MYCOMON

Monitoring systems for mycotoxin contamination

Spadaro D.[1], Dehne H.W.[2], Gullino M.L.[1], Lorè A.[1], Kostov I.[3], Minuto G.[3*] Schlang N.[2], Tinivella F.[3], Trentin R.[3], Garibaldi A.[1]

[1] Centro di Competenza per l'innovazione in campo agro-ambientale (Agroinnova), Università di Torino, Via Leonardo da Vinci 44, 10095 Grugliasco (TO) - ITALY
[2] Rheinische Friedrich-Wilhelms-University, Institute of Crop Science and Resource Conservation, Nussallee 9, D-53115 Bonn, Germany
[3] District Governors Office, 6 Dunavska str, BG-3700 Vidin
[4] Centro Regionale di Sperimentazione e Assistenza Agricola, Regione Rollo 98, 17031 Albenga (SV) – ITALY

*** Corresponding author**
Phone number: +390182554949
Fax number: +390182554949
Email: cersaa.direzione@sv.camcom.it

1. Introduction

Grapevine is susceptible to attacks of different fungi deriving from soil, plant material or store room contamination that may produce mycotoxins, secondary metabolites that are human carcinogens or potential human carcinogens. The presence on grape of mycotoxigenic fungi may eventually result in wine contamination causing a variety of subacute health problems to consumers (Sweeney *et al*., 2000).
For instance, different plant pathogenic fungi, among them *Aspergillus carbonarius,* which is highly mycotoxigenic, produce ochratoxin A (OTA). OTA is a strong nephrotoxin for many animal species, causing kidney disfunctions and alterations of the urinary system as well as appearance of carcinomas (Creppy, 1999).
On grape *A. carbonarius* can colonize bunches very early during the growth stage, often starting before veraison. OTA can easily transfer from grape to wine if proper monitoring systems and prevention/control measures are not established both during cultivation and vinification. It has been proved that both incidence and concentration of the toxin are higher in wines from lower latitude regions and they increase passing from white to rosè to red wines (Battilani and Pietri, 2002).
Some field practices such as the control of certain pests (e.g. powdery mildew and grape moths) or early picking of grapes can play an important role in the development of mycotoxigenic fungi in the vineyards and, therefore, in the subsequent mycotoxin content detected in wines.
It has been observed that, during vinification, the initial content of mycotoxins may vary depending on the different stages. Specifically, it decreases gradually throughout alcoholic fermentation, racking and malolactic fermentation, after which it represents about 1/10 of the starting content value. Therefore a correct management of the vinification process can be of crucial importance in the production of a mycotoxin free wine (Battilani et al., 2003).
The wine chain is an important economical resource for many European regions. Besides producing high quality wines responding to the consumer requests, the wine producers and the wine industry must guarantee for their survival and to keep or increase the current sales and occupational levels, the absence or a low level of ochratoxin A (OTA).
Liguria in Italy, North Rhine-Westphalia in Germany and Vidin in Bulgaria are three wine-producing regions:

- Liguria is specialized in production of Rossese, Ormeasco, Vermentino, Pigato and Sciacchetrà wines, most of them are high quality wines not very well known outside Italy;
- Vidin is situated in the most North-Western part of Bulgaria and is placed on the Danube. Since the ages of the Venetian tradesmen the region has been well-known for its local red wine Gamza, and since the middle of XX century also for its Merlot and Cabernet Sauvignon.

The Regulation (CE) N. 123/2005 fixes the maximum tolerable level of OTA in wines and other grape derivatives to 2,0 μg kg^{-1}, imposing to every European wine region the adoption of effective control strategies in case of OTA contamination potential
Wine represents an emerging production and market sectors even in emerging and developing economies. Wine exports from third producing countries to Europe are increasing (Pantini, 2006). A number of novel methodologies and products for detection and control of mycotoxin contamination in wine are available. Together with more traditional methods such as High Pression Liquid Cromatography, new easy-to-handle methods have been introduced into the market which allow a rapid and accurate detection of OTA in different food preparations. It must be stressed that an efficient control of

mycotoxigenic fungi should be pursued already during cultivation phases through an accurate management of crop viarables and the adoption of control strategies towards mycotoxigenic fungi based on the use of specifical pesticides (Battilani and Pietri 2002).

The management of fungal diseases is one of the main points for a successful cultivation of cereals. Most of the cereal pathogenic fungi only have an influence on the yield quantity. But some of the species are also able to produce mycotoxins. These mycotoxigenic fungi are a big problem for the production of healthy food. Most of the cereal pathogenic fungi which are able to produce mycotoxins belong to the groups *Fusarium*, *Aspergillus* or *Penicillium*. However the most important genus of mycotoxigenic fungi related to cereals is *Fusarium*. Species of this genus occur all over Europe and can cause the *Fusarium* head blight (FHB) disease of small-grain cereals. The most important *Fusarium* species for Europe are *Fusarium graminearum*, *F. culmorum*, *F. poae* and *F. avenaceum*. They are able to produce a broad range of mycotoxins (Table 1)

Table 1: Most important mycotoxigenic *Fusarium* species
(Bottalico and Perrone, 2002, modified)

Species	Mycotoxin
F. graminearum	DON, NIV, ZEA, Ac-DON, FUS
F. culmorum	DON, ZEA, ZOL, NIV
F. poae	NIV, BEA, DAS, FUS, ENS
F. avenaceum	MON, BEA, ENS

Ac-DON: Monoacetyl-deoxynivalnols (3-Ac-DON, 15-Ac-DON); BEA: Beauvericin; DAS: Diacetoxyscirpenol; DON: Deoxynivalenol; ENS: Enniatins; FUS: Fusarenone X (4-Acetyl-NIV); MON: Moniliformin; NIV: Nivalenol; ZEA: Zearalenone; ZOL: Zearalenols

These mycotoxins are a severe risk for human and animal health because of their acute and immunotoxic potential (Deoxynivalenol and other trichothecenes) or their hormone-like effect and genotoxic potential (Zearalenone).
The regulation (EC) No. 1126/2007 fixes the maximum tolerable level of Deoxynivalnol to 1250 µg/kg in unprocessed cereals and 500 µg/kg in processed cereals. The maximum tolerable level of Zearalenon is 100 µg/kg in unprocessed cereals and 75 µg/kg in processed cereals.
The production of mycotoxins cannot be influenced directly and effectively by agronomic actions or use of fungicide. The management of FHB is the key factor to reduce the mycotoxin contamination of cereals. For an effective FHB management it is crucial to know which species are involved in the disease in a particular region because the species are dispersed either by rain splashes or wind which has an impact on the diseases management. To learn more about the species complex of FHB and to deduce recommendations for the disease management wheat samples from Liguria and North Rhine-Westphalia were examined for their infection level and species complex.

2. Materials and methods

2.1 Monitoring of mycotoxigenic fungi

At "Centro Regionale di Sperimentazione e Assistenza Agricola" (CERSAA) trials were carried out in experimental vineyards in order to evaluate the influence of some crop management practices on the development of mycotoxigenic fungi (particularly *Aspergillus* sp.). For this purpose 6 different vine cultivars typically grown in Liguria Region (Ormeasco, Rossese, Vermentino, Vermentino 84, Lumassina and Pigato) were taken in consideration. Three different watering volumes were adopted (no irrigation, 10 mm of water/week, 2x10mm of water/week) through a drip irrigation system. Two different disease management techniques were considered: one foreseeing the use of cyprodynil + fludioxonyl based pesticide (Switch, Syngenta) twice at 800 g/ha and the other one not foreseeing the use of this compound. Vineyard was managed through common practices adopted in Liguria Region.

Four surveys were carried out in 2006, three in 2007. Ten bunches were collected for each replication and treatment, 5 berries per bunch were picked and washed in 500 ml sterile water added with Tween 20. 100 µl of water was plated on selective and semi-selective media (Pollastro, 2006; see annex 1). 3 dilutions were carried out and 3 plates per each dilution were prepared. Plates were incubated for 3 days at 25-27 °C. Number of colony forming unit (CFU) were counted in relation to average berry surface (CFU/mm^2).

A simplified wine making process was carried out according to annex 2. Development of mycotoxigenic fungi was assessed on fresh grape juices plating 100 µl coming from 3 different juice dilutions (10^{-1}, 10^{-2}, 10^{-3}) on the media described in annex 1.

2.2 Statistical analysis

Data collected were first analysed through Kruskal-Wallis test assuming as cluster factors: variety, irrigation and pesticide application. If test showed significant differences among groups ($P< 0,05$), data clustered according to omogenous factors were processed by the analysis of variance and, in the case of significant differences ($P< 0,05$), average values were compared through Tukey's test (varieties) and through T test (pesticide application).

2. Monitoring of OTA in the wines produced from the experimental trial

Fresh must (50g) was mixed with 50 ml of methanol and 5 ml of 0.1 M orthophosphoric acid in a blender for 2 minutes. The mixture was filtered through a glass microfibre filter and the filtrate collected in a graduated cylinder. An aliquot (12.5 ml) of the filtrate was diluted to 100 ml with a solution of polyethyleneglycol and sodium bicarbonate, and 10 ml of the diluted extract was passed through the Immunoaffinity column. OTA was eluted with 2 ml of methanol, completely evaporated with a gentle stream of nitrogen and redissolved in 0.5 ml of mobile phase.

