Ochratoxin A is a food contaminant particularly present in warm climates. It flourishes on grapes and other fruits and vegetables. Climate change scenarios indicate an increase of the occurrence of the toxin in grapes in cool places as Trentino, site of this research.

1. Mycotoxins: origins and effects

Mycotoxins are low-molecular-weight compounds produced by the secondary metabolism of fungi having toxic effects on humans and animals. Mycotoxins are produced by different groups of filamentous fungi, in particular *Aspergillus*, *Penicillium* and *Fusarium* when they invade crops or stored food and they can thus enter the food chain. Around 400 types of mycotoxins have been reported. Major mycotoxins include aflatoxins, ochratoxins, fumonisins, trichothecenes and zearalenone (Bennett & Klich, 2003; Paterson & Lima, 2010a). The severity of their effects (called mycotoxicoses) caused by the ingestion of food contaminated with mycotoxins depends on several factors including the type of toxin, the dose and the extent of exposure (Baht et al., 2010). The ingestion of mycotoxins can have both acute and chronic consequences, including rapid death and induction of cancer.

Key-features

- **Ochratoxin A (OTA)** is a poisoning (nephrotoxic and carcinogenic) substance produced by members of the group *Aspergillus* section *Nigri*, also known as black aspergilli, directly on grapes and frequently found as wine contaminant.

- **Climate** is considered an important risk factor for the increase of the contamination of wines with OTA. In fact, the occurrence of OTA, as well as that of black aspergilli, is higher in grapes grown in Southern European regions compared to those of the North.

- In this booklet, two main aspects are investigated: 1. present and future risk of *Aspergillus* black rot disease of grapes cultivated in environments with a cool climate using the Alpine Valleys of Northern Italy (Trentino region). 2. The development of a method to shorten the process of monitoring the vineyards for the presence of OTA.

- A three years survey shows a reduced presence of black aspergilli in general and OTA-producing strains in particular on grapes grown in the Trentino region.

- However, the projection of temperatures and rains clearly indicate an increase of the occurrence of black aspergilli in the valleys of this region during the next decades.

- Monitoring of OTA-producing black aspergilli on grapes is a time-consuming process. Second main result of this study took to develop an assay with the potential to speed the process of vineyards monitoring for the presence of OTA-producing strains.
Of great concern is the impact of mycotoxins of producing filamentous fungi: it is estimated by FAO that they affect around 25% of the world’s food crops, leading to losses in the range of 1000 million tons of foodstuffs each year (FAO, 2012). The reduction of the occurrence of mycotoxins in agricultural products is based mainly on good agricultural and manufacturing practices including the use of resistant plants, the avoidance of late harvesting, the control of pests, the minimization of times between harvesting and processing and appropriate storage conditions (Bennett & Klich, 2003; Visconti et al., 2008). In the European Community the contamination of foods and feeds with major mycotoxins has been limited with regulations based on the scientific advices of authoritative bodies including the FAO/WHO Joint Expert Committee on Food Additives of the United Nations (JEFCA), the European Food Safety Authority (Egmond et al., 2007).

2. Ochratoxin A, the most important mycotoxin in wine

The only mycotoxin for which there is a limitation in wine is ochratoxin A (OTA; Commission Regulation, 2005). OTA has potent nephrotoxic, immunotoxic, teratogenic and carcinogenic effects when tested on animals and has been classified as a possible human carcinogen by the International Agency for Research on Cancer (Group 2B; IARC; IARC, 1993). Moreover, OTA has been associated to the Balkan Endemic Nephropathy (BEN), a progressive developing renal failure reported in individuals from farming households in geographically limited areas of Balkans (Pfohl-Leszkowicz & Manderville, 2007). Species of Aspergillus and Penicillium isolated from several commodities including cereals, grapes, coffee have been shown to produce OTA (Bayman & Baker, 2006; Perrone et al., 2007).

In an assessment of the dietary intake of ochratoxin A (OTA) by the EU population performed by the Scientific Committee on Food (SCF) of the European Commission it emerged that cereals are the main contributor of OTA intake (50%), followed by wine (13%) and coffee (10%; SCF, 2002). The occurrence of OTA in wines ranged between 0.003 and 15.6 μg kg⁻¹, with a European mean level of 0.357 μg kg⁻¹ (SCF, 2002). OTA content in wine was limited to 2 μg kg⁻¹ in 2005 (Commission Regulation, 2005).
Since the first report on the occurrence of OTA in wines published in 1996 by Zimmerli & Dick, a lot of attention has been paid to the understanding of the origin of OTA in grape products. Some members of the Aspergillus section Nigri, also known as black aspergilli, were identified as the main responsible for the contamination of grapes with OTA (Cabañes et al., 2002). Black aspergilli cause the Aspergillus black rot disease of grapes. Attacked berries appear completely shrunken and dry, presenting a high black sporulation (Visconti et al., 2008).

