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Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape

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Abstract

Fungi responsible for ochratoxin A (OTA) production have been studied especially on cereals, where *Penicillium verrucosum* and *Aspergillus ochraceus* are to be considered the main producers. Until 1998, these fungi were also believed to be responsible for the production of the toxin in grape, but OTA-producing *A. carbonarius* and *A. niger* were identified in dried vine fruits in 1999. Further studies pointed out that mycoflora potentially responsible for the presence of OTA in grapes are present in the field. *Aspergilli* are dominant to *Penicillia*, and among these *Aspergilli* section *Nigri*. *A. carbonarius* probably plays an important role because of the high percentage of positive strains and the amount of OTA produced. *Aspergilli* section *Nigri* are present on grape bunches early in the season and their frequency increases during later growth stages. At early veraison and ripening, the incidence of colonised berries is more related to the year than to the growth stage, but not to visible symptoms, since it is normal to isolate fungi from intact berries. Differences in ochratoxin content of berries have been detected between years, when the same vineyards, managed in the same way, showed high levels (1999) or the absence (2000) of the toxin. The results suggest that meteorological differences between years and grape-growing areas are responsible for differences in OTA levels, but the data are at present insufficient to draw firm conclusions.

Introduction

Epidemiology is the study of changes in the intensity of disease in a population of hosts over time and considers how a plant disease, resulting from the interaction between a host and a pathogen, can be influenced by the environment. When the pathogen is a mycotoxigenic fungus, the disease is not necessarily a visible alteration of the host, but an invisible presence of its metabolites. As a consequence, information has to be acquired not only by monitoring host, pathogen, environment and disease (Campbell and Madden, 1990), but also the toxins which may accumulate. This paper summarises information obtained from published literature regarding ochratoxin A (OTA) in grape and presents further data collected in Italy in order to contribute to the understanding of the epidemiology of the fungi involved.

The host: grapes

Grape is a fruit appreciated by consumers as fresh (table grapes), dried (raisins), or as processed products, such as juice and wine. Grape production in Europe is estimated at 306 million tons per year, which is 54% of world production. Annual yields of table grape and raisin are 2.2 and 0.5 million tons, while 190 million hl of wine are produced (74% of world production). Furthermore, grape and its derivatives play a major role in import–export exchanges (Dutruc-Rosset, 1998). Grape was considered a safe product, free from mycotoxins, until 1996 when OTA was detected during a survey carried out in Switzerland on wine samples of different geographic origins (Zimmerli and Dick, 1996). Until now, OTA is the only mycotoxin to be detected in wine.

The toxin: Ochratoxin A

Ochratoxin A is carcinogenic to rodents and possesses teratogenic, immunotoxic and possibly neurotoxic and genotoxic properties. Furthermore, it has been implicated as a causal agent of Balkan Endemic Nephropathy and the development of urinary tract tumours in humans. In 1993, the International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (group 2B). The worldwide occurrence of OTA contamination of raw agricultural products has been amply documented. It occurs in a variety of plant products such as cereals, coffee beans, pulses, cocoa and spices. It has also been found in beer and in animal products such as pig meat, sausages and other meat products. Human exposure to OTA can be evaluated by blood analysis (FAO/WHO, 2001). During the last 5 years, several papers regarding the presence of OTA in grape and its processed products have been published. The information collected suggests that the intake of OTA related to grape is a real risk, especially for consumers of red wine, dessert wine and dried vine fruits. Red wine is more contaminated than white, especially if produced in Southern Europe. OTA has been detected in wine and in raisins at levels up to 7.6 $\mu g\,l^{-1}$ and 53.6 $\mu g\,kg^{-1}$, respectively (Battilani and Pietri, 2002).

The pathogens

Fungi responsible for OTA production have been studied especially on cereals, where *Penicillium verrucosum* and *Aspergillus ochraceus* (formerly known as *A. alutaceus*) are considered the main producers. Until 1998, they were also believed to be responsible for the production of the toxin in grape (Ospital et al., 1998), but OTA-producing *A. carbonarius* and *A. niger* were identified in dried vine fruits in 1999 (Codex Alimentarius Commission, 1999).