The wine (10 ml) was diluted with 10 ml of a solution of polyethyleneglycol and sodium bicarbonate. The mixture was filtered through a glass microfibre filter and the filtrate was passed through the Immunoaffinity column. OTA was eluted with 3*0.75 ml of methanol, completely evaporated with a gentle stream of nitrogen and redissolved in 0.5 ml of mobile phase.

The lees and the skins were dried for 12 h at 70°C. Five grams of dried sample were extracted with 30 ml of acetonitrile and water (60:40 v/v) by shaking for 60 minutes. After filtration, 6 ml of filtrate were mixed with 44 ml of a water solution of polyethyleneglycol and sodium bicarbonate. The mixture was filtered through a glass microfibre filter and the filtrate was passed through the Immunoaffinity column. OTA was eluted with 3*0.75 ml

methanol, completely evaporated with a gentle stream of nitrogen and redissolved in 0.5 ml of mobile phase.

2.4 Assessment of ochratoxin A (OTA) content in commercial wines

At the Centre of Competence for the Innovation in the Agro-environmental Sector (Agroinnova) 217 samples of wines coming from Liguria Region (years 2003-2004-2005-2006), 77 samples coming from all around Italy and 11 samples coming from Vidin Region, Bulgaria, were analyzed. Analysis procedure was validated according to the existing legislation (EC 2002/26) and some parameters were evaluated in order to confirm the reliability of the procedure. At the same time OTA content was determined on different substrates (wines, skins, fresh musts, lees) coming from the experimental vineyards sited at CERSAA.

The wine (10 ml) was diluted with 10 ml of a solution of polyethyleneglycol and sodium bicarbonate. The mixture was filtered through a glass microfibre filter and the filtrate was passed through the Immunoaffinity column. OTA was eluted with 3*0.75 ml of methanol, completely evaporated with a gentle stream of nitrogen and redissolved in 0.5 ml of mobile phase.

2.5 HPLC

Samples were analyzed in a HPLC Agilent series 1100 formed by a degasser, an autosampler, a quaternary pump, a thermostated column and a fluorimeter. An analytical column RP-18 (150 mm x 4.6 mm i.d., 5 µm) with a pre-column was used. The mobile phase, eluting at 1 ml min-1, consisted of an isocratic mixture of acetonitril:water:acetic acid (45:45:10) for 18 min. 100 µl of sample were injected onto the HPLC column and the retention time of OTA was 6.15 min.

The amount of OTA in the final solution was determined by using a calibration graph of concentration versus peak area and expressed as ng/ml, achieved by injection onto the HPLC column of 100 µl of standard solutions of OTA (Sigma Chemical Co.). The standard solutions had concentrations of 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 µl l^{-1}.

2.6 Monitoring of mycotoxigenic fungi in wheat

Thirty-two wheat samples were examined in 2006 (3 from Italy and 29 from Germany). The German samples were taken from different farming systems. 6 from naturally formed soil and 8 from virgin soil (recultivation after open pit mining). Another 15 samples were taken from one single wheat field to look for the in-field spatial distribution of *Fusarium* spp. and mycotoxins.

Several wheat cultivars were used in this examination (Table 2).

Table 2: Wheat cultivars used in experiments

Location	Soil properties	Cultivar
Italy	Naturally formed soil	Soissons (3)
Germany	Naturally formed soil	Dekan Hattrick (2) Tommi Tuareg Winnetou
	Virgin Soil	Dekan (4) Drifter (4)
	Field with 15 samples	Dekan

Two hundreds kernels of each sample (4 x 50 kernels) were bedded on *Fusarium* selective Czapex-dox-iprodione-dicloran-agar (CZID). The Petri dishes were incubated at 25°C for 5 days. Outgrown mycelium was transferred to Petri dishes with potato-dextrose-agar (PDA) and carnation-leaf-agar (CLA). After another period of growth of 14 days the isolates were identified morphologically according to the method of Leslie and Summerell (2006).
The DNA extraction for the molecular identification was done with the "Wizard Magnetic DNA Purification Kit for Food" (Promega GmbH, Mannheim). 20 mg of ground wheat kernels of each sample were used as starting material. Primers Fp82F (CAAGCAAACAGGCTCTTCACC) and Fp82R (TGTTCCACCTCAGTGACAGGTT) were used to detect *F. poae* (Parry and Nicholson, 1996). All PCRs were done in a volume of 30 µl (2 µl of DNA solution, 0.6 µM of each primer, 80 µM dNTPs). PCRs were carried out using the following protocol: 2 min initial denaturation at 94 °C, 40 cycles of 1 min at 94 °C, 30 s at 60 °C, 1 min at 72 °C and a 10 min final extension at 72 °C.

2.7 Mycotoxin assessment
The 15 wheat samples taken from one field in Germany were examined for mycotoxin contamination with a multimethod based on a LC-MS/MS method . It is possible to detect and quantify over 60 mycotoxins in a single run with this method (Spiteller et al., unpublished). The mycotoxin assessment was done at the Institute of Environmental Research at the University of Dortmund (Workgroup M. Spiteller).

3. Results and Discussion

3.1 Monitoring of mycotoxigenic fungi

Year 2006

Berries result contaminated by *Aspergillus* sp. already at first survey (31/7/06) with higher values (CFU/mm^2) recorded on white berries (Pigato, Vermentino, Vermentino 84 and Lumassina varieties). Contamination tends to increase during time (Figure 1). The application of cyprodinil+fludioxonyl based fungicide, a compound which is also registered for grey mould control, caused a significant reduction of the development of mycotoxigenic fungi (Figure 2). Effect of watering volumes was not significance and this fact, at least in 2006, is due to the heavy rains occurred during the trials and closed to the

surveys. Contamination level by Aspergilli assessed in fresh grape juices tend to become similar in the ones obtained from white as in red berries (Fig. 3); this behavoiur is more clearly observed on MEAB selective medium.

Fig. 1 – *Aspergillus contamination assessed in experimental vineyards during 3 subsequent surveys and expressed as CFU/mm^2 of berry surface (Albenga, 2006). Values expressed by bars marked with the same letter do not statistically differ according to Tukey's test (P=0,05) within the same survey. No letters stands for absence of significance.*

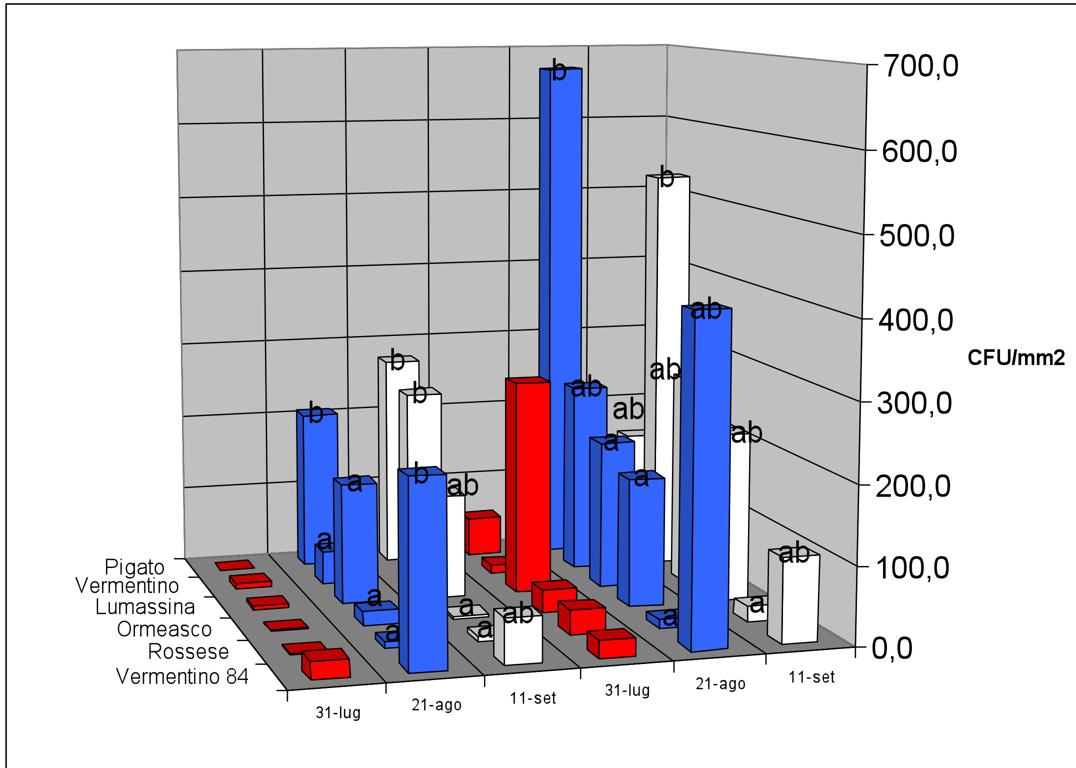


Fig. 2 – *Effect deriving from the application of Switch (a.i. cyprodinil+fludioxonyl, Syngenta; 2 treatments at 800 g/ha) on the development of Aspergilli assessed on MEA and MEAB media and expressed as CFU/mm^2 of berry surface (Albenga, 2006). Data about 6 varieties. Differences between treated and non treated (control) are significant according to T test.*