It was shown that the population of black aspergilli occurring on grapes of the European basin is composed of four main species: A. niger, A. tubingensis, A. carbonarius and A. uvarum (Perrone et al., 2007). Fungal strains belonging to A. carbonarius and A. niger species are potentially ochratoxigenic (Varga et al., 2011).

Fungal strains A. carbonarius is considered the most important OTA-producing black Aspergillus species that can be recovered from grapes, since most of the strains can produce OTA, whereas only 2-20% of the A. niger strains are usually ochratoxigenic (Perrone et al., 2007). Recently, it was shown that strains of the A. niger species can also produce fumonisins, another potent carcinogenic compound, raising the concern of grape and wine producers about the possible contamination of wines with a second major mycotoxin. However, the first studies indicate a negligible level of exposure to fumonisins through wine consumption (Logrieco et al., 2010).

3. OTA and climate

Climate has an important role on the OTA occurrence in grapes, the presence of black aspergilli on berries and also in the composition of black Aspergillus species composition (Zimmerli & Dick, 1996; Battilani et al., 2006). High temperature is an important risk factor for the contamination of OTA in grapes and grape products (Visconti et al., 2008). In fact, surveys on the contamination of wines with OTA evidenced a higher incidence and concentration of OTA in wines from southern regions (Zimmerli & Dick, 1996).

This is in accordance with a geostatistical analysis performed by Battilani et al. (2006) which describes an increase of black Aspergillus incidence on grapes from cooler regions of northern Europe to hotter regions of the south. Moreover, A. carbonarius was shown to be isolated more frequently from grapes grown in Mediterranean regions, while A. tubingensis and A. niger aggregate appear to be widespread (Perrone et al., 2007).
The higher recovery rates of black aspergilli in grapes grown in hot environments can be explained by their ability to grow at higher temperature than other filamentous fungi potentially present on grapes. Since black aspergilli presence on grapes and the subsequent contamination of grapes with OTA appear to be favoured by high temperatures, climate change is likely to cause the migration of OTA producing black aspergilli in regions in which these species had not been widely found previously (Paterson & Lima, 2010b; Magan et al., 2011). Therefore, climatic conditions as well as the presence of OTA-producing black aspergilli must be continuously monitored in vineyards of regions subjected to cool climate to take timely action of avoidance of OTA contamination.

3. Present and future risks

One of the main objectives of this research was to investigate the present and future risk of *Aspergillus* black rot of grapes cultivated in environments with a cool climate using the Alpine Valleys of Northern Italy (Trentino region) as case study to establish a baseline in view of the strengthening of temperature increase (Storari et al., 2012 accepted). To do that, we performed a three-year survey collecting black aspergilli from grapes from different vineyards and cultivars of three areas of the Trentino region (Adige Valley, Rotaliana plan and Sarca valley). Black Aspergilli were never found predominant on grapes grown in the Trentino region, being isolated from around 10% of the sampled berries. This was in agreement with the observed meteorological conditions and it was considerably low in respect to the incidence (20-80%) found on grapes grown in warmer environments of Europe like those in Portugal, Greece, southern Italy or Israel (20% - 80%; Battilani et al. 2006). In fact, the sum of mean daily temperatures and rains we measured in August were associated with a low risk of *Aspergillus* black rot in the geostatistical analysis of Battilani et al. (2006). Most of the collected black aspergilli were classified as *A. uvarum*, *A. tubingensis* and *A. niger*. *A. carbonarius*, the most important OTA-producing black *Aspergillus* potentially present on grapes, could be isolated only once (fig. 1).
OTA production was observed only in the single isolate of *A. carbonarius* and in one isolate of *A. niger*, indicating a low risk of OTA contamination of grapes by black aspergilli. Fumonisins production *in vitro* was detected in most of the *A. niger* isolates, indicating a potential risk of contamination of grapes and the need of an exhaustive assessment of the occurrence of fumonisins in wines produced in the Trentino region. However, the overall low presence of fumonisins-producing *A. niger* isolates we found (below 3%) indicates a low probability of exceeding the low content of fumonisins reported in early studies performed on the natural occurrence of these mycotoxins in wines (Logrieco et al., 2010).

To evaluate the possible effects of climate change on the future presence of black aspergilli on grapes grown in the Trentino region we used the geostatistical analysis of Battilani et al. (2006) together with the projections of mean daily temperatures and rains during August. As shown in fig. 2, maps of Trentino generated with the projections of climate conditions for the two future periods 2021-2050 and 2071-2100 show an increase of the area subject to higher temperatures during August in respect to the reference period 1978-2007. The predicted increase of temperature will likely expose the grapes grown in the Trentino region to a higher risk of black *Aspergillus* occurrence in the next decades.
5. Monitoring of black aspergilli in the vineyard: a new method

Second main objective of the project was to develop a method to shorten the process of monitoring the presence of OTA-producing black aspergilli in the vineyard. In fact, characterization of isolated black aspergilli usually requires the use of molecular techniques such as gene sequencing preceded by the purification of DNA. Moreover, identification of OTA-producing strains requires the detection of toxin production on synthetic media using analytical instruments. A possible shortcut would be to detect the presence of the genes of biosynthesis of the toxin in the genomes of the fungal strains.

