping system in maize-toxin fungi interaction in order to improve the model. The production of these data is very difficult, because the experiments must be managed in field where different parameters interact and it is not easy to quantify separately their effects. Nevertheless, a maize-DSS is now available able to support farmers in decision making regarding aflatoxin and fumonisin management in the maize chain.

Historic, actual (day by day along the growing season) and predicted meteorological data can be used as input in predictive models. Information on past scenarios is the output generated by historic data. Therefore, if the meteorological data net is sufficient, yearly and mean risk maps can be drawn to have a picture of risk of aflatoxin and fumonisin contamination in the area and its variability in time, intended as risk maps referred to different years in the past. Predictions related to actual data describe the situation of the current growing season (day by day, from silk emergence till to harvest). This is the most common application of predictive models, intended for farmers and extension services to support the cropping system choices. Really, very few choices are possible during the growing season in order to prevent mycotoxin
Regarding irrigation, it is commonly planned based on criteria different from mycotoxin risk reduction and regarding pest control it must be applied with short delay from silk emergence, when risk prediction is just started. Trials carried out to study the application of fungicides to reduce Fusarium infection and fumonisin contamination were not encouraging (Mazzoni et al., 2011) and this practice is not applicable because registered active ingredients are not available in Europe. Nevertheless, even if predictions cannot support operational decisions, it is always positive to have this information available approaching crop harvest. In fact, it is well known that mycotoxins cumulate in time and late harvest is related to increased contamination. When the contamination risk is high, it is strongly suggested an early harvest, with high humidity in kernels, especially for Aspergillus flavus, because this fungus is very efficient in aflatoxin production, mainly with kernels humidity below 28%. Predicted meteorological data (WP3) in the future are the last possible input for predictive models. They support the prediction of future scenarios, very interesting in relation to climate change. All possible applications described using historic data are also applicable in this case. Therefore, the most interesting application is the draw of risk maps in different climate change scenarios.

Actual data are the proper input to optimise crop management, harvest and post-harvest/processing, as previously described, but also to correctly address maize to food, feed or energy in relation to the legal limit defined by European Commission. This is very important because, during the growing season, sometimes in large advance respect to crop harvest, it is possible to predict if the production will be contaminated with aflatoxin or fumonisin above the threshold for human or animal consumption. Apart the possibility to anticipate harvest, several other action can contribute to prevent or reduce contamination. In particular, drying, always applied to maize grain post-harvest, should be managed always very quickly, in particular when the crop is harvested with high humidity and when the predicted risk of contamination is very high. Because early harvest imply higher drying cost and quick management requested post-harvest, this is possible with a better organised logistic organisation; these efforts are requested in high risk conditions and it largely justify the additional effort.

Post-harvest

Predictive modeling (WP3)

The predictive models developed and previously described, AFLA-maize and FER-maize were adapted to be used post-harvest, during maize storage.

Around 70 maize samples were collected during true scale storage, both in traditional silos and in silo bags. Storage conditions were those considered suitable for a safety preservation. Fumonisin and aflatoxin content never increased in sampled kernels, some decrease in fumonisins was sometimes observed. Therefore, it was confirmed that the settled conditions guarantee a safe storage, in agreement with models predictions, but it was not possible to validate the model in conditions enhancing mycotoxin synthesis.

Novel post-harvest and storage handling practices (WP4)

Three aspects were considered. Firstly the relationship between mycotoxigenic fungal respiration on maize and its relationship with dry matter and mycotoxin porduction, secondly the effect of Ozone (O3) as a gaseous fumigant and thridly the use of novel natural compounds as a feed treatment to control mycotoxin contamination in situ to substitute existing chemical preservatives.

The relationship between storage environmental factors (WP4) (water activity (aw) (0.70-0.97) and temperature (15-30°C), colonisation of maize by F.verticillioides the dry matter losses (DMLs) caused and the contamination with fumonisins (FUMs) during storage were examined. Fungal growth was assessed by the amount of CO2 produced under different interacting conditions of aw and temperature. DMLs were quantified using the cumulative CO2 data and these were shown to increase as temperature and aw increased. The amount of FUMs (feed maize) produced was significantly affected by the storage conditions. FUMs were produced in higher amounts in maize at 30°C and 0.97 aw, however, at intermediate aw levels (0.955...
The highest production occurred at 25°C followed by 20°C. Polynomial models were developed for the effect of the storage factors on DMLs and toxin production. DMLs under different environmental conditions were significantly correlated with FUMs. At least 75% of the maize samples with DMLs≥0.9% exceeded the EU limits for the sum FB1+FB2 in feed. These results show that it may be possible to use temporal CO2 production during storage of grains as an indicator of the level of contamination of the grain with mycotoxins.

The use of O3 (WP4) as a gaseous treatment showed that for up to 300 ppm O3 (3-6L/hr) for a period of 30-60 mins could completely inhibit spores of A.flavus F.verticillioides and other mycotoxinogenic species. However, mycelial growth was relatively unaffected by exposure to 100-300 ppm O3 over these time periods. Treatment of naturally contaminated maize showed that with 200-300 ppm O3 (6 L/hr) for 1 hr resulted in a significant reduction in the total fungal populations on maize at different aw storage levels. The reduction in total FUMS and FB1 was very effective at intermediate aw levels. Under these conditions (0.92-0.95 aw), very good reduction in FUMs was achieved. However, in wet maize this gaseous treatment was less effective.

The use of natural compounds (WP4) from natural plants, tubers and basidiomycetes were screened for efficacy against A.flavus and F.verticillioides for control of aflatoxins and FUMs in vitro and in maize. This showed that the efficacy to control contamination below the EU legislative limits was influenced by storage temperature and aw. The extracts from Basidiomycetes (Trametes versicolor) was found to be most effective for control of both AFs and FUMs in stored maize. Extracts of Allium species were also very effective, but control to below the EU legislative limits depended on maize storage moisture content and temperature. Under intermediate aw conditions they were more effective. Based on the relative cost-benefit analyses the extracts from the Basidiomycetes were considered to be the most practically and economical to use.

Novel application of food/feed processing technologies (WP5)

The effect of expansion in a true scale study (WP5) The effect of expansion, which is a high temperature-short time process used for the production of foods and feeds, was considered to study the possible effect on Mycotoxins. The expander consists of a heavy-duty screw residing in a barrel section equipped with an adjustable annular discharge gap. In an industrial plant, one ton of maize flour, naturally contaminated with aflatoxins (AFs) and fumonisins (FBs), was accurately mixed with an equal amount of bran, naturally contaminated with trichothecenes (TCTs) and ochratoxin A (OTA); the cereal mixture was sampled before and after the expansion process, with a conditioning step at 90 °C for 4-5 min and a treatment in the barrel, where temperature of 150-160 °C and pressure of 8-10 bar for 30-40 seconds were reached.

The results showed a significant decrease only for DON contamination. For the other mycotoxins, intended as AFB1, FB1 and FB2 HT-2 and OTA, no significant differences were found. Some authors reported that a relevant reduction of the contamination level for some mycotoxins can be obtained at temperatures of about 150-170 °C (Bullerman and Bianchini, 2007); probably, the permanence time of the product in the barrel during the tested expansion process was too short.

The effect of nixtamalization (WP5) (alkaline-cooking used for masa production), thermal processing (heated steam) and maize pasta production on the levels of fumonisins in intermediate and final products was evaluated. Significant reduction of fumonisins was observed during nixtamalization process and the degree of reduction was proportional to the cooking time. However, the process made available fumonisins conjugated with matrix components that where then converted to the relative hydrolysed and partially hydrolysed forms. Thermal processing and pasta production did not have any effect on fumonisins that were completely recovered in the final pre-cooked maize flour and dried spaghetti, respectively.

In vitro selection and efficacy assessment of multi-mycotoxins detoxifying agents (WP5). Fifty two commercial products from 26 industrial partners, including minerals, yeast-based products and blend of components, and 51 agricultural by-products were tested to prepare a nutritional composition intended to reduce bioavailability of a large range of mycotoxins. Preliminary adsorption tests allowed to select 4 commercial products and 4 agricultural by-products as effective in sequestering simultaneously AFB1, ZEA, OTA and FB1. All products failed in binding DON, but activated carbon. Batch adsorption experiments were performed at different pHs with selected binders to determine adsorption parameters (capacity, affinity,
chemisorption index), and kinetic and thermodynamic parameters. Mineralogical analysis (XRD) showed that 3 out of the 4 commercial products selected as best multi-toxin adsorbents (designated by the supplying companies as minerals) were organoclays. All these products showed lower maximum adsorption capacity and chemisorption index than a mineral product containing carbon. Most commercial additives, but two organoclays and one yeast cell wall product, were found to be non-toxic in 2 bioassays. Interestingly, some micronized vegetable dried materials were successful in adsorbing mycotoxins. Contact time curves showed that the simultaneous adsorption of AFB1, ZEA, OTA and FB1 by these materials is rapid, reaching the adsorption equilibrium within 10 min. Toxin adsorption was improved when the sizes of agricultural materials in the form of micro-particles were less than 500 µm. Medium pH affect toxin adsorption depending on the toxin. Single and multi-component adsorption isotherms showed that AFB1, ZEA and FB1 adsorptions is not influenced by the simultaneous presence of different toxins. Thermodynamic parameters suggested that hydrophobic interaction could be associated to AFB1 and ZEA adsorption, while electrostatic interaction could be responsible for OTA and FB1 binding.

Based on data collected, most commercial products, regardless of their composition, are ineffective towards the large range of mycotoxins that can be found in feed. Some commercial products can be even highly toxic in toxicity bioassays. Selected agricultural by-products or mixtures of them can be good candidates for being used as multi-mycotoxin binders.

In vivo evaluation of mycotoxin binders for reducing/preventing mycotoxin absorption (WP5). The urinary biomarkers are good indicators of mycotoxin bioavailability therefore they can be used to assess the in vivo efficacy of binders in reducing gastrointestinal absorption of mycotoxins (Gambacorta et al., 2013). The multi-biomarker approach was used in the Mycored project to evaluate in vivo the efficacy of four promising agricultural by-products (ABP) and two commercial binders (CB) previously selected with in vitro experiments. Groups of piglets were fed with boluses contaminated with mixtures of AFB1, ZEA, OTA, FB1 and DON, with and without the inclusion of binder. Binders that did not show absorption attitude in vitro were also tested as negative controls (1 ABP and 1 CB). The mycotoxin dietary levels were below the maximum permitted levels reported in the directive 2003/100/EC for AFB1 and the guidance values reported in the recommendation 2006/576/EC for DON, ZEA, OTA and FB1. Among the tested ABP and CB binders, one ABP (grape pomace) resulted effective in significantly (P<0.05) reducing the gastrointestinal absorption of ZEA and AFB1. A quantitative reduction was also observed for FB1, DON and OTA although these reductions were not statistically significant (P>0.05). None of the tested CB reduced the gastrointestinal absorption of tested mycotoxins. The two negative controls confirmed their inefficacy in vivo. These results demonstrate that in vitro binding studies seem to have a limited utility in predicting the in vivo efficacy of binders. Therefore in vivo trials are needed to demonstrate the real efficacy of binders. Grape pomace selected as effective both in in vitro and in vivo studies is a safe and low-cost material that can be used as feed additive to mitigate the toxicity of mycotoxins in farm animals (Gambacorta et al., in preparation).