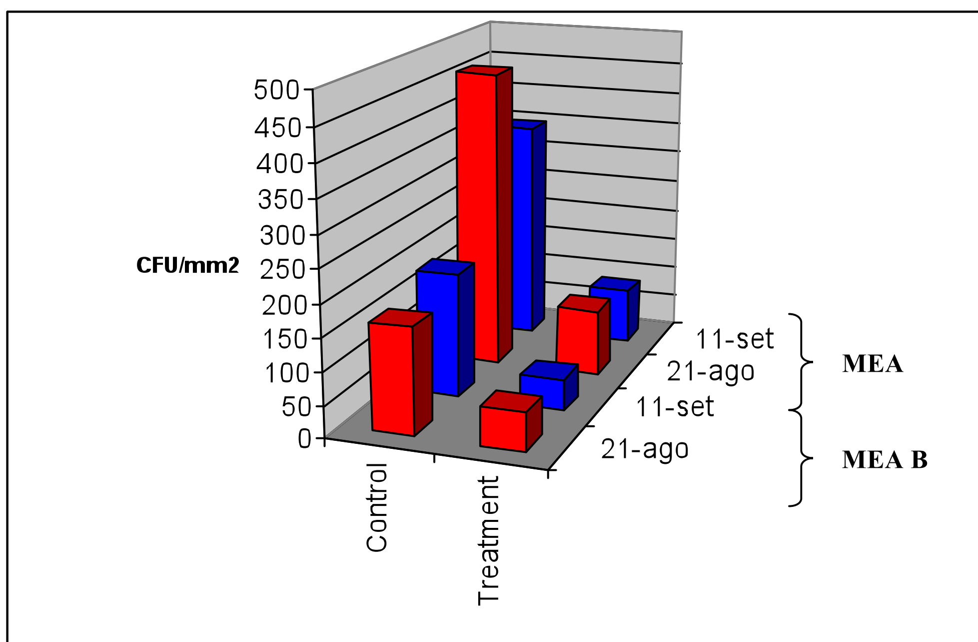
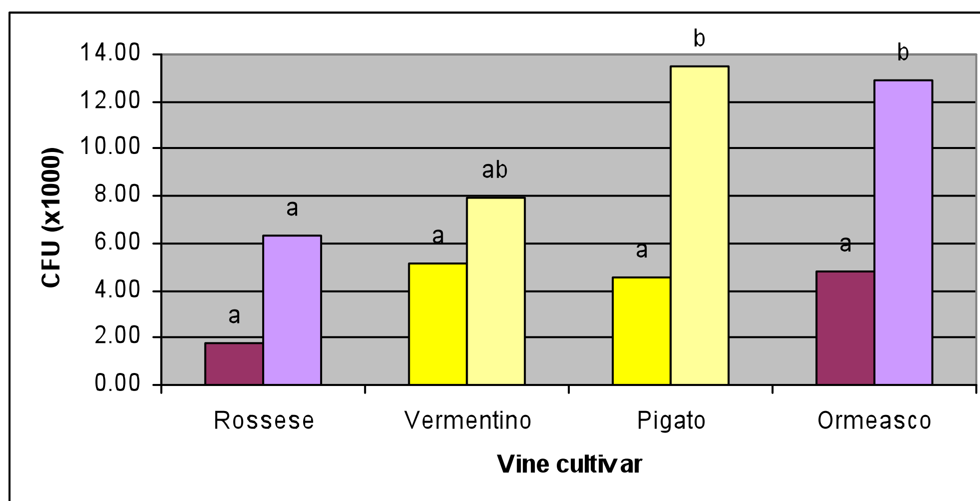


Fig. 3 – *Contamination by Aspergilli in fresh grape juices obtained by different varieties (Albenga, 2006). Values expressed by bars marked with the same letter do not statistically differ according to Tukey's test (P=0,05).*



Year 2007

Contamination of berries by *Aspergillus* sp. in the vineyards were lower than in 2006 and this was due to the less conductive climate conditions registered. Berries of Lumassina variety (white) were the only ones on which *Aspergillus* sp. could be isolated (Figure 4). Application of cyprodinil+fludioxonyl based fungicide confirmed the positive results obtained in 2006 significantly reducing the contamination caused by *Aspergillus* sp. (Figure 5). Relevant to fresh grape juices a higher level of contamination than in 2006 was recorded with particular regards to Rossese, Vermentino and Ormeasco varieties (Figure 6). Neither in 2007 a significant effect caused by different irrigation volumes could be observed.

Fig. 4 – *Aspergillus contamination assessed in experimental vineyards in 2 subsequent surveys and expressed as CFU/mm^{2} of berry surface (Albenga, 2007). Values expressed by bars marked with the same letter do not differ statistically according to Tukey's test (P=0,05) within the same survey No letters stands for absence of significance.*

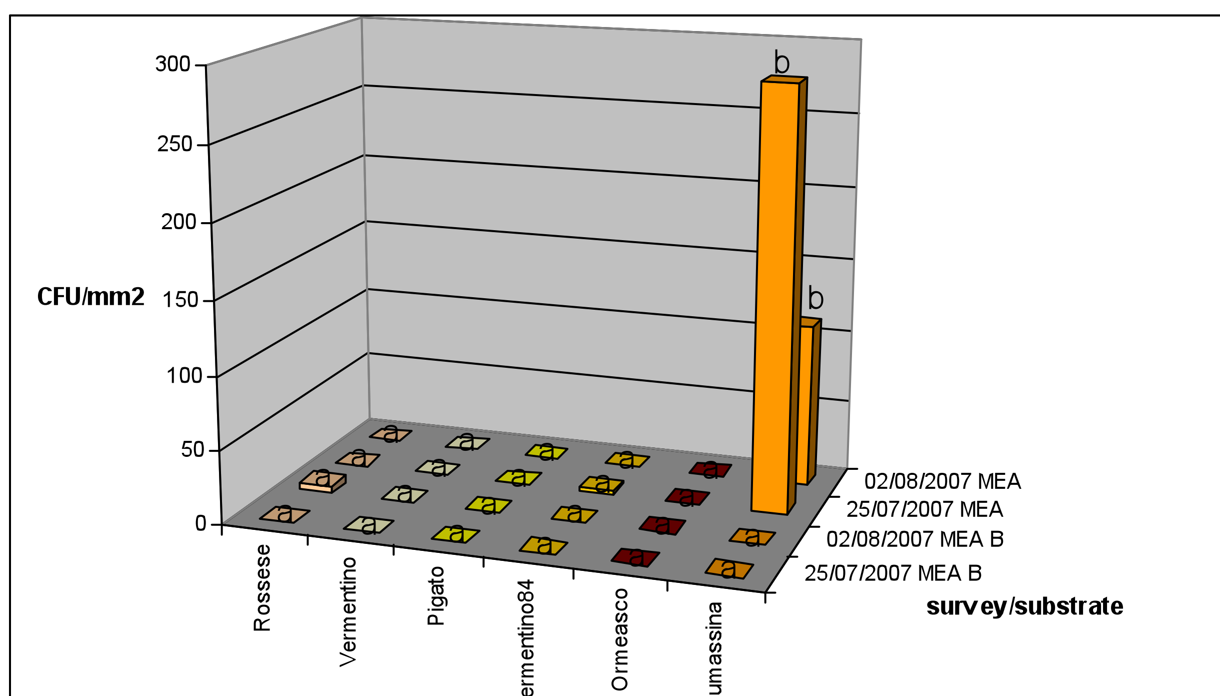


Fig. 5 – *Effect deriving from the application of Switch (a.i. cyprodinil+fludioxonyl, Syngenta; 2 treatments at 800 g/ha) on the development of Aspergilli assessed on MEA and MEAB media and expressed as CFU/mm^2 of berry surface (Albenga, 2007). Data about 6 varieties. Differences between treated and non treated (no Switch – Switch) are significant according to T test.*