Few studies have been published regarding the occurrence of OTA-producing fungi in grapes. During the 1997–98 harvests, 50 grape samples were collected from Malbec and Chardonnay varieties in Argentina and Brazil. *Aspergilli* and *Penicillia* were isolated in both countries. *A. ochraceus* was found only in Brazil with a very low incidence and *P. verrucosum* was not identified. All the *Aspergilli* section *Nigri* that were collected (131 strains) were checked for OTA production and 25% were positive. *A. carbonarius* was isolated only in Brazil and 25% of isolates were ochratoxigenic (Da Rocha et al., 2002). Similar results were obtained in France, where 11 samples of grape and must used in red table wine making were investigated. Several *Aspergilli* and *Penicillia* were identified, but *A. ochraceus* and *P. verrucosum* were absent. Only *A. carbonarius* was tested for OTA production and all the isolates (14) were positive (Sage et al., 2002).

During 1999 and 2000, nine vineyards in Italy were sampled at different growth stages. Five hundred and eight fungal isolates were collected, 477 belonging to *Aspergillus* spp. and 31 to *Penicillium* spp. *P. verrucosum* was not found. Among the *Aspergilli*, species from sections *Fumigati*, *Circumdati* and *Nigri* were identified, with section *Nigri* (464 isolates) dominant. Examples of section *Circumdati*, which includes *A. ochraceus*, were isolated only occasionally. Eighty-six isolates of section *Nigri* were identified as *A. carbonarius* and they represented 19% of the black *Aspergilli* collected in both years. They proved to be the most toxigenic strains: about 60% of the isolates were positive for OTA production (Battilani et al., 2002).

These studies led to some preliminary conclusions. Mycoflora potentially responsible for OTA presence in grapes is present in the field and *Aspergilli* are dominant to *Penicillia*. The identified species did not include *P. verrucosum*. *A. ochraceus* was only found occasionally. As a consequence, they do not seem relevant to grape contamination. *Aspergillus* section *Nigri* was always present and included OTA-producing strains (Abarca et al., 2001). Among the species, *A. carbonarius* probably plays a relevant role, because the percentage of positive strains and the amount of OTA produced *in vitro* were generally higher than in the other black *Aspergilli* (Teren et al., 1996; Heenan et al., 1998; Battilani et al., 2002; Cabanes et al., 2001). The reported results identified *Aspergilli* section *Nigri* *A. carbonarius*, as being mainly responsible for OTA presence in grapes and wine, but no data are available on the dynamics of the fungus in the field or on the relationship between the fungi and OTA content in grapes.

In order to improve knowledge of the epidemiology of black *Aspergilli* in grapes, during a survey carried out in 1999 and 2000 (Battilani et al., 2002), data were collected with the aim of describing the dynamics of fungal populations on berries during grape development, possible interactions between fungal species, and cultural and meteorological factors. OTA content of berries were also investigated.

Materials and methods

Grape sampling

The survey carried out in 1999 and 2000 involved nine vineyards (designated V1–V9). Three were located in Emilia-Romagna (Northern Italy) and six in Puglia (Southern Italy). The vineyards chosen were representative of their grape-growing area as regards grape variety and farming methods (Table 1). Five plants were chosen along diagonal transects of each vineyard and two bunches were taken from each plant. When the training system consisted of two levels of bunches above ground, both were sampled, one bunch per level. In 1999, two growth stages were chosen for sampling: early veraison and ripening. In 2000, samples were also collected at two earlier growth stages, setting and berry enlargement (Table 2). Data on cropping systems were collected and meteorological data (mean daily air temperature, relative humidity and rainfall) were gathered from stations close to the vineyards.

Fungal isolation and characterisation

Samples of berries and rachis were incubated in moist chambers and the growing fungal colonies were transferred to Petri dishes containing Czapek Yeast Agar (CYA). After incubation, the fungal isolates were identified to genus level (Battilani et al., 2002). *Aspergilli* section *Nigri* isolates were identified as uniseriates, biseriates or *A. carbonarius.* Uniseriates are those with uniseriate conidial heads, biseriates are those with biseriate heads and among these *A. carbonarius* isolates were identified at species level. All *Aspergilli* and *Penicillia* isolated were tested for OTA production (Battilani et al., 2002).