Advanced technologies for diagnostics, quantitative detection and novel approaches to control toxigenic fungi (WP6)

Diversity of Fusarium and Aspergillus species colonization of maize worldwide (WP6). The evaluation of the distribution of the main toxigenic species belonging to Aspergillus and Fusarium genera on maize has been carried out in WP6 in order to add detailed information on the current knowledge on the predominance of specific species at global level. The evaluation of the distribution of toxigenic fungi on maize was focused mainly on Fusarium species and on Aspergillus section Nigri. Species belonging to Fusarium genus are responsible of the accumulation on maize of fumonisins B1 (FB1) and B2 (FB2) and deoxynivalenol, while some of the Aspergillus section Nigri species are responsible of the accumulation of FB2. In four years (2009-2012), 500 kernel of maize samples were received from different geographical areas of Europe (Italy and Netherland), Central and South America, Asia (Turkey) and Africa. From these samples we isolated around 1,000 strains identified as Fusarium and we confirmed around 650 representative strains by using molecular tools. Moreover, strains isolated from maize worldwide, deposited in the ITEM Fungal strain collection (http://www.ispa.cnr.it/Collection) were also analyzed. A further study was carried out on 300 strains of Aspergillus section Nigri strains isolated from maize in USA and Italy, in order to evaluate the distribution of the different species of this section in both countries. All strains isolated from maize were firstly identified morphologically and then were studied by a multilocus sequence analysis of four independent gene regions (β-tubulin, calmodulin, RNA polymerase II and elongation factor 1α). In conclusion, we obtained interesting data of toxigenic fungi
distribution in maize. In particular, different mycotoxin producing species were isolated from maize cultivated in geographical areas characterized by significant differences in the environmental conditions. In fact, while the two fumonisin producing species F. verticillioides and F. proliferatum were the predominant species worldwide, the main species isolated in the coldest regions such as Netherlands were the trichotheceone producing species F. crockwellense, F. culmorum and F. graminearum sensu stricto. On the other hand we also showed that F. temperatum and F. subglutinans are both common species in maize worldwide and, although further data are needed to confirm it, we clearly showed the absence of F. temperatum in American Continent. Finally, the study carried out on the Aspergillus section Nigri showed that A. niger, was the predominant species in the total sample, followed by A. tubingensis and A. awamori and they may contribute to FBs accumulation in kernels (Logrieco et al., 2013). Looking at the percentage for each country, it is interesting to notice that A. niger is the predominant species in USA, while the predominant species in Italy were both at the same level A. niger and A. tubingensis. The knowledge of the distribution of these toxigenic species according with the different environmental conditions provides a useful source of information on the toxigenic risk related to the consumption of maize products related to specific geographical areas. Finally the high number of new fungal strains deposited in ITEM collection is an important source of biodiversity of toxigenic fungi useful for further investigations aimed to elucidate genetic, biochemical and pathogenic traits of each single species identified.

Aflatoxin gene detection in Aspergillus flavus from maize in Italy (WP6). A study testing the occurrence in the genome of a population of A. flavus including both aflatoxigenic and non-aflatoxigenic strains of seven well characterized AFL biosynthetic genes in relation to AFL production was carried out: the genes were the two regulatory genes aflR and aflS and the structural genes aflD, aflM, aflO, aflP, and aflQ. The result was the grouping of strains into four different amplification patterns, characterized by seven, four, three, or no DNA bands. All the aflatoxin-producing isolates exhibited the complete set of genes, whereas the non-aflatoxigenic isolates lacked the PCR products corresponding to three, four or even all seven genes. Thus, data confirmed that lack of amplification of AFL biosynthetic genes is correlated with non-aflatoxigenicity.

The relationship between FUM gene expression and fumonisin production in F. verticillioides (WP 6) under different environmental conditions (pH, T, water activity and salinity) was studied. The good adaptability of this species to diverse environmental conditions was demonstrated, with the production of moderate amounts of fumonisins under a wide range of conditions. A post transcriptional regulation mechanism that could account for the difference between optimal temperature for FUM gene expression and fumonisin production was also hypothesized (Lazzaro et al., 2012; Fanelli et al., 2013). These data improved the knowledge on the molecular mechanism and the conditions that promote fungal growth and fumonisin production, providing crucial information to reduce fumonisin contamination.

The role of light (WP6). The analysis of the expression of genes involved in mycotoxin production demonstrated that several stressing condition, caused by biotic and abiotic factors, are responsible for the activation of mycotoxin biosynthetic pathway. Among these, light has proven to be a very important signal for fungi, since it influences many different physiological responses such as pigmentation, sexual development asexual conidiation, the circadian clock and secondary metabolism. A light system was developed to evaluate the effect of different light wavelength and intensity on fumonisin production by three important fungal species contaminating maize: F. verticillioides, F. proliferatum and A. niger. The analysis demonstrated that red and blue lights are the most effective in modulating gene expression and fumonisin biosynthesis, leading to an increase in fumonisin production (Fanelli et al., 2012a-c). Other light sources instead, e.g. pulsed light or short wavelength lights, exerted an inhibiting effect on fungal growth and mycotoxin biosynthesis. This new technology, based on the matrix exposure to different type of light, could represent a new tool to control the production of certain mycotoxins, at least for surface growing fungi. Pulsed light and specific wave length exposure could be easily applied in different steps of food processing and management as contribute to minimize mycotoxin contamination.

GRAPE-WINE CHAIN

Introduction

In most countries, the grapes grown today are varieties of just one vine species, Vitis vinifera L.. The production of wine grapes, in terms of quantity and quality, is highly influenced by three key factors: 1) Climate, 2) Soils, Grapes can be produced on a number of soils – fertility is not as important as soil structure, 3) Viticulture practices.

Aspergillus section Nigri are described as the main source of ochratoxin A (OTA) contamination in grapes and wine worldwide with A. carbonarius as main producer (Somma et al., 2012). Fumonisin B2 represents an additional potential mycotoxin risk in
the grape-wine chain (Logrieco et al. 2010, 2011) with A. niger/A. awamori as main FB2 producers (Logrieco et. al., 2009). The knowledge of the factors affecting grape contamination by species included in this section and OTA production is essential to be able to reduce their presence, not only to improve wine quality, but also to maintain their safety. Aspergillus section Nigri colonise grape berries in field and ripening period is crucial for OTA contamination (Battilani et al., 2003). The role of wine making was also studied and it was confirmed by several authors that OTA content could only increase post-harvest for fungal activity before processing. During maceration, in red wine making, a release of OTA from pomaces is possible, but only a decrease of contamination was reported due to following operations (Grazioli et al., 2006; Lasram et al., 2008; Leong et al., 2004; Leong et al., 2006a; Solfrizzo et al., 2008, Ponsone et. al 2010).

The grapes growing period is therefore crucial for OTA contamination and the crucial role of weather conditions was underlined in several papers and quantitative data generated in in vitro trials. It was also confirmed that geographic areas can be shared between those prone to OTA contamination and those were the contamination never take place, with the former showing different level of contamination based on annual meteorological trends (Battilani et al., 2003; Battilani et al., 2006a). The role of cropping system and grape varieties were poorly studied but they suggested that both grape variety and trellising system could play a relevant role. Light exposure, canopy humidity and bunches distance from the soil were considered relevant aspects (Battilani et al., 2003; Chiotta et. al 2013, Tjamos et. al, 2006).Vineyard soil was considered as the main source of black aspergilli inoculum ( Kazi et al., 2006).

Several studies demonstrated that damaged grapes are more commonly infected than undamaged ones (Bellí et al., 2006, Cozzi et al., 2006). Damages on berries can be due to the presence of other pathogens, like Lobesia botrana larvae and powdery mildew, and their support to black aspergilli conidial dispersion and fungi penetration, through berry wounds they cause, with consequent increase in OTA content in grapes at harvest (Cozzi et al., 2006). Isolates of Bacillus thuringiensis showed to attack L. botrana larvae and to inhibit significantly the growth of OTA-producing fungi on grapes (Varga et al., 2006), while strains of Ampelomyces ssp. were tested against Erysiphe necator, the grapevine powdery mildew causative agent, with encouraging results (Caffi et al., 2013). Another study has shown that the skin-damaged berries by Planococcus ficus affected the incidence of ochratoxigenic species and OTA contamination in grapes (Chiotta et al., 2010).

Moreover, pre-harvest treatments, like the application of fungicides and insecticides were efficient in reducing black aspergilli development and toxin accumulation (Bellí et al., 2007a).

In conclusion, the crucial period for OTA occurrence is grape growing in field, ripening in particular, and the fungi involved in OTA contamination are black aspergilla, with a main role of A. carbonarius; ecological conditions are crucial for fungi growth and OTA production and also determine the prevalence of different black aspergilla species. During the Mycored project -advances in the knowledge of the biodiversity on Aspergillus section Nigri was done in the grape growing area from Argentina, where selected potential biocontrol agents were evaluated to reduce the entry of ochratoxin to the food chain.- Good Agricultural Practices for ochratoxin A (OTA) management in vineyards were established. -Also in order to predict the level of OTA contamination in grapes and derived products a modelling approach was considered.

Biocontrol as an Eco-friendly Strategy (WP2)

Although chemicals have been commonly used to reduce fungal proliferation and mycotoxin production under field conditions, nowadays a strict legislation about their use has been established in the European Union, due to the increasing number of resistant fungal strains and the impact of fungicides on the environment and human health (De Costa & Bezerra, 2009). Maximum residue levels of pesticides have been regulated in many products, including grapes (EC, 2006). Therefore, alternative methods are necessary to substitute or complement fungicide treatments to control toxigenic fungi at pre- and postharvest stages. Pre-harvest treatments, like the application of fungicides and insecticides were efficient in reducing black aspergilli development and toxin accumulation (Bellí et al., 2006). These results were confirmed both by in vitro and in vivo trials; the efficacy of cyprodinil + fluoxidxonil combination was particularly underlined. Control tools alternative to chemicals were also tested. Dimakopoulou et al. (2008) compared the efficacy of the yeast isolate Aureobasidium pullulans Y-1 to cyprodinil + fluoxidxonil and results demonstrated that the biocontrol agent was as effective as the commercial fungicide, in reducing sour rot infection.

Yeasts are considered one of the most potent biocontrol agents due to their biology and non-toxic properties (Pimienta et al.,...
The mechanism most probably involved in filamentous fungi biocontrol by yeast is competition. Competition among microorganisms for essential factors, such as nutrients and space, is expected to have a dramatic effect on the secondary metabolism of filamentous fungi (Spadaro & Gullino, 2004, Zhou et al., 2007).