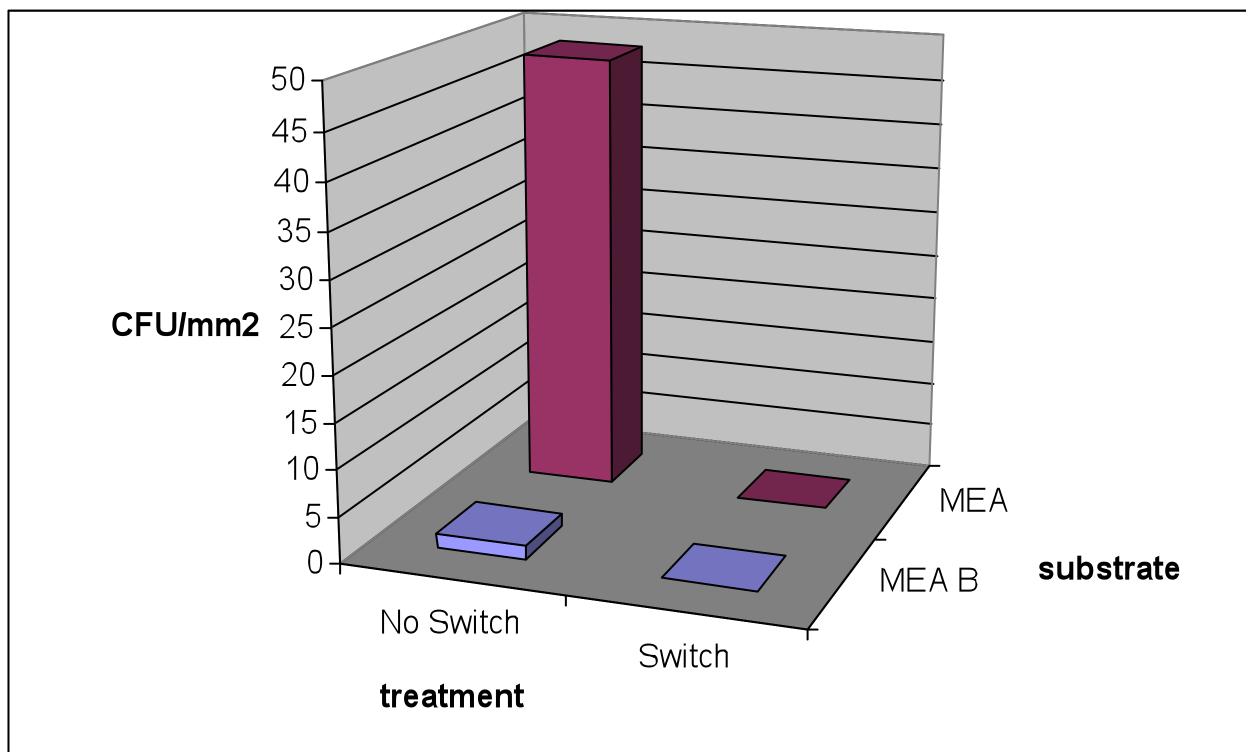
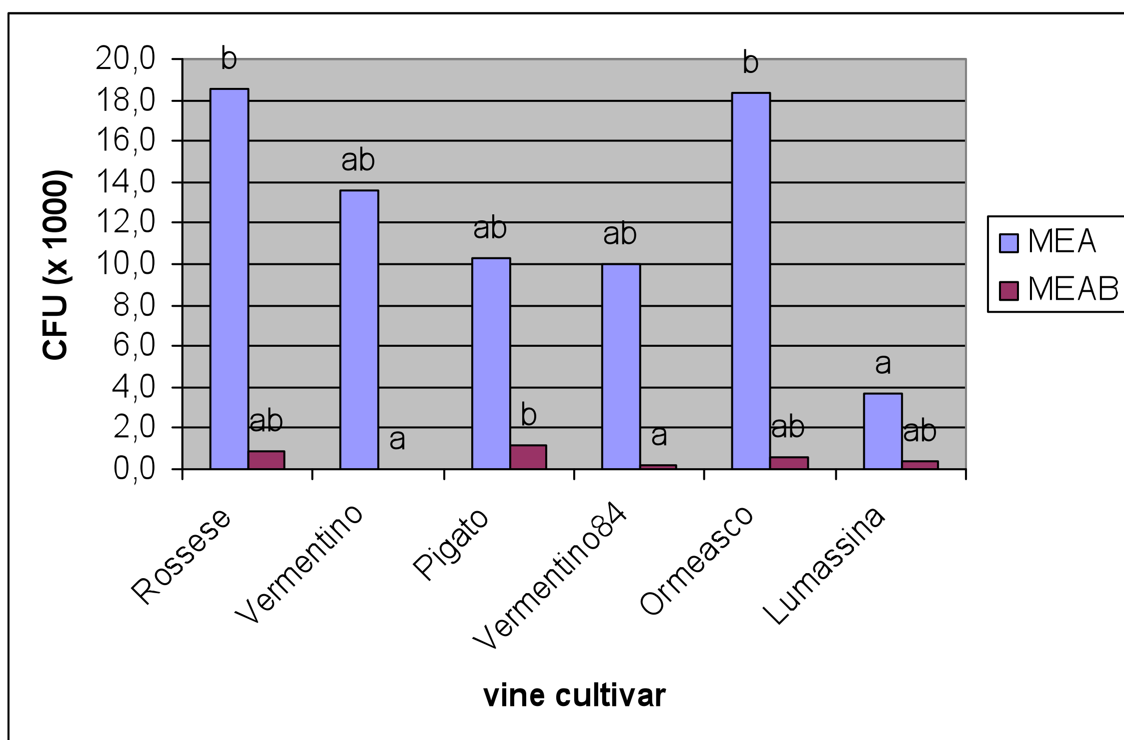


Fig. 6 – *Contamination by Aspergilli in fresh grape juices obtained by different varieties (Albenga, 2007). Values expressed by bars marked with the same letter do not differ statistically according to Tukey's test (P=0,05).*



3.2 Monitoring of OTA in the wines produced from the experimental trial

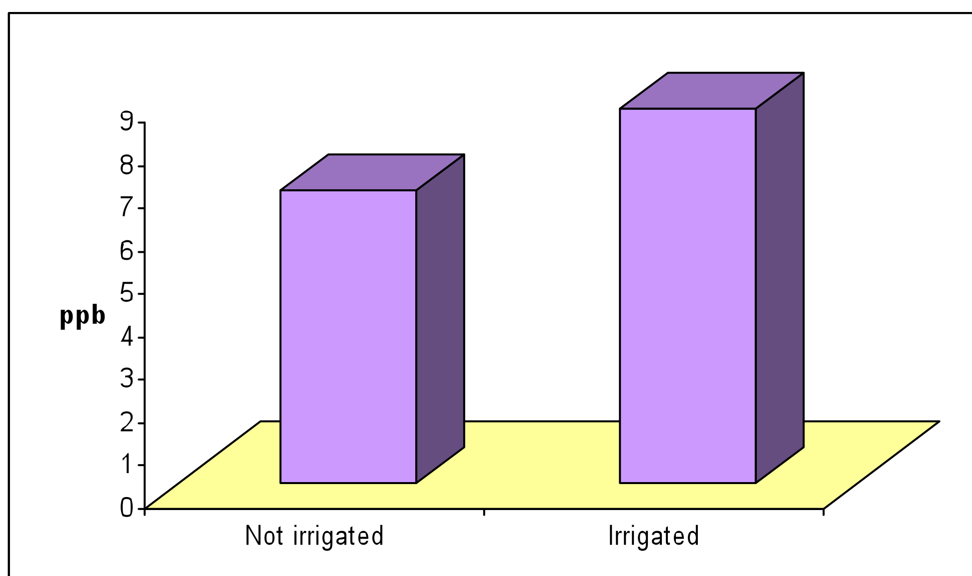
The analysis carried out on samples coming from the wines produced from the experimental trials showed that chemical treatments with cyprodinil and fludioxonil, have a strong effecton the reduction of the *Aspergillus* spp. growth, and in particular on the development of *Aspergillus carbonarius*, causing also a reduction of the OTA in grapes and in the derived products (Figure 7).

Fig. 7 – *Contamination by ochratoxin A (OTA) assessed in different matrixes obtained during wine making process carried out in Albenga (2006) and depending on the application of the a.i. cyprodinil+fludioxonyl (treated – not treated).*

	Variety	*Must*	*Wine*	*Lees*	*Skins*
Not treated	Vermentino	7.68	10.67	17.14	-
	Pigato	3.07	8.45	12.18	-
	Rossese	5.87	8.81	36.60	36.15
	Ormeasco	1.77	-	2.5	14.4
Treated	Vermentino	0.70	1.14	-	-
	Pigato	1.50	2.98	-	-
	Rossese	0.87	1.78	8.46	8.35
	Ormeasco	0.85	-	1.55	6.00

From the table, lees, together with skins (for red wines), appear to be the substrate where OTA tend to accumulate during the wine making process. Yeast and bacteria involved in the wine making process are normally able to adsorb OTA, so that with sedimentation and lees elimination, OTA content is significantly reduced.
Considering the effect of the level of irrigation on the OTA production (Figure 8), the limitation of is a positive factor in order to contain the OTA production, but in our experiments, due to adverse climatic conditions, it is difficult to evaluate this parameter. From the analysed samples, the not irrigated samples have an OTA concentration lower (6.810 $\mu g\ Kg^{-1}$) than the irrigated ones (8.7221 $\mu g\ Kg^{-1}$).

Fig. 8- Mean concentration of OTA in the irrigated and not irrigated samples.



3.3 Assessment of ochratoxin a (OTA) content in commercial wines

OTA content in Italian and Bulgarian wines as well as in the wines collected in Liguria was lower than the threshold established by law with the exception of a very few percentage of Southern Italian red wines correspondent to a percentage of 1% of overall samples which showed an OTA concentration higher than 2 ppb. No significant differences in OTA content between red and white wines produced in Liguria region were observed.
Considering the 217 Ligurian wines analysed, 76% of the samples had detectable levels of OTA, although the mean concentration was low (0.079 ppb). The most contaminated varieties were two red ones: Ciliegiolo, followed by Sangiovese.
Considering the area of production, the wines produced in the province of La Spezia generally had higher levels of OTA, followed by the wines produced in the province of Savona.
Concerning the comparison between red and white wines, the last ones present a slightly higher but not significantly different level of contamination.
Looking finally at the year of production (2003-2004-2005-2006), the wines produced after harvesting 2006 and 2003 have higher OTA levels (Figure 9).

Fig. 9 – Mean concentration of OTA in wines produced in the years 203, 2004, 2005 and 2006.