Unfortunately, OTA biosynthesis in black aspergilli was still almost unknown when the Envirochange project started. To find suitable markers for OTA production, we cloned different polyketide synthase gene fragments from DNA of different black aspergilli strains isolated from grapes (Storari et al., 2010).

Deduced amino acid sequences were then compared to two PKS genes involved in OTA biosynthesis in A. westerdijkiae. Two PKS gene fragments showing a relatively high identity with one of those, called aolc35-12, were found in the DNA of OTA-producing A. carbonarius (acpks_OTA) and A. niger (anpks_OTA). All A. niger strains isolated from grapes of Trentino region and some reference strains were screened for the presence of anpks_OTA using specific primers. This gene could be amplified in each OTA-producing isolates, but not in the non-ochratoxigenic A. niger strains.

The ability of a fungal strain to produce a toxin can be evaluated in different ways. Usually, the compound of interest is extracted from the organism and detected using analytical instruments. However, this is a laborious procedure which is not ideal if the isolates to be investigated are numerous. Another indirect way is to detect the genes responsible for the production of the toxin: fungal strains which do not possess these genes will not be able to produce it. Thanks to the new techniques of DNA amplification this represents a faster and simpler alternative in respect to the direct detection of the toxin. However, there are several examples of fungal strains possessing biosynthesis genes which are not able to produce the toxin. An evaluation of the degree of association between biosynthesis genes and

**Polyketide synthase genes (PKSs) are a family of complex, multidomain genes involved in the biosynthesis of several fungal secondary metabolites including many mycotoxins such as OTA, aflatoxins and fumonisins.**
The strong correlation between inability to produce OTA and the absence of this PKS makes it a potentially suitable marker to discriminate between OTA-producing and non-producing *A. niger* strains. A similar approach was used to find molecular markers to discriminate between *A. niger* strains able and not able to produce fumonisins. All *A. niger* strains were screened for the presence of three genes essential for the production of these mycotoxins. The three biosynthesis genes were detected in several fumonisins-nonproducing isolates, excluding their possible use as markers for fumonisins production (Storari et al., 2012 accepted).

Anpks OTA and acpks OTA were then used as molecular targets in two assays based on the loop-mediated isothermal (LAMP) reaction to identify ochratoxigenic *A. carbonarius* and *A. niger* strains (Storari et al., submitted).

Fig. 4. Detection of decreasing amounts of *A. carbonarius* DNA using the loop-mediated isothermal amplification (LAMP) assay.

The LAMP reaction was developed in 2000 by Notomi et al. and is based on a continuous amplification of target DNA at constant temperature. A positive reaction can be indicated by a simple change in the colour of the solution. The strength of the LAMP reaction lies in its simplicity since it has the advantage of not needing expensive requirements such as real-time machines, gel electrophoresis apparatus or a complex purification step before DNA amplification. The specificity and the limit of detection of the two LAMP assays were tested using DNA of different black aspergilli isolated from grapes (Fig. 4). The two assays were then applied for the identification of *A. carbonarius* and OTA-producing *A. niger* grown in pure cultures without the need of a prior DNA extraction step.

The positive outcome of this work suggests a useful application of the two LAMP assays in the process of vineyards monitoring for the presence of black aspergilli able to produce mycotoxins. Through this method it would be possible to identify the collected ochratoxigenic black *Aspergillus* strains without the need of DNA extractions, analytical instruments or great knowledge in *Aspergillus* taxonomy, speeding up the screening process.

After providing a detailed description of the population of black aspergilli on grapes grown in a wine-producing cool region, it is evident how the low occurrence of OTA-producing strains indicates a low risk of OTA contamination of grape products by black aspergilli. However, the strengthening of temperatures expected in the future is likely to increase the presence of OTA-producing strains, invoking the development of specific control strategies which include the continuous monitoring of vineyards. The two novel LAMP assays presented here have the potential to speed up the process of vineyards monitoring for the presence of OTA-producing black *Aspergillus* strains.
To know more


IARC. 1993. Ochratoxin A. IARC, 56:489-521. From:


THE ENVIROCHANGE PROJECT

The EnviroChange project focuses on global change and sustainable management of agriculture in highly developed mountain environment. It aims at assessing the short-term biological, environmental and economic impact of climatic change on agriculture at the level of the Trentino region particularly on quality and pest management that are more likely to be influenced by climate change in the short term. The final aim is to preserve and improve the quality of life of habitants, protecting environment and biodiversity for the future generations, as well as to represent a model for sustainable development of mountain areas.

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