Nowadays, several yeast species included in different genera are considered potential biocontrol agents towards ochratoxigenic Aspergillus (Bleve et al., 2006). Dimakopoulou et al. (2008) compared the efficacy of the yeast isolate Aureobasidium pullulans Y-1 to cyprodinil + fludioxonil and results demonstrated that the biocontrol agent was as effective as the commercial fungicide, in reducing sour rot infection. Ponsone et al. 2011 described two epiphytic strains of Kluyveromyces thermotolerans that were able to control the growth and OTA accumulation of ochratoxigenic fungi both “in vitro” and “in situ”. It was demonstrated that Kluyveromyces thermotolerans strains can control both, growth and ochratoxin A accumulation by Aspergillus section Nigri strains. It was observed that K. thermotolerans strains have a strong influence on the expression of the mycotoxin biosynthesis genes. According to the data the production of mycotoxins can be regarded as an adaptation to imposed biotic and other stress conditions by these mycotoxigenic species. If the activation of the mycotoxin biosynthesis is the cause or the consequence of a stress reaction cannot yet be concluded (Ponsone et al. 2013)

The data reported until now indicate that the yeasts that occur naturally on grapes, are promising ecological fungicides, because they can survive and colonize grape berries in the vineyards and also maintain the equilibrium of the natural environment.

The future use of biological control agents for controlling ochratoxigenic fungi and ochratoxin production will depend on the cost production and the field effectiveness of the formulated product.

In conclusion, an integrated strategy based on the combination of biological control agents with natural compounds or reduced dosage of fungicides appears to be one of the most reliable options for large-scale utilization of microbial antagonists in the control of ochratoxigenic fungi and reduction of the entry of OTA to the food chains Field trials done during two vintages showed reduction in ochratoxin A accumulation in grapes (unpublished results)

Modelling (WP3)

The modelling activity done followed a step by step approach: (i) development of the prototype of a mechanistic model for Aspergillus section Nigri infection cycle; (ii) collection of quantitative data from literature; (iii) development of mathematical equation to describe the steps of the infection cycle, (iv) collection of quantitative data for model validation and (v) model validation using probabilistic approach.

Available information on the Aspergillus section Nigri – grape patho-system was organised in a relational diagram according to the principles of “system analysis” (Leffelaar and Ferrari, 1989). The interest was focused on A. niger and A. carbonarius, the main OTA producing species involved in grapes contamination in warm climates like southern Europe (Pitt, 2000).

The relational diagram developed by Battilani and Silva (2010) was considered as starting point for the model development. Specific equations for sporulation, spore dispersal, spore germination, fungal infection, fungal growth and OTA production rates were developed, both for A. carbonarius and A. niger aggregate, in relation to temperature and water activity (aw). Most of literature available regarded fungal growth and OTA production, while information is poor on other growth steps of infection cycle and several assumptions were mandatory to allow the model development.

The effect of temperature and aw regimes on the growth of different strains of black aspergilla was very similar, based on published papers; therefore, functions developed described well fungal behaviour (Astoreca et al., 2007; Battilani et al., 2003a; Belli et al., 2004, 2007, Spadaro et al. 2010; Leong et al., 2006b). Regarding OTA production, only A. carbonarius was considered because of its relevance in practice; almost all the strains are OTA producers and the amount produced is largely higher compared to other species.

Different optimal temperatures were reported for diverse A. carbonarius strains, not related to their geographic origin (Alborch et al., 2011a; Alborch et al., 2011b; Astoreca et al., 2009a, 2009b; Belli at al., 2004 Esteban et al., 2004; Esteban et al., 2006; Leong et al., 2006b; Romero et al., 2010; Selouane et al., 2009; Spadaro et al., 2010)

Therefore, two different equations were developed to take into account the two behaviour observed. The modelling approach needs to take into account both equations, but it is not possible to define the contribute of the 2 sub-populations in different vineyard conditions. Based on data collected in a previous project (Battilani et al, unpublished data), it is expected a very similar presence and 50% contribute was considered in the modelling approach. OTA production rate as function of aw was similar between A. carbonarius strains and it was well described by the developed function.
The berry status is a very important aspect; in case of damages, it is very easy for the fungus to invade the berry and it was confirmed in many trials that infection is very rapid and effective (Bavaresco et al., 2003). Many factors can determine skin damages, but rain, especially during ripening, and pest and disease attacks (P&D) play a main role. Infection of healthy berries was observed in vitro, but time and the inoculum concentration requested suggest that this way of infection should probably be not frequent in field, at least till to the last period of berry ripening (Battilani et al., unpublished data).

The prediction of grape phenology is crucial because it was confirmed that black aspergilli become relevant in berries from veraison and OTA can be rarely detected before harvesting and its increase take place between veraison and ripening. Therefore, a sub-model able to predict growth stages, veraison in particular, is a mandatory support for OTA grapes predictive model. The models considered suitable, as sub-model for OTA grape modelling, was developed by Mariani et al., (2013) and it is based on Normal Heat Hours (NHH) approach.

All the equations developed to describe each steps of the infection cycle were linked between them in a coherent framework; also the grape phenology model was integrated in the OTA-model as independent sub-model to predict the achievement of the four grapevine growth stages relevant for black aspergilla infection: BBCH 71 – fruit set, 75 – berries pea-sized, 81-beginning of ripening, 89 berries ripe for harvest. Stage BBCH 75 drives the starting point of model runs along the season till harvest.

A total of 267 grape samples was collected by beneficiaries. OTA contamination was detected at very low level (as mean value) in all the sampling season. Only few samples collected were found above the UE legal limit; only 6% of total samples were detected above the EU legal limit.

Since model validation using probabilistic approach requires independent variables (OTA contamination) distributed above and below a fixed threshold, and due to a low amount of samples above the legal limit (less than 5% of the total dataset), it was not possible to elaborate the data in order to obtain the probabilistic equation and validate the model.

The future step is the model validation with data collected in different countries, possibly with a reasonable amount of vineyards with contamination above the legal limit. This will confirm if the assumptions defined are correct and a reliable prediction can be delivered or if the collection of further data, related to the underlined lack of information, will be necessary to improve the predictions. The role of pest and disease, even if relevant, is actually not included in the model, but it will be considered especially if model validation is not satisfactory.

Biodiversity of Aspergillus section Nigri (WP6)
The grape-growing area in Argentina has a wide latitudinal extension which combined with the topography of its valleys determine ecological variations that allow the classification of well-demarcated regions. In general, the regions have cold winters, hot summers and good sunshine. Low precipitation requires artificial irrigation from rivers or groundwater, forming oases perfectly defined and separated. The altitude varies between 450 m to 1,800 m above sea level (Catania et al., 2010). Aspergillus section Nigri species are frequently isolated in Argentinean vineyards showing different potential of OTA production (Magnoli et al., 2004; Chulze et al., 2006; Ponsone et al., 2007). In regions with higher mean temperatures such as La Rioja and San Juan, the highest biodiversity of species and A. carbonarius incidence have been observed (Chiotta et al., 2009). The incidence of Aspergillus section Nigri species harvested in different grape-growing regions from Argentina, their ability to produce OTA was evaluated. The morphological identification showed that A. niger aggregate species were the most prevalent ones, followed by A. carbonarius and Aspergillus uniseriate. These populations were confirmed through using AFLP markers and A. tubingensis was separated from A. niger aggregate (Chiotta et. al 2011a, 2011b). Climatic factors, altitude, longitude and latitude have influenced on the distribution of species included in the section. A. carbonarius and A. niger were OTA producers but differed in their OTA producing ability. Temperature was the factor that influenced the most over the highest incidence of A. carbonarius in La Rioja and San Juan regions. The trellis system in vineyards and drip irrigation also influenced the species isolation. The OTA levels detected in grapes and wines were low, but grape variety was more important in susceptibility to fungal infection and OTA levels (Chiotta et. al 2013).

Biodiversity of Aspergillus section Nigri was represented by A. niger aggregate species, A. carbonarius and Aspergillus uniseriate in Argentinean vineyards during 5 vintages. Altitude, longitude and latitude have shown some influence on the species distribution. Isolation frequency of A. niger aggregate species increases towards the south and west of grape-growing area, while the isolation frequency of A. carbonarius and A. uniseriate was higher in the northern regions (Chiotta et. al 2013).
The highest Aspergillus section Nigri species incidence does not necessarily imply OTA contamination on grapes. Ecological conditions that influence the ochratoxigenic species growth are different from those that allow optimum OTA production. Most OTA production by A. carbonarius isolated from grapes from different geographical regions occurred in the range from 0.95 to 0.99 aW, and a temperature of 20 °C, followed by 15 °C, and decreased remarkably in the range of 30-37 °C (Mitchell et al., 2004; Belli et al., 2004, 2005a). A. carbonarius and A. niger have been OTA-producers in this study. These species differed in their OTA producing ability, as the percentage of A. carbonarius OTA-producing strains and the levels produced were higher than those observed by A. niger strains. Potential producers of OTA belonging to A. carbonarius were isolated from regions with higher temperatures (La Rioja and San Juan) suggesting an OTA contamination potential risk as a wide temperature range has been observed in these regions. Higher temperatures during the day could favor fungal growth and lower temperatures during the night could favor OTA production (Chiotta et. al 2013).

The cropping conditions could explain the highest A. carbonarius and A. uniseriate incidence in Parral and A. niger aggregate species in high VSP. The highest A. niger isolation in VSP could probably be due to its faster growth rate and increased tolerance to high temperatures and low water activity as it was observed by Battilani et al. 2003, Leong et al. 2004, Serra et al. 2003, 2005. Great species biodiversity isolated, also under low VSP could be attributed to bunch position which is closer to the soil (Chiotta et. al 2013).

The results obtained provide relevant information to establish the potential risk areas for OTA contamination in Argentinean vineyards. Moreover, the data will be useful for the application of appropriate management strategies to reduce or prevent the development of ochratoxigenic species (Chiotta et. al 2013).

Good Agricultural Practices for ochratoxin A (OTA) management in vineyards (WP2-WP6)
Grape berries attacked by Lobesia botrana larvae are more easily infected by Aspergillus section Nigri (black aspergilli) ochratoxigenic species. Results of field trials confirmed L. botrana as an important ochratoxin A risk factor for grapes in the pre-harvest during seasons with heavy natural infestations. Effective biocontrol of the last generation of moth larvae is useful to reduce indirectly the health hazards of OTA. The entomopathogenic fungal strain of Beauveria bassiana, ITEM-1559, showed high entomotoxic activity on L. botrana larvae of the third generation and can thus be regarded as a good candidate in biocontrol in vineyard.

Powdery mildew (PM), caused by Erysiphe necator, is one of the most widespread fungal diseases of Vitis vinifera and infects all green tissue on the grape wine, including young berries. Severe PM infection leads to splitting of the berries, which may predispose grapes to attacks by various fungi including black aspergilli. Trial results indicate that grape berries affected by PM are more susceptible to black Aspergillus growth and to production and/or accumulation of Fumonisin B2 and OTA. Thus, preventive control of E. necator on grape berries could reduce the mycotoxicological risk from black Aspergillus species, maintaining the safety of grape-derived products.(Cozzi et al., 2013 a and b)

NUTS AND DRIED FRUITS CHAIN

Nuts and dried fruits have been an important part of the diet in many cultures and civilizations during centuries due to their high energy and nutritional value, as well as their variety of flavours and tastes. Nuts and dried fruit production and consumption is increasing year by year mainly due to their linkage to several health benefits, specially the prevention of cardiovascular diseases (Estruch et al., 2013, Kris-Etheron et al., 2008) and all-cause mortality (Bao Y et al., 2013).