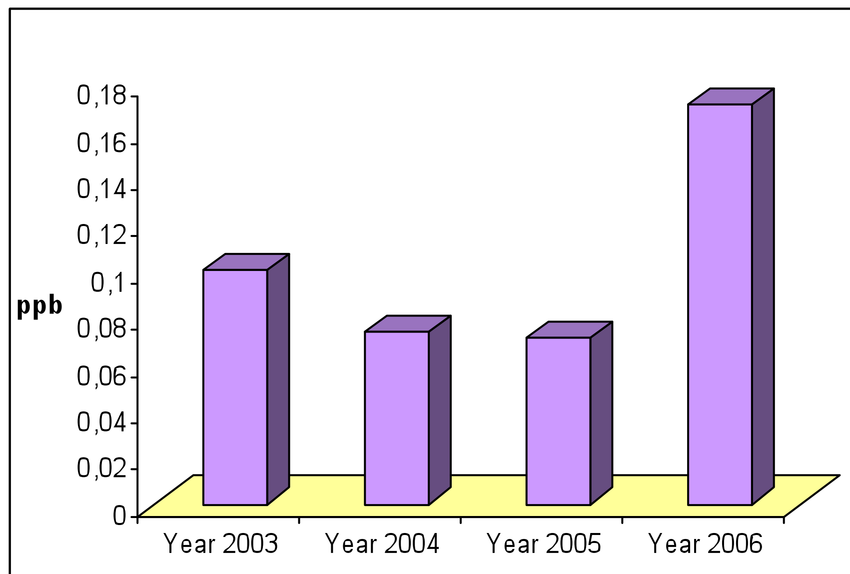
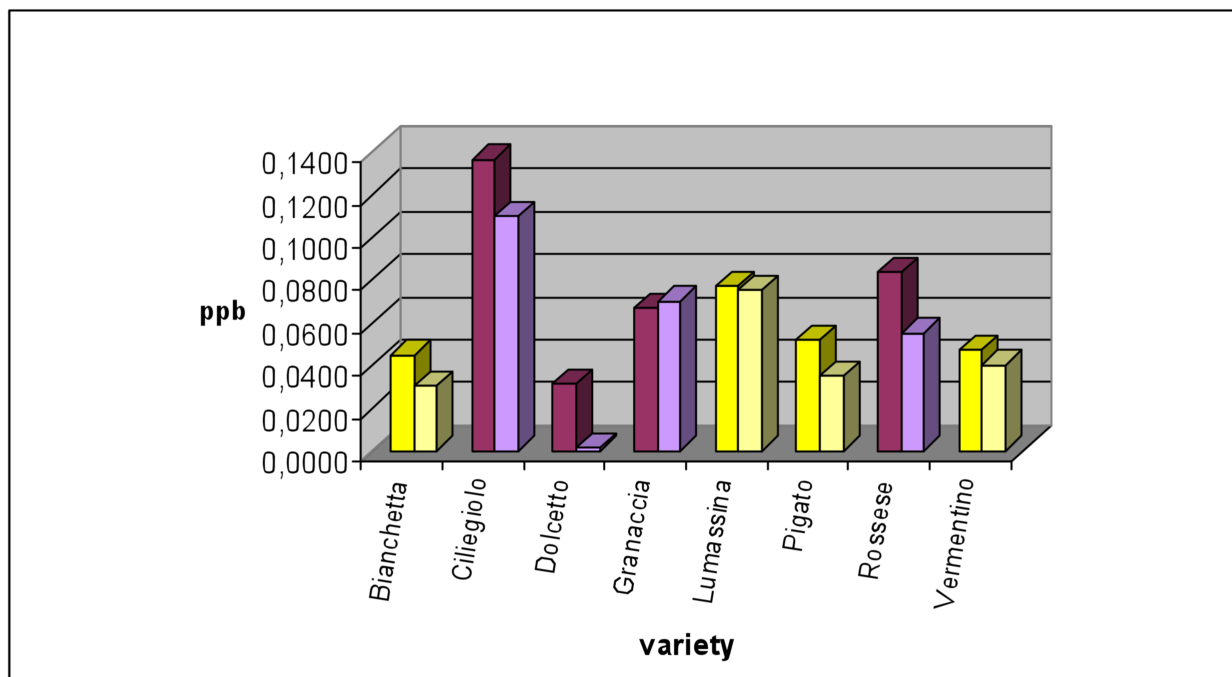


Fig. 10 – *Contamination by ochratoxin A (OTA) assessed in different wines obtained from varieties typically grown in Liguria Region.*



Analysing the results of the wines coming from different Italian regions, most of the samples are contaminated by OTA, but rarely above the threshold impodsed by the European Regulation 123/2005. Only few samples of wines produced in Southern Italy (Nero d'Avola or Cirò) had high mean concentrations, sometimes superior to 2 ppb.
The samples analysed present a contamination level and incidence lower in the white than in the red wines and with a decreasing value from 2003 to 2005.

Fig.11 – Comparison of the mean concentration of OTA in wines produced in Northern, Central and Southern Italy.

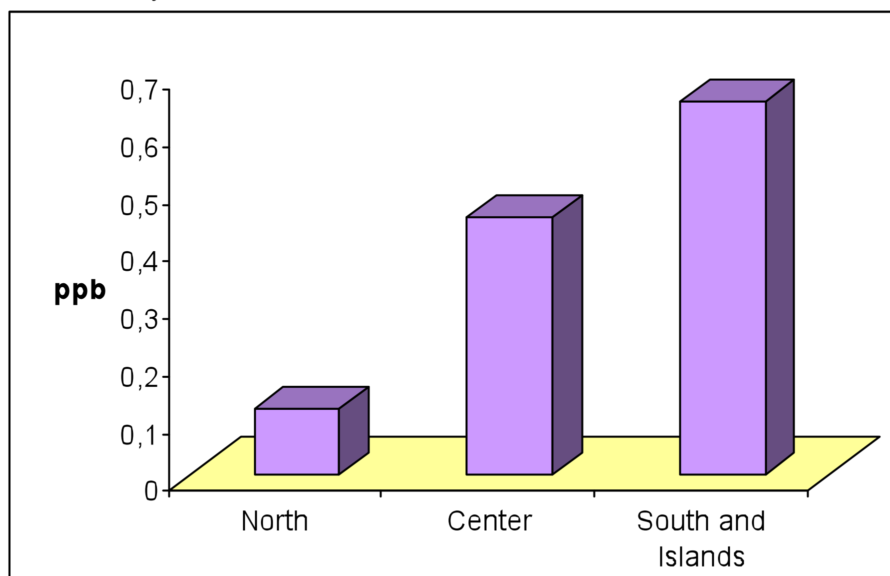


Fig. 12 – *OTA levels in different wines produced in Bulgaria*

WINE	YEAR	COLOUR	OTA ppb
Gamza	2006	red	0.053
Cabernet Sauvignon	2004	red	0.038
Cabernet Sauvignon	2006	red	0.044
Merlot	2006	red	0.054
Cabernet Sauvignon	2005	red	0.057
Gamza	2006	red	0.023
Merlot	2006	red	0.057
Merlot	2006	red	n.d.*
Merlot	2004	red	0.038
Cabernet Sauvignon	2006	red	n.d.
Gamza	2004	red	n.d.

*** n.d. not possible to be determined**

3.4 Wheat samples from naturally formed soil, Germany

Six wheat samples were taken from conventional farms in North Rhine-Westphalia. The infection level ranged between 4% and 11,5% (Fig. 13). *Fusarium avenaceum* and *F. proliferatum* were found in all samples, whereas *F. poae* and *F. graminearum* were only found at low levels in 3 and 4 samples, respectively. There was a co-occurrence of *F. poae* and *F. verticillioides* in 3 samples and neither *F. poae* nor *F. verticillioides* were found in one sample singly.

The early summer of 2006 in Western Germany was relatively dry. Only low and short rainfalls were recorded. The climatic conditions at the stage of wheat flowering were not optimal for infection by *Fusarium* species. The late summer was very hot and dry, no rainfall was recorded in last three weeks before harvest. The dryness in the early summer and at stage of flowering resulted in a low incidence of *Fusarium* head blight. *F. graminearum* and *F. culmorum*, which are normally the predominant species in Western Germany, were only found at a very low incidence. Other species, which are normally typical for maize, like *F. proliferatum*, were found more frequently. It is likely that the maize *Fusarium* species are better adapted to higher temperatures than the wheat *Fusarium* species. The climatic conditions of the

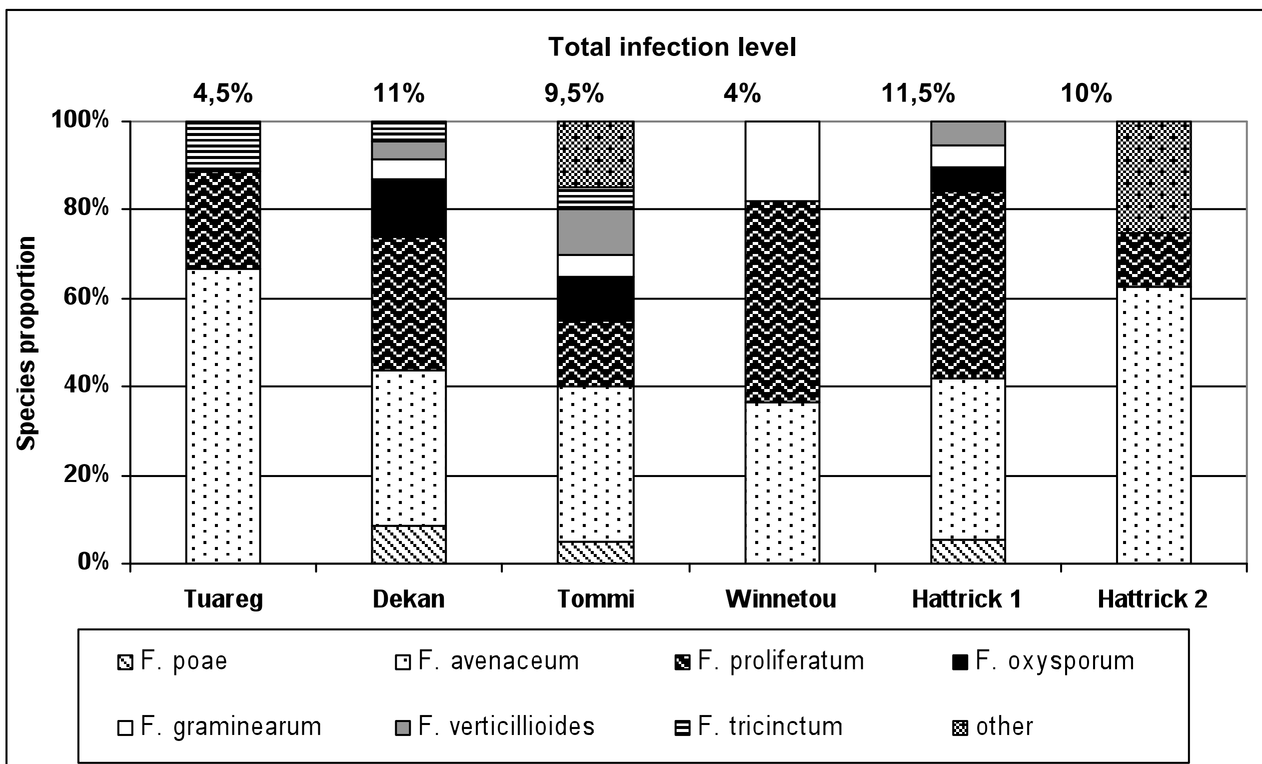


Fig 13: Level of *Fusarium* infection and species complex, 6 samples from naturally formed soil, Germany