Ochratoxin content in berries

All bunches collected, after selecting samples for fungal isolation, were manually crushed and OTA content was determined. The toxin was extracted with a mixture of acetonitrile : water (60:40) and, after dilution with water, an aliquot of the solution was purified using an immuno-affinity column and analysed by reverse-phase HPLC with fluorescence detection (Battilani et al., 2002).

Statistical analysis

Data on samples colonised by *Aspergilli* section *Nigri* were analysed using non-parametric Kruskal–Wallis

Table 2. Date of bunches sampling during the 2 years considered in the study

Year	Setting	Berries increase	Early veraison	Ripening
1999	—	—	2 August	10 September
2000	8 June	6 July	2 August	10 September

Table 1. Data on vineyards sampled in Italy in 1999 and 2000

Vineyard	Latitude	Longitude	Place	Grape variety	Training system	Height of bunches
V1 (South)	40°23′N	17°58′E	Salice salentino (LE)	Negroamaro	Bilateral spur pruned cordon	60
V2 (South)	40°23′N	17°58′E	Salice salentino (LE)	Malvasia nero	Bilateral cane pruned cordon	60
V3 (South)	40°23′N	17°58′E	Salice salentino (LE)	Negroamaro	Head-trained spur pruned	40–50 100–120
V4 (South)	40°24′N	17°57′E	Guagnano (LE)	Sangiovese	Overhead trellis, cane pruned (*Tendone*)	170–180
V5 (South)	40°24′N	17°38′E	Manduria (TA)	Primitivo	Cane pruned cordon (*Guyot*)	40–50
V6 (South)	40°44′N	17°25′E	Cisternino (BR)	Verdeca	Cane pruned cordon (*Guyot*)	60
V7 (North)	44°17′N	11°53′E	Faenza (RA)	Trebbiano	Overhead trellis, cane pruned (*Pergoletta*)	130–160
V8 (North)	44°17′N	11°53′E	Faenza (RA)	Trebbiano	Cane pruned cordon	40 170
V9 (North)	44°17′N	11°53′E	Faenza (RA)	Sangiovese	Spur pruned cordon	140–150

Italian provinces: LE: Lecce; TA: Taranto; BR: Brindisi; RA: Ravenna.

analysis of variance in order to study the significance of the considered factors (year, vineyard, growth stage and plant). Correlation analysis was used to check the relationship between the presence of fungi and the OTA content in berries.

Results

Fungal isolation and characterisation

Aspergillus and/or *Penicillium* strains were present on grapes, starting from setting in two vineyards, V3 and V9. One month later these fungi were detected in more vineyards: V1, V3, V4, V8 and V9. Vineyard V6 was free from fungi at early veraison in both years, as was V8 in both samplings in 1999 and V7 at ripening in 2000 (Figure 1). The number of colonised samples (berries or pieces of rachis) was low (4–10%) during early growth stages, but it increased up to 70% at ripening. Five hundred and twenty samples were colonised by *Aspergilli*, 506 of them by *Aspergilli* section *Nigri*. The height of bunches above ground level was not related to the fungi present. In fact, almost the same number of moulded samples was obtained in V3 and V8, independently of the distance of bunches from the ground. The percentage of colonised samples was higher in berries than in pieces of rachis. The percentage of colonised berries was 9% and 15% in 1999 and 2000, while the percentage of colonised rachis was 7% in both years.