Furthermore, many nuts are cultivated in low income countries representing one of the biggest economies in such growing areas, helping to develop the region and improving life quality of its inhabitants.

In 2012, global production of tree nuts achieved 3.4 million metric tons which represents a 24.5% increase during the last seven years. Dried fruit production in 2012 was 9.4 million metric tons, 23.2% up from 2006, while peanuts reached 32.2 million metric tons, 11.3% higher than in 2006 (INC, 2013).
As many other crops, nuts and dried fruits can be colonized by mycotoxin producing moulds, being an important concern for some commodities.

Aflatoxins are mainly found in maize, peanuts, tree nuts and dried fruits. Ochratoxin A is mainly found in cereals and in a smaller proportion in wine, coffee, spices and dried fruits. Fumonisins are mainly found in maize and maize based products, while tricothecenes and zearalenone are chiefly associated with grains.

Mycotoxins are toxic and their regular ingestion can cause mycotoxicosis (Bryden, 2007). Several studies revealed that mycotoxins are immunosuppressive (Corrier, 1991) and carcinogenic (Marasas et al., 2001; Henry et al., 1999). Many illnesses are associated and can be modulated by mycotoxins, causing direct losses to governments on health expenditures.

Worldwide regulations on maximum allowable levels of mycotoxins modulate the global trade of goods, and cause high costs due to the amount of destroyed goods at the borders, and block many exports, the countries of origin of which end up transforming the goods into feed or redirecting them to other markets.

In 2001, Otsuki and Wilson calculated that a 10 percent lower maximum allowable level of contamination in the European countries would reduce trade flow from Africa to Europe by 11 percent for cereals, 4.3 percent for fruits, nuts and vegetables, and 13 percent for groundnuts (Otsuki et al. 2001).

Pre-harvest aflatoxin contamination of crops is associated with drought stress and hot temperatures. Drought stress was found to increase peanut (Wotton and Strange, 1987) and pistachio (Hadavi, 2005) susceptibility. Special risk of contamination is found where a combination of hot dry stress conditions followed by warm moist conditions occurs (Cotty et al., 2007). Predictive models are becoming very important due to the importance of climate on crops contamination.

Good practices are not enough to prevent mycotoxin contamination in nuts and dried fruits. Climate change is increasing the frequency of extreme events, such as severe droughts, which is worsening the issue.

The focus of the MycoRed research was to find out novel strategies to implement from farm to retail package aimed at reducing mycotoxin contamination and occurrence. Investigations were carried on pre-harvest: biocontrol and modelling; and post-harvest actions: post-harvest and storage handling practices, novel application of food/feed processing technologies, advanced technologies for diagnostics, quantitative detection and novel approaches to control toxigenic fungi and advanced analytical tools for rapid multi-toxins detection of Mycotoxins and relevant biomarkers.

Pre-harvest

Biocontrol of aflatoxins in peanuts (WP2)

Aflatoxin contamination of peanuts results from growth in peanut kernels by toxigenic strains of Aspergillus flavus Link and A. parasiticus Speare. Soil is the main source of inoculum for A. flavus/A. parasiticus and since peanut fruits develop underground, pods are in direct contact with the soil fungal populations (Horn and Dorner, 1998).

Pre-harvest aflatoxin contamination of peanuts is associated with severe late-season drought stress. Contamination can also occur after peanuts are dug if they are not quickly harvested, dried and maintained at safe moisture level; or during storage when improper conditions of moisture and temperature exist. Contaminated lots of peanuts cannot be used for human consumption and therefore represent great economic losses for the peanut industry (Lamb and Sternitzke, 2001).

Peanut (Arachis hypogaea L.) is an economically important crop in Argentina. Since 2006 and so far, the exports of edible peanuts from Argentina went over 400,000 tones and actually our country ranks first as peanut exporter in the world. Around
65% of the Argentine peanut exports go to the European Union (mainly The Netherlands, Germany, UK, France, Greece and Poland), other consistent importers are the USA and Canada (Cámara Argentina del Maní, 2012). Aspergillus species from section Flavi have been isolated from soil and peanuts cultivated in the main peanut production area in Argentina and characterized in relation to their toxigenic profile and genetic diversity (Barros et al., 2003, 2005, 2006 a,b, 2007). Aflatoxin control in peanuts relies on several approaches both pre-harvest and postharvest such as good cultural practices, irrigation, use of drought resistant cultivars and postharvest sorting by electronic devices and blanching (Dorner, 2008). However, these procedures are expensive and not always effective. One strategy that has been developed for reducing preharvest aflatoxin contamination of crops is biological control, which is achieved by applying competitive non-toxigenic strains of A. flavus and/or A. parasiticus to the soil of developing crops (Dorner and Cole, 2002). This approach is based on the premise that when spores of the nontoxicogenic strains are added to soil, they will compete with naturally occurring toxigenic strains for infection sites for growth on peanut and for essential nutrients.

A 2-year study was carried out to determine the efficacy of an A. flavus strain as biocontrol agent to reduce aflatoxin production in peanuts under field conditions in Argentina. The competitive strain used was a nontoxicogenic A. flavus (AFCHG2) naturally occurring in peanut from Córdoba Province. The inoculum was produced through solid-state fermentation on long grain rice and applied at rate of 50 kg inoculum/ha. During 2009/2010 growing season, treatments produced significant reductions in the incidence of toxigenic isolates of A. flavus/A. parasiticus in soil and peanuts. However, no preharvest aflatoxin contamination was observed. In 2010/2011 growing season, plants were exposed to late season drought conditions that were optimal for aflatoxin contamination. Significant reductions in aflatoxin levels averaging 71% were detected in treated plots with different inoculation treatments. This work represents the first report using the strategy of competitive exclusion to reduce aflatoxin contamination in Argentinean peanuts.

Use of nontoxicogenic strains of A. flavus to competitively exclude aflatoxin-producing strains has emerged as the best management practice for reducing aflatoxin contamination in several crops. The present study demonstrated that the nontoxicenic A. flavus AFCHG2 have the potential to become management tools for the biocontrol of aflatoxin contamination of peanuts in Argentina. Additionally, nontoxicenic A. flavus AFCHG2 does not produce CPA, which is a concern for the safety of crops products to be used livestock feed and human food. This work represents the first report using the strategy of competitive exclusion to reduce aflatoxin contamination in Argentinean peanuts.(Alaniz Zanon et al., 2013)

Modelling and development of DSS (WP3)

A mechanistic weather-driven model based on the infection cycle of Aspergillus flavus on maize to predict the risk of aflatoxin contamination in field on a daily basis from silk emergence to harvest was developed; hourly data of temperature, relative humidity and rain were used as model input (Battilani et al., 2013). The model developed follows a mechanistic approach; it means that the infection cycle of A. flavus is considered and each step of infection is quantified with mathematical function using meteorological parameters as input. Therefore, the model can be transferred to a different crop, taking into account the new host. In particular, crop phenology is also a crucial part of the system and a predictive model also for this aspect is mandatory.

Krishnamurthy et al. (2007) used data collected in 1990-2004 to develop models for prediction of different phenophases for normal and late sown peanuts crop, based on the growing degree days with base temperature 10°C. This model is appropriate to be linked with A. flavus predictive model because it is based on the degree day, as that one chosen for maize phenology. Therefore this was linked to AFLA-maize models changing the support crop from maize to peanuts.

A prototype model to predict aflatoxin contamination in peanuts was then developed. In the future, this prototype model should be validated with field data, both on peanuts phenology and aflatoxin contamination, and possibly used to support farmers in defining the risk of contamination in different areas and year with several advantages for farmers.
Novel application of food/feed processing technologies (WP5)

Maximum permitted levels for aflatoxins in almonds, pistachios and apricot kernels to be subjected to sorting, or other physical treatment, before human consumption are higher as compared to direct human consumption (EC n. 165/2010). The fate of aflatoxins during processing of almonds, apricot kernels and pistachios was evaluated in the MycoRed project.

Electronic sorting, blanching/peeling followed by manual colour sorting were evaluated for apricot kernels and almonds. Transformation processes such as production of nougat, pastries and almond syrup (blanching, roasting, water infusion and cooking) were evaluated for almonds. The mass balance approach was used to determine levels and distribution of aflatoxins in each fraction collected during processing steps. Experiments were conducted on naturally contaminated apricot kernels and almonds inoculated with a toxigenic strain of Aspergillus flavus.

The efficacy of electronic sorting of shelled apricot kernels was limited and results were highly variable because rejected fractions contained 13-59% of total aflatoxins. The manual colour sorting of peeled apricot kernels gave excellent results because the removal of discoloured kernels removed 97-99% of total aflatoxins. These results were confirmed in another laboratory since after peeling the removal of discolored kernels gave 97% reduction in aflatoxin levels.

Blanching processes by steaming or boiling in water did not reduce aflatoxin levels in blanched almonds and apricot kernels. Negligible amounts of aflatoxins (<1%) were found in boiling water analysed after blanching contaminated almonds or apricot kernels. Aflatoxins were substantially stable during preparation and cooking of pastries and a slight reduction of aflatoxins (10%) was observed when pastries were cooked at 180°C. A significant reduction of aflatoxins were observed during roasting of almonds. However, a variability of results was observed when the process was repeated at different aflatoxin levels. The reduction of aflatoxins in roasted almonds was proportional to their initial levels. Higher aflatoxin reduction could be obtained by roasting at high temperature for long time (150°C for 120 min), but almonds lose their organoleptic characteristics. Nougat prepared with roasted almonds mixed in melted caramelized sugar produced a further consistent reduction of aflatoxins. The caramelisation of sugar was identified as the critical step for aflatoxin reduction. Almond syrup was prepared from peeled almond paste that was infused in water. After infusion the exhausted almonds were discarded and the infuse was sugared and boiled until reaching the consistency of syrup. The whole process of almond syrup preparation produced a marked increase of total aflatoxins probably due to the involvement of enzymes during the infusion step that released free aflatoxins from masked aflatoxins. About 20% of total aflatoxins passed in the final syrup during the whole process of almond syrup preparation (Solfrizzo et al., manuscript in preparation).

The reduction of aflatoxin contamination during pistachio roasting was higher as longer times and higher temperatures. Pistachios were roasted for 30 minutes at 90,120 and 150 ºC and for 60 minutes at 90, 120 and 150 ºC. Total aflatoxin reduction ranged from 47.6 to 98.5 %. Mechanical optical sorting of pistachios reduced by 92.25±9.76 % (n=8) total aflatoxin contamination of pistachios inshell and 97.49±3.7 % (n=10) for shelled pistachios.