summer 2006 in Germany would then have favoured the maize *Fusarium* species compared to the wheat *Fusarium* species. This may be an explanation for the relative low incidence of *F. graminearum* and *F. culmorum* in North Rhine-Westphalia in 2006. In comparison to *F. graminerum* and *F. culmorum*, *F. avenaceum* was found more frequently

in this samples. *F. avenaceum* is known to be an important species in North Rhine-Westphalia. Good adaptation of this species and a wide dispersion in the region may result in a high inocolum level. This could lead to higher infection levels although the climatic conditions were not optimal for an infection with *Fusarium*.

3.5 Wheat samples from virgin soil, Germany

Eight samples (2 cultivars) were taken from virgin soil. This soil results from recultivation after open put mining. The soil is a nutrient-rich, mineral substrate with a low content of organic matter. The total infection level showed a broad range from 0,5% to 30% (Fig. 14). *F. poae* were present in nearly all samples. *F. avenaceum* and *F. proliferatum* co-occurred in 3 samples and were not found singly. *F. tricinctum* occurred in 6 out of 8 samples but was predominantly found in cultivar Drifter. *F. graminearum* occurred in 3 out of 8 samples and was predominantly found in cultivar Dekan. This could mean that the cultivar Drifter is more susceptible to *F. tricinctum* and the cultivar Dekan to *F. graminearum*. The higher the total infection level was the more species were found in the sample (Dekan 4 and Drifter 1). Overall the incidence of *F. graminearum* was relatively low. For the low incidence of *F. graminearum* the same explanation should be valid like in the above-named samples.

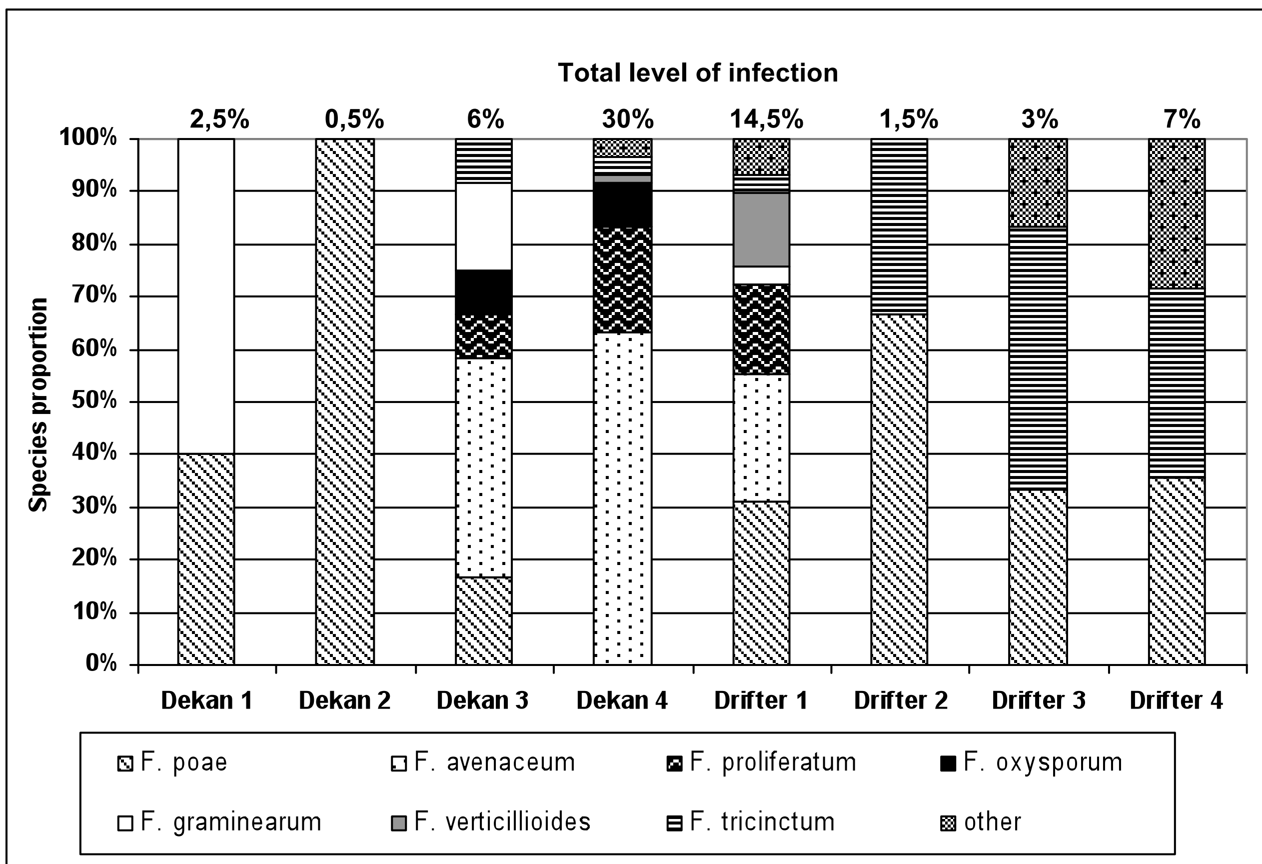


Fig. 14: Level of *Fusarium* infection and species complex, 6 samples from virgin soil, Germany

Comparing the species complex of the wheat samples from naturally formed and from virgin soil one can see that the complex consist of nearly the same species. Only the proportion of the species changes. *F. avenaceum* and *F. proliferatum* were predominant in the samples from naturally formed soil whereas *F. poae* occurred most often in the samples from virgin soil. After recultivation the substrate of the virgin soil has an organic matter content of nearly zero. Also the rate of soilborne pathogens is close to zero. Infections of plant pathogens have to establish first. *F. avenaceum* and *F. proliferatum* are soilborne plant pathogens whereas *F. poae* is airborne. It is likely that the climatic conditions are more favourable for the former species but the spores were not dispersed over the fields. The spores of *F. poae* may have been distributed much more faster by wind and spread over larger areas. This could be an explanation for the predominance of *F. poae* on virgin soils.

3.6 Wheat samples from Italy

Three wheat samples from Italy were examined for infection with *Fusarium* species and the species complex. Samples were taken from the same cultivar to avoid a inter-cultivar effect. The total infection level was lower than in Germany and ranged between 3% and 4,5% (Fig. 15). *F. proliferatum* and *F. verticillioides*, two typical maize *Fusarium* species, were found in all three and in one sample, respectively. *F. poae* were also found in all samples. *F. culmorum* and *F. graminearum* were not found in any sample.

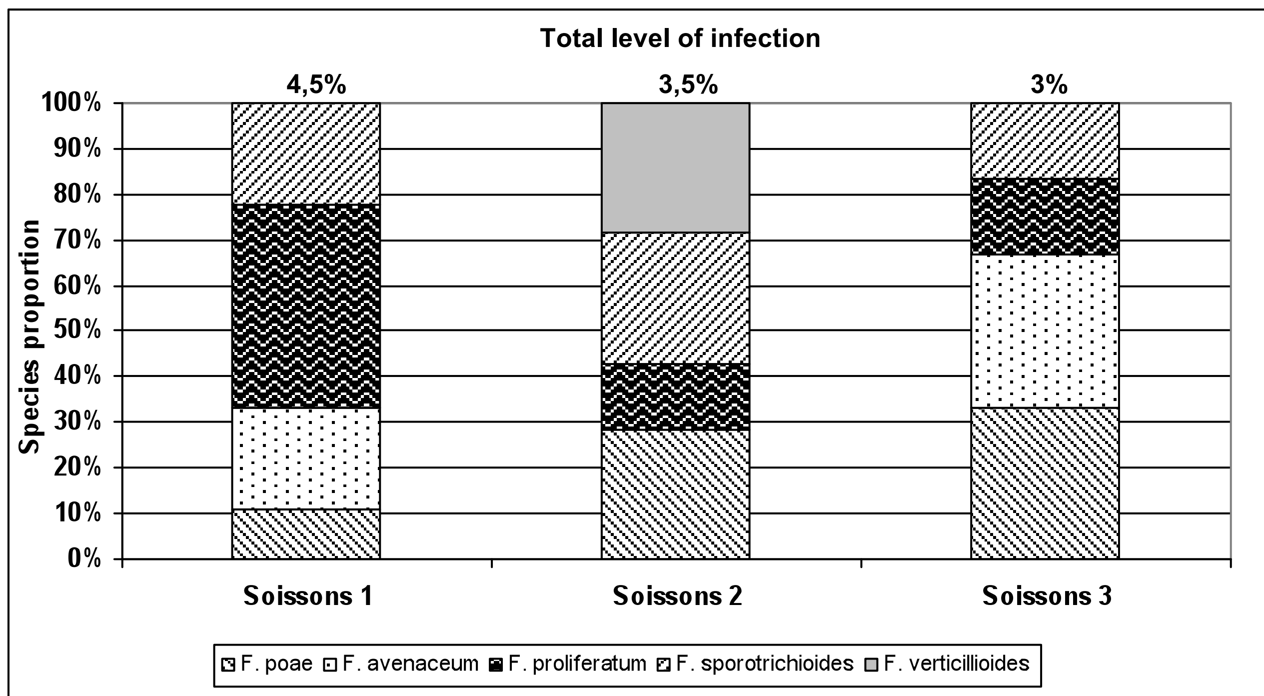


Fig. 15: Level of *Fusarium* infection and species complex, 3 samples from conventional farming, Italy