The incidence of samples colonised by black *Aspergilli* was significantly influenced by the vineyard in both years and by the growth stage in 2000. Also, the year showed a significant effect when data were considered together (Table 3). The dynamic of samples colonised by black *Aspergilli* is shown in Figure 1 for early veraison and ripening, the most relevant growth stages. Fungal incidence was higher in 2000 than in 1999, especially at ripening. Most samples were colonised by *A. niger* aggregate in both years and at both growth stages. *A. carbonarius* was relevant at early veraison in 1999 and at ripening in 2000. In both

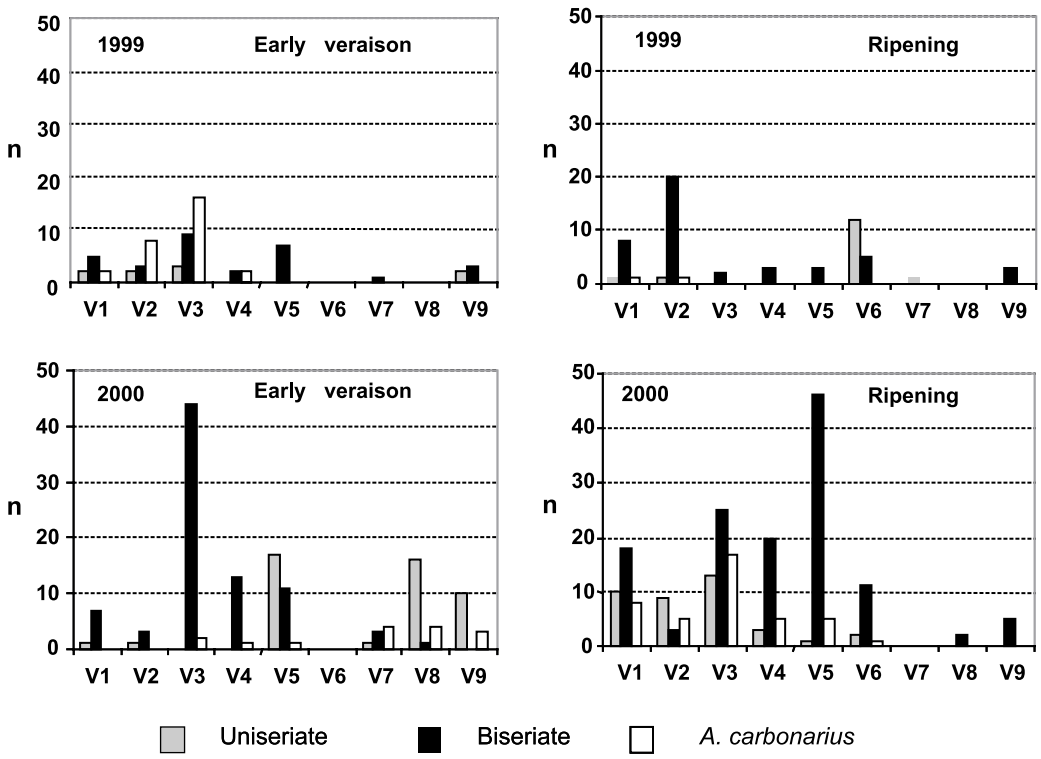

Figure 1. Dynamic of *Aspergilli* section *Nigri* identified as uniseriate, *A. niger* aggregate and *A. carbonarius* in nine Italian vineyards (V1–V9, see Table 1) at early veraison and ripening in 1999 and 2000.

cases, vineyards V1, V2 and especially V3 were the most colonised. The incidence of *A. carbonarius* was higher at early veraison in 1999 and at ripening in 2000, in both cases in Southern Italy. In Northern Italy, fungal contamination was always low, especially at harvesting.

The results on fungal dynamics are important, both because these genera are usually considered post-harvest moulds and because they were all isolated from berries without visible symptoms. Taking into account the dynamic of berries colonised by OTA-producing fungi, the higher incidence at early veraison in 1999 and at ripening in 2000 was confirmed. Several vineyards, including V7 and V8, were free of OTA-producing fungi at ripening in both years, and V6 which was free of toxins in both samplings in 2000 (Figure 2).