Multilocus sequence analysis of Aspergillus Sect. Nigri in dried vine fruits of worldwide origin (WP6)

Black Aspergilli are one of the more difficult groups in terms of classification and identification, and several taxonomic schemes have been proposed. New molecular approaches have shown that there is a high biodiversity, but that species are occasionally difficult to recognize based solely on their phenotypic characters (Samson et al., 2007). Dried vine fruits may be heavily colonized by Aspergillus species. The molecular biodiversity of an Aspergillus population (234 strains) isolated from dried vine fruit samples of worldwide origin were analyzed by investigating four housekeeping gene loci (calmodulin, β-tubulin, elongation factor 1-α, RPB2). A multilocus analysis for 4 housekeeping genes covering 2556 nucleotides was performed on the
234 strains isolated from raisins worldwide in comparison with 24 reference type strains from Aspergillus Sections Nigri and Flavi. The evolutionary history was inferred using the Neighbor-Joining method. Aspergillus Sect. Nigri was dominant and the strains were identified as A. tubingensis (138), A. awamori (38), A. carbonarius (27), A. uvarum (16) and A. niger (11). Four Aspergillus flavus strains were also identified from Chilean raisins. Two clusters closely related to the A. tubingensis species with a significant bootstrap (60% and 99%) were identified as distinct populations. Among the four loci, RPB2 showed the highest genetic variability. This is the first complete study on the worldwide distribution of black Aspergilli occurring on dried vine fruits identified by a molecular approach (Susca A et al., 2013)

Influence of varying temperature and water activity on fungal growth, production of AFB1, and expression of aflatoxin biosynthesis genes in Aspergillus flavus on almond medium (WP 6)

Almonds may be infected by Aspergillus flavus, the major responsible of aflatoxin contamination, representing a serious food safety hazard, other than a concern for the economic losses due to the border rejection of contaminated products. Aflatoxins are produced both on the field and during storage under various environmental conditions. Water availability and temperature are among the most significant environmental factors influencing aflatoxin production. In this study we have monitored the influence of varying temperature (20°C, 28°C, and 37°C) and water activity (0.99, 0.96, 0.93, and 0.90 aw) on fungal growth, aflatoxin B1 (AFB1) production and expression of four aflatoxin biosynthetic genes (aflR, aflS, aflD, and aflO) in A. flavus grown on almond enriched medium. Analyses were performed on fungal mycelium collected at different time points along the incubation period. The highest growth rate, in terms of quantity of fungal biomass, and the highest AFB1 production were observed when A. flavus grew at 28°C at 0.99 and 0.96 aw. At 0.96 aw AFB1 production was slightly higher, even though the mycotoxin production process was slower and the maximum of accumulation was reached later than at 0.99 aw. It was observed that with lowering of aw the risk of AFB1 contamination may increase at prolonged time of incubation, anyway at 28°C and at 0.90 aw a remarkable loss of AFB1 production capability was observed. At 0.99 aw, both high (37°C) and low (20°C) temperatures caused a lowering of AFB1 production, with the high temperature affecting more than the low temperature. When the two parameters were contemporaneously changed, their influence was greater. In fact, at 0.96 aw and at 20°C, mycotoxin production was reduced compared to when only temperature was changed (0.99 aw and 20°C). When aw was set at the lowest levels (0.93 and 0.90) the growth was completely inhibited at 20°C, while at 37°C the growth was reduced in comparison to 28°C. At 37°C AFB1 production was slightly higher at 0.93 than at 0.96 and it was repressed at 0.90 aw. Regarding the expression of aflatoxin biosynthesis genes, the evidence was that in general the activation trend of the two regulatory genes (aflR and aflS) and of the two structural genes (aflD and aflO) mirrored the AFB1 accumulation profile. In correspondence of AFB1 synthesis onset the structural genes were highly upregulated compared to their basal level and in greater degree than regulatory ones. Generally, when conditions were unfavorable for aflatoxin production, the transcription levels decreased, above all those of structural genes. The regulatory genes were always expressed, regardless of aw and temperature values, also in absence of AFB1 production.

Biodiversity of toxigenic fungi isolated from dried fruits (WP6)

Microbial diversity is viewed as a valuable, yet invisible, resource for science and industry with implications for economy and finance. Differences in environmental conditions could influence the distribution of various toxigenic fungi and related mycotoxicological risks worldwide and therefore they can have consequence on the biodiversity of toxigenic fungi. Emerging problems due to climate change and new mycotoxin/commodity combinations add further concern and seem confirming the importance of keeping monitoring the biodiversity of toxigenic fungi. In addition, trans-global transposition and trade exchanges of plant products significantly contribute to the spreading of toxigenic fungi worldwide. For the identification of toxigenic fungi from dried fruits a multi-locus gene approach based on 4 genes sequence analysis was used. The genes were: β-tubulin (βt), calmodulin (caM), RNA polymerase II (RPB2) and elongation factor 1α (EF-1α). Fungal strains belonging to Aspergillus and Penicillium were isolated and identified from samples collected worldwide as reported below. The Penicillium species identified are also reported below.
Samples of Pistachio (18 Iran, 10 USA, 5 Turkey, 3 Italy) with 276 strains belonging to P. crysogenum; P. citrinum; P. commune; P. coryophilum; P. crustosum; P. cyclopium; P. glabrum; samples of almonds (11 Italy, 14 USA, 6 Spain) with 89 strains belonging to P. glabrum; samples of walnuts (2 Chile, 2 Moldavia, 1 Italy, 1 China, 1 USA, 1 Brazil) with 63 P. crustosum; P. expansum; P. melanoconidium; P. freii; samples of cashew nuts (3 Brazil, 2 India) with 58 strains belonging to P. crustosum, P. Citrinum; samples of Hazelnuts (4 Italy) with 14 strains identified as P. crysogenum.

For the Aspergillus genus the most important data were the following a population of 171 strains from pistachios was isolated and the identification by using the 4 genes mentioned above evidenced the prevalence of Aspergillus Sect. Nigri, with 80 strains of A. tubingensis, 9 atypical strains of A. tubingensis, 1 strain of A. acidus, 12 strains of A. awamori, interestingly 1 strains of A. ibericus (never found outside from the Portugal and Spain) was identified on pistachios from Iran. Additionally, only 2 A. carbonarius and 4 A. japonicus were found. Within the 56 strains of the Flavi group 53 belong to the A. flavus type strains and only 2 were A. parasiticus and 1 A. nomius.

Economic Evaluation of the Impact of Mycotoxin Contamination in the nut and dried fruit industry (WP 8)

As many other crops, nuts and dried fruits are affected by mycotoxin contamination, which besides of the health related consequences due to its regular ingestion and its linked economic costs, causes direct losses to the industry. Within the group of nuts and dried fruits, Aflatoxins are found in peanuts, tree nuts and dried figs, while Ochratoxin A is found in dried fruits.

Worldwide regulations on maximum allowable levels of Mycotoxins cause high costs to the industry due to destroyed goods, demurrage fees at ports, extra transport and conversion of the product into feed. Furthermore, both exporters and importers perform routine controls for aflatoxins, and the whole nut and dried fig industry is in charge of this expense which includes the sampling, the sampled product’s loss, shipping the sample to the lab, laboratory materials and the staff involved.

We carried an estimation on the Governments and industry losses due to mycotoxin contamination. The toxicity of the mycotoxins and their implication in some diseases and the food safety expenditures were evaluated to calculate the Governments costs. Border rejections of the commodities, destruction of goods, and routine analysis were evaluated for the industry losses estimation.

The industry losses were evaluated with the real border rejections of the EU-27 countries plus Norway, Liechtenstein, Switzerland and Iceland, Japan, United States of America and Australia, which all of them account for a high share of global imports. A questionnaire was distributed to the main exporters to estimate the costs of routine analysis, fees, demurrage at ports, staff and extra costs on transportation due to border rejections. The total costs due to mycotoxin contamination for the nuts and dried fruit industry reaches up to 60.000.000 US$ annually.

MULTI-MYCOTOXIN ANALYSIS AND METABOLITE PROFILING (WP7)

Introduction

A transition from single to multi-mycotoxin approaches, such as the use of liquid chromatography/tandem mass spectrometry (LC/MS/MS), has dominated the last decade in mycotoxin analysis. Most of the published methods cover all mycotoxins dressed by regulatory limits (as well as some of their derivatives such as nivalenol or zearalenols), which drastically reduces analysis time compared to multiple analysis using conventional methods based e.g. on LC coupled to fluorescence detection, which cover only single classes of mycotoxins. The goal within MYCORED was more ambitious as we intended to develop LC-MS based methods covering hundreds of fungal metabolites, both for metabolite screening in fungal cultures (to investigate the metabolic potential of some of the most prevalent fungal species occurring in food and feed) as well as for quantitative analysis in food samples generated by the project consortium. In that aspect, it is important to consider the “emerging mycotoxins” from Fusarium (moniliformin, fusaproliferin, fusaric acid, aurofusarin, beauvericin and enniatins), Alternaria (tentoxin, alternariols, and altertoxins), Penicillium (roquefortine C, meleagrin, citrinin, cytochalasins), and Aspergillus (sterigmatocystin, kojic acid, emodin, 3-nitropropionic acid and cytochalasins) as well as potential synergistic effects caused by
the co-occurrence of different mycotoxins (reviewed by Grenier and Ostwald, 2011). Extension and validation of the quantitative multi-mycotoxin method (WP7)

As the multi-mycotoxin methods based on the dilute and shoot approach do not include any sample-preparation other than a single extraction step they preserve the pattern of metabolites and any new compound can be added as long as it is compatible to the acidic conditions determined to be the best compromise (Sulyok et al., 2006). A new data acquisition mode, the “scheduled Multiple Reaction Monitoring” mode enables acquiring several hundred MS/MS transitions with dwell times of 25 ms and above. The method has been transferred to a more sensitive mass spectrometer and has been validated for 270 metabolites in four model matrices from different matrix classes commonly used for validation of multi-residue methods for pesticides (SANCO 12495/2011): Apple puree for infants (high water content), hazelnuts (high fat content), maize (high starch or protein content) and green pepper (“unusual/challenging matrices). LODs and LOQs were generally in the sub-µg/kg to the low µg/kg range and were below the regulatory limits except those for aflatoxin M1 and the respective limits for aflatoxins and Ochratoxin A set for baby food. Extraction efficiencies were in the target range of 70-110% in case of 80% of the investigated analytes in apple puree, hazelnuts and maize, whereas as recoveries lower than 70% were observed for approximately 30% of all analytes in pepper. Significant signal suppression of > 30% was observed only for 10% of the investigated analytes in apple puree, hazelnuts and pepper, whereas the fraction of analytes suffering from severe matrix effects was approximately 50% in case of the “difficult” matrix pepper. For further verification of the accuracy method, samples deriving from proficiency tests were analysed and z-scores between -1 and 1 were obtained in approx. 80% of the results submitted for aflatoxins (including M1), ochratoxin A, fumonisins B1 and B2, deoxynivalenol, zearalenone, nivalenol, T-2 and HT-2 Toxin in maize, wheat, oats, peanuts, peanut paste, peanut cake, pistachio paste, pepper, raisins, coffee, animal feed, baby food, milk powder, (Malachova et al., to be submitted).

Application within MycoRed (WP7)
The above described LC-MS/MS based multi-mycotoxin method was applied to numerous food and feed samples arising from different MycoRed work packages and research questions. For instance, wheat and barley samples were analysed for WP4 to investigate mycotoxin production under different storage conditions (Mylona et al. 2012). Many samples obtained from African partners were analyzed and revealed the ubiquitous co-contamination many African populations are exposed to (Abia et al., 2013; Ezekiel et al., 2012b; Ezekiel et al., 2012c; Matumba et al., 2012; Shephard et al., 2013; Warth et al., 2012a). Single kernel analyses of maize kernels showed that sorting of maize could reduce exposure significantly and that some kernel could contain up to 1500 ppm fumonisins (Mogensen et al., 2011). Multitoxin analysis and mycological investigation showed that Talaromyces concavorugulosus and Aspergillus wentii were some of the major post-harvest contaminants in South African maize.