The climatic conditions in Italy, Region Liguria, are warmer and more humid than in North Rhine-Westphalia. The warmer conditions may favour maize *Fusarium* species, which are better adapted to a warmer climate. The total infection level is low and it is known from the German samples that more species are found if the total infection level is higher. In the Italian samples only a small spectrum of species can be found. This may be a sign for

unfavourable conditions for a *Fusarium* infection. In this case it is likely that only the best adapted species or the species with highest inoculum level will occur.

3.7 Spatial distribution of Fusarium species

Fifteen Samples (cv. Dekan) were taken from one wheat field. The samples were examined for infection with *Fusarium* species, both microbiological and for one species also with a species-specific Polymerase Chain Reaction method (PCR). The wheat fields were sampled in a row (Fig. 16). In most cases an infection with *Fusarium* species were found. The highest incidence was found at sampling point 1 with 19,6 % on the other sampling points the incidence ranged between 1,8 % and 7,1 %. Only five different species were found at the 15 sampling points (Fig. 17). *F. verticillioides* had the highest incidence at a single sampling point (namely 16% at sampling point 1). *F. poae* was the species which occurred in the most cases when *Fusarium* was found. The other 3 species were found infrequently. The infection level was relatively low in this field but it is observable that the same species were found all over the field. The high incidence at sampling point 1 could be explained with the spatial position of the sampling point. The field border characterized by a hedgerow at the south-western edge. The main wind direction in this region is west. The lower wind exposure on this point could lead to a more humid microclimate which is favourable for fungal infections.

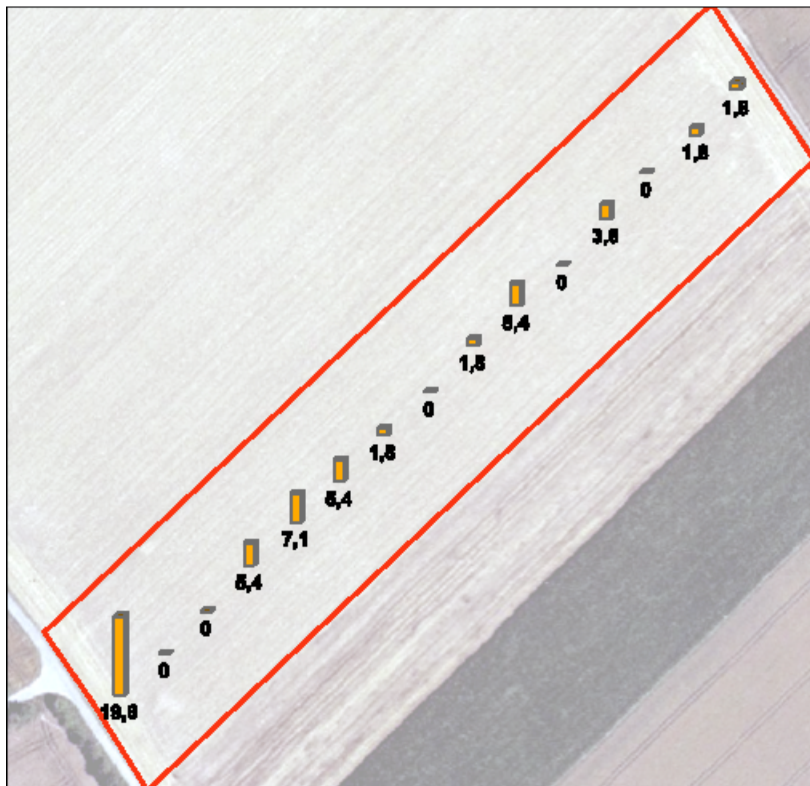


Fig 16: *Fusarium* infection (%) of 15 wheat samples taken from field K6, spatial distribution of the disease

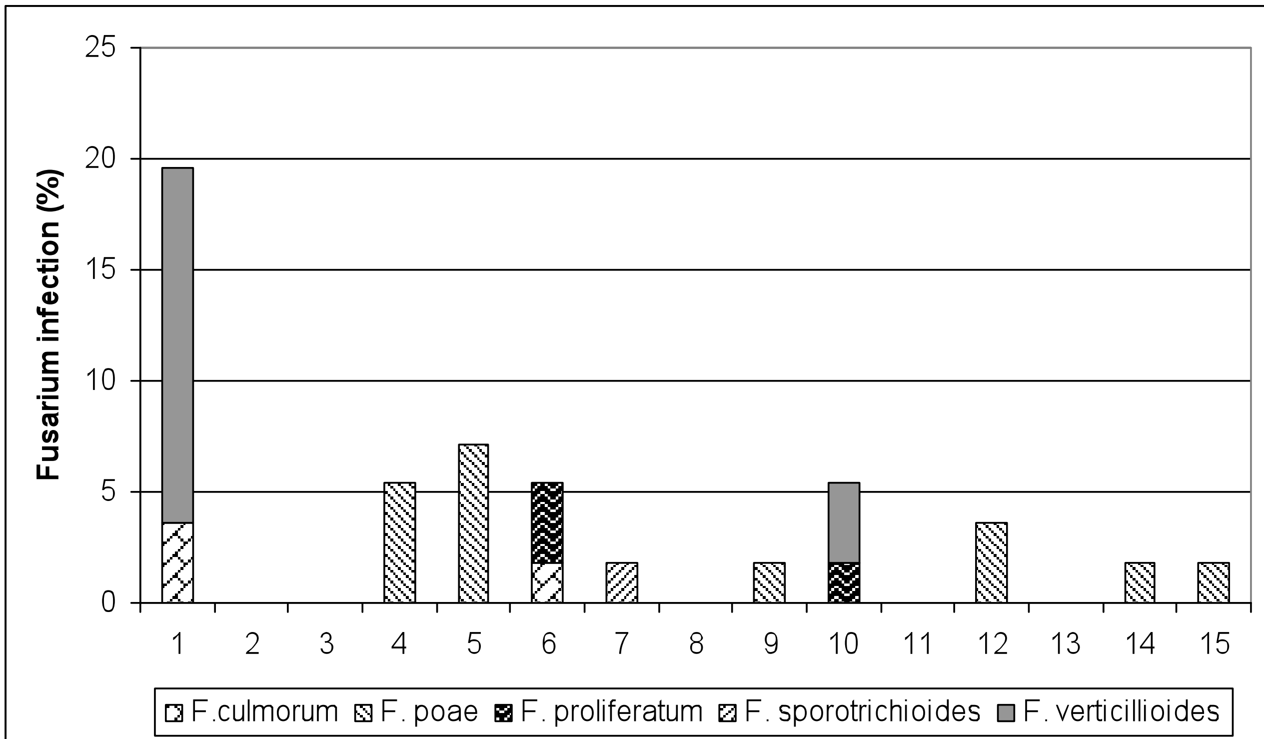


Fig. 17: *Fusarium* infection and species complex of the 15 wheat samples taken from field K6

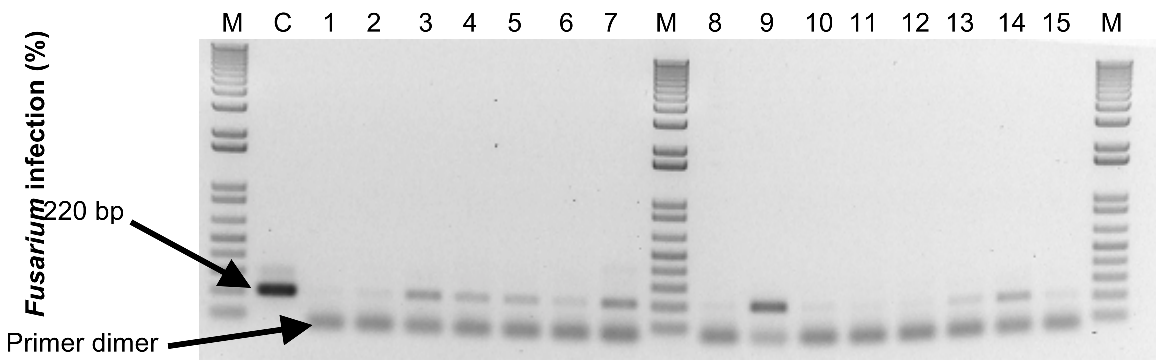


Fig. 18: Species-specific PCR for *Fusarium poae*; M: 1kb molecular marker; C: positive control; 1-15: samples 1 to 15 from field K6

With a DNA based assay, a PCR with species-specific primers for *F. poae*, the pathogen *F. poae* was found at every sampling point (Fig. 18). With a microbiological assay F: poae was only found at sampling point 4, 5, 9, 12, 14 and 15. The last three weeks before harvest dry and warm weather conditions predominated in this region. With a microbiological assay only living organism can be detected. Due to the unfavourable weather conditions it is possible that most of the *Fusarium* species die off in the last weeks before harvest. They could not be detected by microbiological assay but the DNA of the dead organisms still remain in the plant an can be detected with a molecular assay.