Table 3. Kruskal–Wallis analysis on incidence of berries colonised by *Aspergilli* or *Penicillia* in nine Italian vineyards in 1999 and 2000

Year	Vineyard	Plant	Growth stage	
1999				
χ^2	28.13	0.62	1.23	
Sign	0.00	0.43	0.27	
2000				
χ^2	19.70	0.54	233.22	
Sign	0.00	0.82	0.00	
1999–2000				
χ^2	39.62	0.31	107.20	49.42
Sign	0.00	0.58	0.00	0.00

Cropping system

Six grape varieties were included in the study. Varieties Negroamaro and Trebbiano were cultivated in two vineyards, the former in the South and the latter in the North, while Sangiovese was present in both grape-growing areas (Table 1). The training system was variable, as was the height of bunches from the soil. Four vineyards in the South (V1, V2, V5 and V6) had bunches close to the soil (40–60 cm) while in the North bunches were commonly at a greater height, around 150 cm above the soil level.

Chemicals were normally sprayed in all vineyards (Table 4), but the total number of sprays was greater in the North, with a mean of 15 applications in each cropping season, compared to 10 in the South. Active ingredients used were normally different between vineyards, especially if North and South are compared, but copper

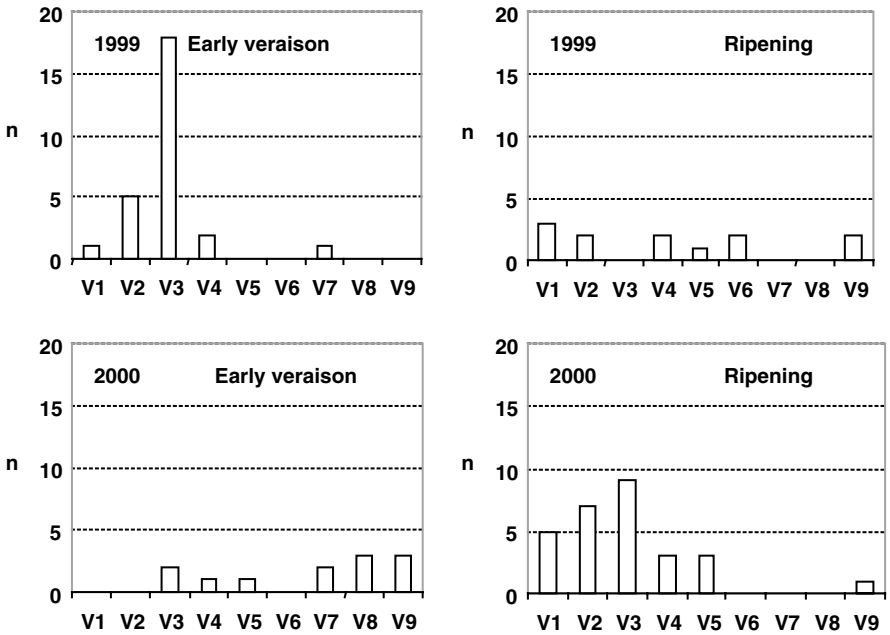

Figure 2. Dynamic of *Aspergilli* section *Nigri* positive for OTA production in nine Italian vineyards (V1–V9, see Table 1) at early veraison and ripening in 1999 and 2000.

Table 4. Active ingredients and total number of sprays distributed in the nine vineyards sampled in 1999 and 2000

	Vineyard																	
	V1		V2		V3		V4		V5		V6		V7		V8		V9	
Year	99	00	99	00	99	00	99	00	99	00	99	00	99	00	99	00	99	00
Azoxystrobin											■						▒	
Benalaxil											■	▒						
Cymoxanil	▒	▒	▒	▒	▒	▒	▒	▒		▒	■							
Dimethomorph									▒		■			▒		▒		
Dinocap	▒		▒		▒				▒		■							
Famoxadone											■							▒
Fenarimol	▒	▒	▒	▒	▒	▒			▒		■	▒	▒		▒			
Mancozeb	▒		▒		▒		▒		▒	▒	■						▒	▒
Metalaxyl	▒	▒	▒	▒	▒	▒				▒	■							
Metiram											■			▒		▒		
Penconazole											■							▒
Procymidone											■							▒
Propiconazole											■			▒		▒		
Quinoxifen											■							▒
Tetraconazole									▒	▒	■							
Thiophanate methyl	▒		▒		▒		▒	▒			■							
Copper	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	■	▒	▒	▒	▒	▒	▒	▒
Sulphur	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	■	▒	▒	▒	▒	▒	▒	▒
No. sprays	10	10	10	10	10	10	15	11	10	9	■	8	17	16	17	16	11	15

■: No data available; ▒: Sprays; □: No sprays.

and sulphur were always applied. Vineyards V1–V3 were managed in the same way, being included in the same farm, as were V7 and V8. None of the active ingredients sprayed is known to be active against *A. niger* (Khatri and Shekhawat, 1989; Suryawanshi and Deokar, 2001).