Particular attention should be given to the work of several MYCORED short-term visit fellows who had the opportunity to analyse samples from their home countries and obtained comprehensive data on mycotoxin contamination (Abia et al., 2013; Ennouari et al., 2013; Ezekiel et al., 2012a, Mikusova et al. 2013). Maize samples of good and bad quality, obtained within the South African biomarker study mentioned below, confirmed extraordinary high fumonisin exposure in the former Transkei region (Shephard et al., 2013). As a general conclusion it can be stated that frequent aflatoxin and fumonisin contamination remains the most severe problem in most of those countries while also potential synergistic effects of cocktails of those toxins should be investigated in the future.

Metabolite profiling of plants (WP7)
Metabolomics is the latest of the so called “-omics” disciplines and aims at the comprehensive qualitative and quantitative determination of all low molecular weight metabolites of a biological system. Although metabolomics shows great potential to provide a deeper understanding of biochemical processes on a system level, there is still a limited number of applications of this technology for the investigation of plant-pathogen interactions, which is mainly due to technical problems. Within MYCORED a previously developed powerful workflow has been applied for the study of the metabolomes of maize and wheat varieties (resistant and susceptible) and their interaction with F. verticillioides (maize) and after treatment with the mycotoxin deoxynivalenol (wheat). As a key feature, the presented approach involved the measurement of mixtures of both non-labelled as well as in vivo 13C stable isotopically labelled maize and wheat plants by LC-HRMS (Bueschl et al.. 2012; Bueschl et al.,...
submitted) and subsequent database searches for compound annotation. For the experiment maize / Fusarium verticillioides
the putatively identified metabolites exhibiting differentially up- or down-regulation in the resistant and susceptible line,
respectively, could be linked to metabolic pathways that are well known to be correlated to resistance: linoleic acid
metabolism, alpha-linolenic acid metabolism, phenylpropanoid biosynthesis, tryptophan metabolism, phenylalanine
metabolism and flavonoid biosynthesis. For the wheat / DON interaction elevated metabolite concentration levels were
successfully linked to compound classes of phenolic acids, phenylpropanoids, flavonoids, C18 fatty acids and oxylipines.

Masked mycotoxins (WP7)
The occurrence of different forms of Fusarium toxins, such as conjugated or bound forms arising from resistance processes
during plant growth and/or food processing, could lead to a significant underestimation of the amount of a mycotoxin actually
ingested. It is under discussion whether these compounds might have a similar toxic potential to those of their precursors
when ingested with food, as attached functional groups like glycosylic or sulfate residues might be enzymatically cleaved
during digestion. Indeed, it was found in in-vitro experiments that deoxynivalenol-3-glucoside is stable in acidic medium, but is
partially cleaved by enzymes and bacteria that are present in the human gastro-intestinal tract (Berthiller et al., 2011).
However, in vivo experiments in rats suggested a low bioavailability and lower toxicity compared to DON, although a partial
hydrolysis to DON was observed (Nagl et al., 2012). As the hydrolysis is depended on the composition of the intestinal bacteria
the toxicity of the compound is very likely not only species-specific can also be differ on the individual level. As considers
occurrence, a dedicated method for masked DON- and ZON-species was applied to various commodities and revealed the
prevalence of ZON-4-sulfate and DON-3-glucoside in various food samples. However, the respective concentrations were at
least an order of a magnitude lower than the respective values of the parent toxins (Vendl et al. 2010). The fate of fumonisins
during maize heat treatment was investigated and interestingly none of the glucose-conjugated product reported in the
literature was detected. With heat treatment at up to 130˚C at high moisture contents fumonisin reduction of up to 50% was
observed giving partially hydrolysed fumonisins (loss of one TCA chain). At low moisture conditions significant reduction was
first observed at temperatures above 130˚C, which led in parallel to a significant change of the maize matrix.

Advanced analytical tools for rapid multi-analyte detection of fungal and plant metabolites and some relevant biomarkers
(WP7)
Prevention and minimisation strategies require effective analytical tools to monitor the effectiveness of the practical
approaches employed. Therefore, the activities of the horizontal analytical workpackage of MycoRed were crucial for the
conclusions being drawn in all of the other workpackages through providing advanced LC-MS based methods and support
analyses. In addition, valuable insights into plant-fungal interactions was provided as well. These insights are a major impact
from this WP dealing with analytical tools for mycotoxin detection. The identification of conjugated (masked) mycotoxins by
LC/MS/MS that escape routine analyses are of interest to ensure the proper toxicological evaluation of these metabolites in
 cereals and food products since the toxins can be converted from conjugated to free toxins in the intestinal tract of humans
and animals. Conventional techniques to monitor the level of mycotoxins are not answer the pivotal questions regarding the
availability of mycotoxins present in the bio-available fraction in the exposed organism. Monitoring of specific biomarkers in
bio-fluids and tissues is the most reliable means to assess human and animal exposure to mycotoxins. The related activity
within MycoRed provided advanced tools to measure mycotoxin contamination in commodities and biomarkers in biological
fluids serving horizontally the other workpackages. Finally, the application and optimisation of easy-to-use rapid tests helped
to obtain rapid information about the level of mycotoxins in unprocessed and processed cereals but also will contribute to the
availability of improved test-kits as screening tools that can be used at different stages in the production chain and possibly
incorporated into HACCP protocols.

LC-MS/MS based multi-biomarker analysis (WP7)
The measurement of biomarkers in blood or urine offers an elegant and complementary tool for advanced human and animal
exposure assessment besides the traditional approach of food/feed analysis (Baldwin et al., 2011; Turner et al., 2012; Warth et
al., 2013b).
First biomarker methods in the area of mycotoxin research were developed around 20 years ago and since then most work
focused on aflatoxins (Kensler et al., 2011), fumonisins (Van Der Westhuizen et al., 2013), ochratoxin A (Duarte et al., 2011)
and deoxynivalenol (Turner, 2010). Since 2010 multi-analyte methods, with the potential to detect a number of mycotoxins and/or their metabolites simultaneously, are available for human exposure assessment due to increased sensitivity and selectivity of modern mass spectrometers (Warth et al., 2013b).

Within the MycoRed project two innovative multi-biomarker methods were developed using complementary analytical approaches. At CNR-ISPA (Bari, Italy) an advanced cleanup protocol using enzymatic hydrolysis of conjugates, a novel multi-toxin immunoaffinity column in combination with a reversed-phase SPE column was established for simultaneous determination of biomarkers of DON, AFB1, FB1, ZEA and OTA in human and animal urines (Solfrizzo et al., 2011). The use of multi-toxin immunoaffinity and SPE columns enriched the sample 30 times which permitted to inject in the LC-MS/MS apparatus up to 0.6 ml equivalent of urine with acceptable matrix effects. Good values of LOD, ranging between 0.02 – 2.2 ng/ml, were obtained for the tested biomarkers even by using a normal mass spectrometer (QTrap 2000 MS/MS system). The method was successfully applied in an Italian small-scale pilot survey (n=10) that showed the first co-occurrence of OTA and DON in human urine with percentages of positive samples of 90 and 70%, respectively (Solfrizzo et al., 2011). The use of enzymatic hydrolysis of conjugates and immunoaffinity cleanup provides a high sensitivity and allows the determination of the total amount of mycotoxin biomarkers excreted in urine avoiding the use conjugated standards which are not commercially available. The sensitivity of this method was further improved by using UPLC, a more sensitive mass spectrometer (API 5000 MS/MS system) and the “Scheduled Multiple Reaction Monitoring”. With these conditions the values of LOD ranged between 0.002 – 0.5 ng/ml for the tested biomarkers. The improved method was used in an advanced exposure survey among subsistence farmers in a high esophageal cancer region in the former Transkei region, South Africa (Shephard et al., 2013).

The high sensitivity of the improved method permitted to identify and measure DON, FB1, β-ZEL, α-ZEL, ZEN and OTA in 87%, 96%, 75%, 92%, 100% and 98% of the samples, respectively. The absence of AFM1 in all tested samples confirmed previous results showing the absence of aflatoxins in food consumed in South Africa (Shephard et al., 2013).

Beneficiary IFA-BOKU (Tulln, Austria) followed a direct, so-called ‘dilute and shoot’ approach. Here, a urine sample is simply diluted and injected into a highly sensitive LC-MS/MS system (Warth et al., 2012c). This approach offers the advantage that virtually all analytes of interest can be integrated including polar conjugates such as deoxynivalenol-3-glucuronide (DON-3-GlcA) and zearalenone-14-glucuronide (ZEN-14-GlcA), which are otherwise frequently lost during sample preparation. However, this direct approach requires the availability of authentic, NMR confirmed reference standards of these metabolites which we achieved through in-house synthesis (Fruhmann et al., 2012; Mikula et al., 2012). Consequently those two important conjugates (DON-3-GlcA, ZEN-14-GlcA) could be quantified for the first time in naturally contaminated human urine (Warth et al., 2011; Warth et al., 2012c). Besides the glucuronide conjugates also other mycotoxins, which could serve as potential new exposure markers but have not been examined yet, were included in the ‘dilute and shoot’ method, including nivalenol, T-2 toxin, HT-2 toxin and fumonisins B2. This method was applied in several human pilot surveys: In Austrian volunteers (n=27; Warth et al. 2012b) and pregnant women from Croatia (n=40; Šarkanj et al. 2013) high concentrations of urinary DON and DON-glucuronides were determined with a significant number of volunteers (Austria 33%; Croatia 48%) estimated to exceed the tolerable daily intake set by the JECFA/WHO (FAO/WHO, 2010) under certain assumptions. In four Croatian women DON and OTA co-exposure was detected and in one woman it was estimated that the TDI value of DON was exceeded by a factor of approximately 33, the highest reported exposure level so far (Šarkanj et al., 2013). In the South African study mentioned above, besides FB1 the first evidence for the co-exposure to DON and its metabolites (DON-3-GlcA and DON-15-GlcA) as well as to nivalenol could be provided for this region (Shephard et al., 2013). The most severe case of mycotoxin co-exposure reported as yet in human urine was found for a HIV positive volunteer from Cameroon, where AFM1, FB1, OTA, DON, DON-15-GlcA and nivalenol could be verified simultaneously (Abia et al., in press; Warth et al., 2012c). Furthermore, in this survey (n=175), urinary nivalenol and zearalenone-14-glucuronide were quantified for the first time in naturally contaminated human urine samples and frequent co-exposure among the participants was discovered.