Fig. 19: Deoynivalenol content (µg/kg) of 15 wheat samples taken from field K6

3.8 Mycotoxin contamination

The 15 samples taken from field K6 were examined for Mycotoxin contamination with a multi- analysis-method. Three mycotoxins were mainly found: Deoxynivalenol (Fig. 19), Ochratoxin A (Fig. 20) and Enniatin B (Fig. 21).

Deoxynivalenol (DON) were found at every single sampling point (Fig. 19). The contamination ranges from 93,5 µg/kg to 234,5 µg/kg. No sample overran the maximum tolerable level for DON fixed by Regulation (EC) 1126/2007. It is noticeable that DON also were found were no *Fusarium* was detected by microbiological assay (cp. Fig. 17, sampling point 2, 3, 8, 11 and 13). A reason why *Fusarium* mycotoxins are found were no *Fusarium* species seem to be is the same reason like why *Fusarium* was detected by molecular but not by microbiological methods. Apparently the DON contamination is not clustered in the field but seem to be distributed relatively homogenously. This could be of great interest for mycotoxin monitoring.

Ochratoxin A (OTA) is not a *Fusarium* mycotoxin but as explained in former chapters it is one of the most dangerous mycotoxins found in wine and cereals. In field K6 it was only found at three sampling points in the south-western part of the field (Fig. 20). The maximum tolerable level for OTA is 5 µg/kg according to Regulation (EC) 1126/2007. Two of the three contaminated samples overran the threshold. The highest concentration of OTA was 25 µg/kg, a five time overrun. This contamination occurred at sampling point 1 where also the highest *Fusarium* incidence was recorded. The same reason which seem to be causative for the high *Fusarium* incidence may be an explanation for the high OTA contamination, too - The changed microclimate due to the hedgerow at this field border.

OTA contamination is apparently not distributed homogenously but aggregated in hot spots.

Fig. 20: Ochratoxin A content (µg/kg) of 15 wheat samples taken from field K6

Fig. 21: Enniatin B content (µg/kg) of 15 wheat samples taken from field K6

Enniatin B (ENB) is a mycotoxin produced by a broad range of fungi. Also some *Fusarium* species are able to produce it. In field K6 ENB was found in two-thirds of the samples (Fig. 21). The ENB content of the contaminated samples ranged from 6,3 to 281,7 µg/kg. No maximum tolerable level is defined for ENB but it is known to be cytotoxic. In comparison to DON and OTA which are apparently distributed homogenously and aggregated, respectively, ENB seems to tend to occur more aggregated. Too little is known about production of ENB by *Fusarium* species to give an definite answer. Also the potential of danger by ENB has to be assessed more accurately to decide whether a contamination as high as 280 µg/kg is tolerable or not.

Conclusions

Mycotoxins can be a severe problem in wine and cereal production which must be carefully monitored although all samples analyzed in the frame of this project, with very few exceptions, proved to be contaminated by OTA at a level which is higly lower than the threshold established by the European law. The survey carried out in Liguria Region showed that no significant differences in OTA content between red and white wines could be assessed, but that different levels of contamination by ochratoxigenic fungi can be observed depending on vine cultivar. It is stressed the efficacy of pesticide application for the control of mycotoxigenic fungi in the vineyards since the activity of these compounds can have a positive effect even during the wine making process reducing the OTA content. Anyway this practice should be carefully evaluated and based on a real disease pressure for a more sustainable management of the vineyard.
Relevant to cereals, although the maximum tolerable levels of mycotoxins were not overran in most cases, mycotoxins were obviously present in nearly every sample. This means that there is a potential of high and severe mycotoxin contamination given in the field. In Years where the weather conditions are more favourable for fungal infections it is possible that there will be much higher concentrations of mycotoxins. The results taken out of these examinations also showed that not only the typical expected plant pathogens can lead to problems but also rather unexpected species which are normally found on different but close related plants. It is worth to take a closer look at these species like the maize *Fusarium* species *F. proliferatum* and *F. verticillioides* in the future since they are important mycotoxigenic plant pathogens. It seems also reasonable to expand the monitoring to mycotoxins which are rather unexpected in cereals like Ochratoxin A and Enniatin B.
Some recommendations for a mycotoxin monitoring system can therefore be drawn:

a) The results show that the focus in mycotoxin monitoring should not only be on the ‘common’ mycotoxins Deoxynivalenol and Zearalenon but also on rather unexpected mycotoxins in wheat like Ochratoxin A

b) In the case of Deoxynivalenol, where the distribution seemed to be homogenously, it should suffice to examine a mixed sample for the whole field

c) In the case of Ochratoxin A, which seemed to be rather aggregated in foci, a different sampling system is recommendable:

    - risk factors, favouring an infection with ochratoxigenic species in wheat have to pointed out more clearly

- these risk factors can than be mapped, for example with geographical information systems (GIS)
- mycotoxin monitoring can then firstly be done in the defined high risk areas

d) The recommended Ochratoxin A monitoring system could also be used for other mycotoxins for a first estimation of the contamination level

e) In practical monitoring multi mycotoxins methods should be used where it is possible to better overview the contamination status of rather uncommon or unexpected mycotoxins

f) The Research should, where it is possible and reasonable, not focus on one or two mycotoxins but look for more mycotoxins

Experimental activities were followed by different initiatives aiming at improving the knowledge of the European legislation related to mycotoxins, promoting its implementation, and proposing solutions to prevent or reduce the risks of mycotoxins in the food chain, both at the regional and local, level, along the whole food chain, involving all the stakeholders, from the farmers to the consumers. Meetings, seminars, infodays, exchanges of students were organized for a better practice transfer among partners. On a major extent the wide variety of occasions for meeting and knowledge and technology exchange will be able to promote the economic activities – agriculture, food industry and tourism - of the three regions involved.

References

Abd-Elsalam K.A., Aly I.N., Abdel-Satar M.A., Khalil M.S., Verreet J.A. (2003). PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. Afr. J. Biotechnology, 2: 82-85

Battilani, P., Pietri, A., Bertuzzi, T., Languasco, L., Giorni, P., & Kozakiewicz, Z. (2003). Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. Journal of Food Protection, 66: 33-636.

Battilani P., Pietri A. (2002). Ochratoxin A in grapes and wine. European Journal of Plant Pathology, 108, 639-643.

Bottalico, A. and Perrone, G. (2002). Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. European Journal of Plant Pathology, 108: 611-624

Creppy, E. E. (1999). Human ochratoxicosis. Journal of Toxicology Toxin Reviews, 18: 277-293.

Leslie J.F., Summerell B.A. (2006). The *Fusarium* Laboratory Manual. Blackwell Publishing, Ames, USA

Nicholson P., Simpson D.R., Weston G., Rezanoor H.N., Lees A.K., Parry D.W., Joyce D. (1998). Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assay. Physiol. Molecular Plant Pathology, 53: 17-37

Pantini, D. (2006). Cambiano i contorni del mercato del vino. Informatore Agrario, 46: 31-34.

Parry D.W., Nicholson P. Year (1996). Development of a PCR assay to detect *Fusarium poae* in wheat. Plant Pathology, 45: 383-391

Pollastro, S., De Miccolis Angelini, R.M., Faretra, F. (2006). A new semi-selective medium for the ochratoxigenic fungus *Aspergillus carbonarius.* Journal of Plant Pathology, 88: 107-112.

Sweeney M. J., White S., Dobson A. D. W. (2000). Mycotoxins in agriculture and food safety. Irish Journal of Agricultural and Food Research, 39: 235-244.

Turner A.S., Lees A.K., Rezanoor H.N., Nicholson P. (1998). Refinement of PCR-detection of *Fusarium avenaceum* and evidence from DNA marker studies for phenetic relatedness to *Fusarium tricinctum*. Plant Pathology, 47: 278-288.

Annex 1

Receipt for the preparation of selective and semi-selective media

MEA
Malt extract 20 g/l
Agar 20g/l
Cloramphenicol 50 mg/l
Clortetraciclin 50 mg/l
Dichloran a.i. 0,2% w/v in 1ml ethanol

MEA-B
As described for MEA plus boscalid a.i. 10 mg/l

Annex 2

Wine making process

Fermentation starter was produced using glucose (100 g/l) and yeast *Saccharomyces cerevisiae* (Zymoferm, Franke prodotti enologici, Susa (TO), Italy) (20 g/l) in sterile and deionised water. It was put at 25 °C for 72 hours. Juiced obtained from smashed bunches were filtered through a sieve (0,5 mm), then distributed into plastic vessels suitable for food storage and the starter was added (200 ml/l of must). Juices were stored in a climatic chamber at 25 °C till the fermentation process was naturally over. Red berry skins were kept in contact with musts during fermentation while white ones were removed. Once fermentation stopped, temperature was lowered to 4 °C for 24 hours in order to facilitare the natural separation between musts and lees.