Meteorological data

Meteorological data show differences both between the places and years considered in the study (Figure 3). In Faenza (Northern Italy), the mean daily temperature ranged between 13 and 23 °C in both years and the summation in degree-days from 1 April to 30 September was 3650. In Brindisi (Southern Italy), the temperature was higher, with mean daily values varying between 15 and 30 °C in 1999 and between 19 and 30 °C in 2000, with a summation in degree-days of 4500 and 4800, respectively. The two grape-growing areas were also quite different regarding rainfall, especially in 2000. The total April–September rainfall in Faenza and Brindisi was 330 and 305 mm in 1999, and 182 and 62 mm in 2000. August and September were rainy months in 1999, with more than 70 mm/month in both areas, whereas they were dry in 2000, with 0 mm in August in the South.

Ochratoxin content in berries

The 2 years were quite different for OTA content in berries. In 1999 (Table 5), OTA content at early veraison was below $1\,\mu g\,kg^{-1}$ in all vineyards except V1 and V4. The situation remained more or less the same at ripening, except for V3, where OTA concentration was $13\,\mu g\,kg^{-1}$. In 2000, only traces of OTA (maximum $1\,ng\,kg^{-1}$) were occasionally detected in bunch samples. According to correlation analysis, the number of samples colonised by black *Aspergilli* and the OTA content in berries were not significantly related ($r = 0.46$), while the correlation was significant when only samples colonised by OTA-producing fungi were considered ($r = 0.94$).

Discussion

Aspergilli section *Nigri* are present on bunches early in the season and their frequency increases in later grape growth stages. At early veraison and ripening, the incidence of colonised berries is similar and the differences seem to be more related to the year than to the growth stage. It was normal to isolate fungi from intact berries. Independently of the growth stage considered, OTA was not always detected in berries, even