Another main outcome of the MYCORED project was the first interlaboratory comparison of different LC-MS/MS based biomarker methods. For this purpose the performance of the two novel multi-biomarker methods cited above and two established single target methods for the determination of total urinary DON and FB1, developed at the University of Leeds (Gong et al., 2008; Turner et al., 2008) were compared (Solfrizzo et al., 2013). Each laboratory analysed common urine samples spiked with up to eight biomarkers (DON, DOM-1, AFM1, ZEN, α-ZEL, β-ZEL, OTA and FB1) at two levels of each biomarker. The results of each laboratory were evaluated for their z-score values. Good method performances were obtained
for most biomarkers, at the levels tested in this study, as demonstrated by the overall percentage of satisfactory z-scores for all analytes (85%, 68 of 80 results). Excellent results were obtained for DON, DOM-1, AFM1, ZEN, α-ZEL, β-ZEL whereas for FB1 and OTA the performances were variable depending of the laboratory and calibration solution used. These promising results demonstrate the high accuracy of the employed methods for the majority of analytes and suggest further efforts in this direction including more laboratories, analytes and samples of different origin.

Sophisticated biomarker methods can also aid to advance our understanding of human metabolism, toxicokinetic patterns the detoxification potential at an individual level. This was elegantly exemplified by deoxynivalenol in vivo and in vitro experiments using the “dilute and shoot” method, which is able to directly quantify glucuronide conjugates. As opposed to previous studies, DON-15-GlcA, and not DON-3-GlcA, was determined by us to be the main human conjugation product of DON (Warth et al., 2012b), an observation that was later confirmed by exposure studies of other populations (Abia et al., in press; Šarkanj et al., 2013; Shephard et al., 2013; Ezekiel et al., unpublished) and using a liver microsome in vitro assay (Maul et al., 2012). In addition, the structure of a third DON-conjugate, putatively identified as DON-7-GlcA (Maul et al., 2012), was investigated in detail in a recent publication (Šarkanj et al., 2013). Furthermore, the first detailed in vivo case study on human deoxynivalenol and zearalenone metabolism was carried out within the MYCORED initiative. In this experiment the urinary excretion patterns were investigated over a period of eight days, the fate of ingested masked DON forms (3-acetyl-DON and DON-3-glucoside) was preliminary assessed in humans and the mean excretion rate of total ZEN was estimated to be 9.4% (Warth et al., 2013a). The work conducted within the consortium contributes to an advanced understanding of mycotoxin metabolism and exposure and therefore clearly supports sophisticated exposure and risk assessment to increase global food safety.

The immunoaffinity based multi-biomarker method was used to validate the urinary mycotoxin biomarkers in piglets administered boluses contaminated with mixtures of DON, AFB1, FB1, ZEN and OTA at different levels. Mean percentages of dietary mycotoxins excreted as biomarkers in 24 h post dose urine were 36.8% for ZEA, 28.5% for DON, 2.6% for FB1, 2.6% for OTA and 2.5% for AFB1. A good correlation was observed between the amount of mycotoxins ingested and the amount of relevant biomarkers excreted in 24 h post dose urine. Linear dose-response correlation coefficients ranged between 0.68 and 0.78 for the tested couples of mycotoxin/biomarker (Gambacorta et al., 2013).

Human pilot study (WP7)
The novel urinary multi-biomarkers developed within the MycoRed project were applied in a study of mycotoxin exposure in a rural subsistence community in the former Transkei region of South Africa. Fifty-three female participants donated part of their stored home-grown maize, maize-based evening meal and first void morning urine. The multi-biomarker ‘dilute-and-shoot’ method indicated deoxynivalenol-15-glucuronide was predominantly present. The multi-biomarker method developed at CNR-ISPA with β-glucuronidase and immunoaffinity clean-up determined (results corrected for recovery with not detected samples assigned a value of LOD) zearalenone (100%; 0.529 ± 1.60 ng/mg creatinine), FB1 (96%; 1.52 ± 2.17 ng/mg creatinine), α-zearalenol (92%; 0.614 ± 1.91 ng/mg creatinine), deoxynivalenol (87%; 11.3 ± 27.1 ng/mg creatinine), β-zearalenol (75%; 0.702 ± 2.95 ng/mg creatinine) and ochratoxin A (98%; 0.041 ± 0.086 ng/mg creatinine). These results demonstrate the value of multi-biomarker methods in measuring exposures in subsistence populations exposed to multiple mycotoxins and in which the collection of food samples and food consumption data is difficult. This was the first finding of deoxynivalenol, zearalenone, their conjugates, ochratoxin A, nivalenol and zearalenols in urine from volunteers in Transkei (Shephard et al., 2013a). The stored home grown maize, previously sorted into good and mouldy parts by the farmers themselves, was analysed by previously developed LC-MS/MS methods. Participant CNR-ISPA used an immunoaffinity clean-up method, whereas IFA-BOKU used a ‘dilute-and-shoot’ method. Significant correlations were observed between the two methods for FB1 and FB2 (correlation coefficients, R², of 0.9144 and 0.8859 respectively). Although no aflatoxins, OTA, T-2 or HT-2 toxins were detected, 50 other mycotoxins and secondary metabolites, mainly from Fusarium and Penicillium species, were found.

Potential Impact:
MYCORED solutions could give significantly contribution to reduce mycotoxin contamination in food and feed chains in Europe and worldwide for some targets. The reduction of principal mycotoxins by novel, multidisciplinary, integrated strategies proposed by MYCORED along the maize, wheat, grape and dried fruit chains may have the following socio-economical impacts:
• A potential decrease in the number of acute and chronic pathologies in Europe and in ICPC Countries due to the consumption of mycotoxin-contaminated products, obtained through the dissemination activities and awareness campaign addressed to farmer, industries and stakeholders. This reduction could reduce the costs in the human and animal health care systems too.

• A decrease in the costs of rejection of contaminated raw crop materials (especially dried fruits and cereals) and processed products. This impact will be very important for farmers, food/feed producers and retailers. Over the past years, mycotoxin regulations in the European Union continued to change rapidly and to proliferate, posing significant challenges to more sectors of the food industry than ever before. Failure to comply with the latest standards can have major repercussions for exporters and importers, ranging from costly retesting and reprocessing to impounded or rejected shipments.

• Improved ability to reduce safety problems due to mycotoxin contamination including in the early stage in the production chain (pre-, post- harvest and processing).

• An increase in the safety of feed and foodstuffs due to the application of various innovative technical solutions developed by MYCORED for an effective interconnection and communication of sensing systems and decision making bodies. The corrective actions could be implemented during preliminary phases, in order to reduce contamination effects during storage/shipment and production processes.

• New opportunities for agro-food industries by exploitable foregrounds (safety food/feed, predictive models, antagonist formulations, new detection kits) able to improve SMEs competitiveness.

• Major awareness and knowledge about mycotoxin problem at global level, obtained through a series of dissemination actions carried out in four Continents.

• Major knowledge arised from MycoRed project on AFLAs management using biocontrol agents, in tropical countries, mainly affected by contamination, could be used for solving emerging mycotoxin problems due to ongoing climate changes, as AFLAs occurrence in maize in South East Europe;

• Improved skills in the food safety area by young students and researchers who participated at MycoRed initiatives.

• Knowledge asset available for scientific and industrial community, useful to boost mycotoxin research and exploitation of results in industries.

• Outcomes of conferences, workshops and training courses could represent a support to develop worldwide action plans to reduce the adverse effects of mycotoxins.

This project has a significant impact on food and feed safety by combining an education and dissemination programme with training, including information on HACCP and risk analysis to facilitate a significant whole food chain approach to improve international trading. Thus, the MYCORED outcomes may have significant economic impact in relation to food/feed safety both for the agro-food international community (farmers, animal and plant breeders, etc.) and for European agro-food industries. In addition, the project produced a study obtaining an estimation on the Governments and industry losses due to mycotoxin contamination. The toxicity of the mycotoxins and their implication in some diseases and the food safety expenditures were evaluated to calculate the governments costs. Border rejections of the commodities, destruction of goods, and routine analysis were evaluated for the industry losses estimation. The estimation considered the real border rejections of the 27 European Community countries, EFSA countries, Australia, Japan and United States, which they publish regularly their border rejections and they represent a large share of the global imports. The total costs due to mycotoxin contamination for the processing industry reaches up to 60.000.000 US$ annually, besides the costs of wheat and maize growers and livestock farmers. Government’s cost on public health related to mycotoxin ingestion was approached considering the possible incidence of mycotoxins on different illnesses, the rate of implication of those illnesses on the disability-adjusted life year (WHO) and the health expenditure per capita of the most affected countries by uncontrolled mycotoxin exposure. Estimating the lowest percentage of implication (1%), this cost reaches 2 billion US$. Government’s costs by controls at borders are mainly charged to the imports industry. Nevertheless, MycoRed novel integrated strategies could be applied to mycotoxins minimizations, particulary in dried fruits, that were still one of the most notified risks in the 2012 RASFF Annual Report, reaching 24% of total border rejections on the RASFF Member States (56% in 2008, 38% in 2009, 36% in 2010, 27% in 2011). Thirty-nine percent of the total mycotoxin notifications were for nuts, nut products and seeds, 25% for dried figs and 12% for peanuts for feed purposes. Nuts, nut products and seeds accounted for 16% of the total border rejections in 2012. Of the total notifications for
mycotoxins in 2012, 92% were for aflatoxins, which are the most toxic and occurring ones. Information generated by MycoRed and other sources (existing projects at a global level, especially in the EU and USA) have been utilised to maximize impact and the dissemination plan among the EU and the global beneficiaries.

The dissemination strategy has been finalized to enforce the communication process to reach the goals of: a) awareness (informing general public and scientific community about the outcomes of the research activities, in order to reinforce the knowledge source in the field of mycotoxins and the position of the Consortium and the enlarged group of experts); b) understanding (making usable and applicable the outcomes of our research for certain target groups such as farmers, labs, companies and researchers too, who can apply novel methodologies); c) dissemination for action (involving policy makers and industries, associations, decision makers to apply the novel methodologies to reduce the mycotoxin occurrence in food and feed chains).

Since the beginning, several dissemination actions were taken to introduce the project in the international scenario opening the road to further events, by project’s presentations worldwide at international conferences and workshops, project’s website, newsletters and promotional materials. International conferences, workshops and training activities, together with Short Term Visits have been successfully conducted; in parallel networking actions gave a considerable impact of MycoRed on the international community, by creating an interesting scientific network focused on mycotoxins and sharing knowledge. This strategy allowed MycoRed to reach the identified target audiences communicating the project and its results. The communication plan has been developed on the basis of the overall communication strategy with different levels of communication addressed to target groups. This process was managed in the framework of a specific WorkPackage (WP8 - Information/ dissemination and education) lead by CNR with contribution and active participation of all the MycoRed Beneficiaries, who addressed their communication actions (on press, radio, tv, web, meetings etc.) to improve awareness and advanced knowledge of MycoRed contents and results all over the world, favouring several social levels (ie. African rural populations having restricted access to media) to access useful information for their health and development. International MycoRed beneficiaries, such as CGIAR institutions such as CIMMYT and IITA, who work with many beneficiaries and have major initiatives on mycotoxins worldwide, enforced the dissemination in Asian and African countries, together with other national and regional organizations.

In this respect the contamination of crops with mycotoxins is a widespread problem in the world and is especially important including developing countries in Africa and South America, from which the EU is a major food importer. A key hurdle for export of economically important crops into EU from other countries is meeting the EU regulations and standards.

MYCORED represents an application model of the EU integrated food safety and quality programme, aiming to assure a high level of food safety, animal health, animal welfare and plant health within the European Union through coherent farm-to-table measures and adequate monitoring, while ensuring the effective functioning of the export/import markets. In such a context, the MYCORED project contributed to enforce this vision by disseminating an integrated approach based on:

• The development of an integrated prevention model for production and distribution food chain, from producers to consumers, with safer foods reducing the risks of mycotoxin contamination.
• The promotion of new technical tools for the control, reduction and monitoring of mycotoxins contamination in the food chains.
• The attention to aspects connected to control and improvement of public health.

These points have been effectively implemented through the dissemination of outputs of the project itself and of technical cooperation projects and working groups for prevention and control of mycotoxins contamination in which several MYCORED beneficiaries are directly involved.

Impact on standards and policy development
At mid-term project’s results may contribute to European and International standards on mycotoxins and toxigenic fungi in food safety through recommendations or deliverables of the conferences. Regulation or standardisation bodies could integrate the outputs of the conferences in their general framework. Moreover the interaction during information, education and dissemination activities between developers and end-users of safe food products can help to establish consistent standards or regulations. The conclusions and the recommendations of MYCORED could be used as background material for any political
report and can deeply contribute significantly to the evolution of food regulation. In this respect, the contribution of INC (Beneficiaries 21) to the Codex Alimentarius Committee on Contaminants (of which INC is member) is quite relevant for setting standards, guidelines and codes of practice for mycotoxins at global level. Moreover, a direct transfer of the MYCORED outcomes to the Commission and to CEN (European Committee for Standardization) through the EU beneficiaries has been provided by involving Beneficiaries participating at EU meetings and External Advisory Board members, including representatives from both European and International regulatory bodies.

Due to the increasing number of mycotoxin regulations and regulating countries, the project will contribute to the European and International social and policy objectives, through the evaluation of the obtained results, with respect to the needs of industry, politicians, risk evaluation institutions and decision-makers. The results from MYCORED could influence EU legislation and other actions:

• To assure effective control systems and evaluate compliance with EU standards in food safety and quality, animal welfare and animal nutrition sectors within the EU and in Third Countries in relation to their exports to the EU, as those regions explore ways to expand export markets and accelerate domestic regulatory reform;

• To manage international relations with Third Countries and international organisations concerning food safety, animal welfare and animal nutrition: developing nations face additional constraints in absorbing best-practice information on standards and mobilizing resources necessary to adopt appropriate process and production methods. They have fewer resources and expertise on hand to become informed of newly enacted safety laws.

• To manage relations with the European Food Safety Authority (EFSA) and promote science-based risk management.

Mycotoxins are one of Food Safety’s biggest concerns in Europe. A considerable decrease of mycotoxin notifications was produced during the last years, mainly due to the good practices implemented in the field and industry, where MycoRed dissemination could have played an important role, and the implementation of the Commission Regulation (EU) No 165/2010 increasing the maximum allowed level of aflatoxins in almonds, apricot kernels, Brazil nuts, hazelnuts and pistachios that entered into force on 9th March 2010 changing maximum levels from 2ppb to 5ppb (hazelnuts and Brazil nuts) and 8ppb (almonds, apricot kernels and pistachios) for aflatoxin B1 and from 4ppb to 10ppb for total aflatoxins.

Specific Impacts

The activities performed under MycoRed technical WPs gave a contribution to advance the cutting edge of science by integrating advanced technologies, including molecular and chemical ones, into traditional agricultural practices to generate novel insights that can result only from interdisciplinary team-oriented research.

Pre-harvest phase (WP1, WP2 and WP3)

The research applied to pre-harvest processes focused on the optimization of plant resistance and fungicide use, giving a contribution to mycotoxin reduction and related losses through the exclusion of highly susceptible genotypes, by applying additional fungicide use. The impact of this action could be enforced by a policy supporting cultivar registration positively: the fungicides are part of current, everyday practice and if done they should be used more effective than now. The better understanding of host/pathogens interaction and the QTL functions will help in breeding and control of diseases in wheat and maize plants. The fungicide optimization and new nozzle combination will make much more powerful tools to decrease toxin contamination than before. These new technologies can be introduced into the European countries in 1-2 years, so a significant mycotoxin reduction can be achieved in maize and wheat in short time. The research on biocontrol reducing mycotoxins in cropping system will contribute to the development of new cost effective preventative measures in maize production such as novel good agricultural practices that lower the risks of mycotoxin formation in the pre-harvest stage. Such cultural measures stimulate antagonistic organisms on-site could be easily implemented directly by farmers and may be applied in other crops and cropping systems. Based on MycoRed outcomes, contamination by FUM in maize and trichothecenes in wheat, OTA in grapes and AFLA in peanuts and maize may effectively be reduced by using antagonists applied in the field.

As project’s results, several antagonists with proven efficacy against toxigenic fungi are available for further development of economically feasible biocontrol products, following the successful MycoRed application case of atoxigenic strains of Aspergillus in Nigeria, enlarged to many Central African countries, mainly exposed to mycotoxin risk. Also naturally occurring antagonists of Fusarium spp. in maize stubble are identified. SME and industries could be provided with antagonists that are
suitable for development into biocontrol products and the implementation of the developed control strategies. Growers, SME’s as producers of the biocontrol products and consumers could benefit from this strategy.

Finally, the Advanced modelling and rationalized logistic and development of DSS represent effective tools to support crops management pre- and post-harvest. The developed models provide support for policy-makers and regulators, as they, with different applications, help to define risk areas, based on both historical knowledge and yearly data. This approach may have important economic impacts because of the rationalization of crop management and the advance knowledge of risk level. Predictive models for DON and ZEA contamination in wheat at harvest, FUM and AFs in maize, OTA in grapes are available as well as surveys on the contamination of these crops at harvest in different geographic areas. They will be a relevant support in decision making regarding the cropping system of the crops in order to minimise mycotoxin contamination at harvest. Besides, those tools will be very useful in the optimisation of post-harvest management. Risk maps based on collected data and predictive models developed will be useful for evaluation of mycotoxin risk exposure to specific crops at global level.

Post-harvest phase (WP4, WP5 and WP10 )

Novel post-harvest and storage handling practices developed under MycoRed could give significant benefits to stakeholders including farmers, co-operatives, crop management, stored grain drying and storage and handlers, to food chain managers, processors and consumers. Relevant transfer of technology from the EU to developing countries for improving quality assurance and storage systems should maximize export to Europe and reduce exposure of rural populations to mycotoxins. The models for Dry Matter Losses relative to the EU legislative limits will be useful for better stored grain management and be useful for millers and for grain processors. We expect O3 or other physical treatments, and novel application of treatments to stored grain, could be adopted for practical application in both developed and developing countries for minimising contamination of wheat, maize and hazelnuts with DON, FUM and AFLA respectively.

The efficacy of the wireless sensor system (CO2, temperature, humidity) in prevention strategies was demonstrated by WP10 activity. The application of the ambient intelligence system will significantly improve the post-harvest management of stored commodities and be useful for indicating high and low risk conditions for better storage management.

Novel application of food processing technologies developed during MycoRed (WP4) will represent practical tools (1) to reduce mycotoxin content in cereals and dried fruits by handling and food/feed processing procedures and (2) to prevent/reduce mycotoxin absorption through the use of detoxifying agents, dietary fibres and microorganisms. The long-term objective of this activity is to improve the knowledge and technology that promotes food safety by reducing human and animal exposure to mycotoxins in grain-based processed foods and dried fruits. The use of efficient decontamination treatments will reduce the cost of monitoring programmes, by reducing the number of sampling and mycotoxin analyses. The selection of effective dietary fibres and microorganisms will increase the value of the relevant agricultural by-products used as a source of fibres. Efficient mycotoxin-detoxifying agents studied by MycoRed can be widely used in the livestock industry to reduce livestock losses and to improve the safety of animal food products. The use of these agents also will reduce the carry-over of mycotoxins in animals and thus the mycotoxin contamination of food-products of animal origin intended for human consumption. After the in vivo trial, agricultural by products able to adsorb mycotoxins and reduce their bioavailability and toxicity in animals identified in MycoRed, together with other microorganisms able to detoxify and/or bind together aflatoxins B1 and OTA, represent the efficacy and safety of several food processing procedures in reducing mycotoxin content, with potential applications to industrial processes to rationalize the food safety processing.

Moreover, the knowledge of the fate of mycotoxins during different food processing processes represents the basis to optimize food processing technologies at industrial level and to increase mycotoxin degradation, obtaining safe end products.

Horizontal technologies (WP6 and WP7) based on advanced technology for diagnostics & quantitative detection of toxigenic fungi and mycotoxins may have several impacts to: i) provide tools based on new approaches for controlling fungal contamination and related mycotoxins in raw materials and in the subsequent food chain, ii) provide the necessary know-how to study toxigenic fungi populations and novel mycotoxicological problems at global level due to climate change and trade exchange; iii) provide opportunities for industry to use the research results for developing new detection kits and novel control measures against toxigenic fungi; iv) support strategies based on influence of various stress conditions, applied during transport and storage on the activation of mycotoxin biosynthesis and develop predictive models based on these data; and v)
develop new approaches based on receptor or signalling molecules to prevent mycotoxin biosynthesis. In particular, after building up the mycological risk map in conjunction with meteorological data from WP3, clear statements are available about new emerging toxigenic species due to climate changes or changes in agronomical practices. Moreover the development of specific detection systems for these species could allow a very careful and detailed monitoring of these population changes, which is important for the correct assessment of these risks and for the possible application of counter active measures. The knowledge of stress regulation of mycotoxin biosynthesis genes could enable to assess correctly the influence of new agricultural and storage practices on the control of the mycotoxin biosynthesis. Moreover knowledge about the regulation of mycotoxin biosynthesis at the molecular level in correspondence to environmental conditions could lead to new principles to control mycotoxin biosynthesis, which can already be foreseen in the application of light of specific wave length. Advanced analytical tools developed in MycoRed may provide valuable insights in food safety and into plant-fungal interactions. The identification of conjugated (masked) mycotoxins by LC/MS/MS that escape routine analyses are of interest to ensure the proper toxicological evaluation of these metabolites in cereals and food products since the toxins can be converted from conjugated to free toxins in the intestinal tract of humans and animals. The novel analytical screening tools could provide a more comprehensive picture of the spectrum of toxic fungal metabolites in the food chain. At the same time, a better understanding of their role during the plant/fungus interaction will be obtained, which will be of great support in novel approaches for pre-harvest mycotoxin minimization in the field (linked to WP1). Finally, data on the concentration of biomarkers is of great help to assess the actual exposure to mycotoxins as well as to evaluate the success of interventions.

List of Websites:

www.mycored.eu
http://www.mycored.eu/d/3/Contacts/
Antonio F. Logrieco - CNR ISPA - antonio.logrieco@ispa.cnr.it
Nunzia M. Cito - CNR ISPA - nunzia.cito@ispa.cnr.it

Related information

Result In Brief  Limiting fungal contamination of food
Documents and Publications  final1-mycored-publishable-summary.pdf

Reported by

CONSIGLIO NAZIONALE DELLE RICERCHE
Italy
See on map

Subjects

Food

Last updated on 2015-02-05
Retrieved on 2019-08-21

© European Union, 2019