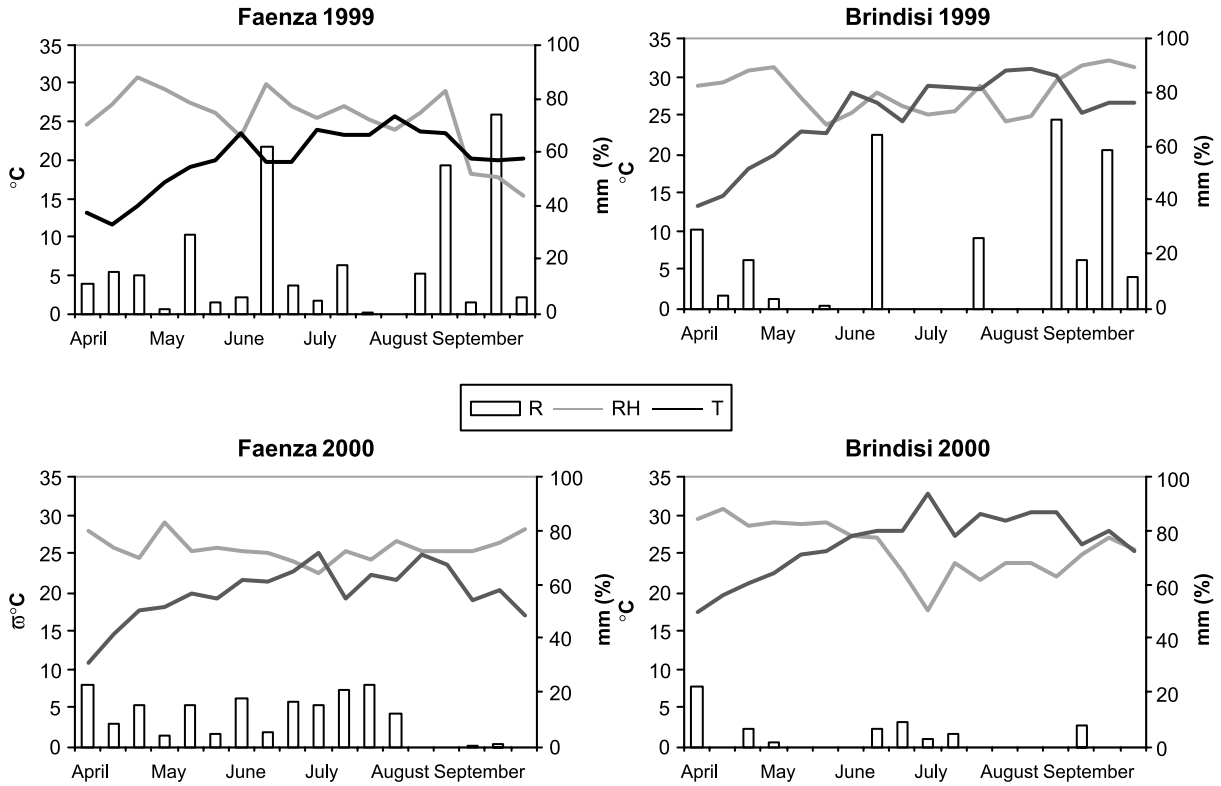

Figure 3. Temperature (T), relative humidity (RH) and rain (R), computed on 10 day periods, from April to September in Faenza and Brindisi in the 2 years of sampling.

Table 5. Ochratoxin A content ($\mu g\,kg^{-1}$) in bunches collected in nine Italian vineyards in 1999

Vineyard	Early veraison	Ripening
V1	1.29	1.51
V2	0.01	0.15
V3	0.03	13.08
V4	2.30	1.78
V5	0	N.A.
V6	0.12	0.03
V7	0.03	0.02
V8	0.00	0.01
V9	0.01	0.00

N.A. = Not available.

in those colonised by ochratoxigenic fungi. When OTA was present, it seemed to be correlated to the nature of the fungal isolates which were present. The presence of a fungus does not mean OTA synthesis. There are other factors that clearly play a role. Vineyards V1–V3, located in the same farm and managed following the same annual practices, showed differences in OTA contamination. OTA was practically absent in V2, but was detected at concentrations of 1.51 and 13.08 $\mu g\,kg^{-1}$ in V1 and V3, vineyards at the same site but trained with a different system. In addition, the Sangiovese grape variety was present both in V4 and V9. However, while the former vineyard contained OTA ($1.78\,\mu g\,kg^{-1}$), the latter was practically free from OTA.

The most remarkable difference was detected between years, where the same vineyards, managed in the same way, showed high levels of OTA in 1999 but OTA was absent in 2000. It appears that meteorological differences between years and at different grape-growing areas may be responsible for differences in OTA levels. Unfortunately, the existing data are not sufficient to draw final conclusions. Knowledge of the epidemiology of OTA-producing fungi in grape is actually very poor. A milestone has been gained: the pathogen is defined, but the role of the host, in particular of different grape varieties, as well as environment is still not clear.

Because of the importance of the problem and of the wide geographic area interested in it, a project supported by EU, Quality of Life, Key Action 1, started in 2001. In this project several European Countries in the Mediterranean Basin, relevant for grape growing, are involved. The overall objective of the project is the risk assessment of OTA presence in grapes and wine in Europe and protection of the consumer's health by decreasing the amount of toxin with the aid of integrated management of production and processing. The objectives will be followed by defining the critical control points for OTA synthesis during grape production and processing, modelling the effect of ecological conditions on fungal growth and OTA synthesis, and assessing possible preventive and corrective actions. All the information available about the pathosystem will be put together in a Decision Support System that will identify the actions likely to reduce the risk of OTA accumulating in grapes and its processed products.

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