MYCOLOGICAL RESEARCH 110 (2006) 971–978

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Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species

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ARTICLE INFO

Article history:
Received 2 February 2006
Received in revised form
26 April 2006
Accepted 18 May 2006
Published online 7 August 2006
Corresponding Editor:
Stephen W. Peterson

Keywords:
Aspergillus
Grapes
Mycobiota
Penicillium

ABSTRACT

A three-year study was undertaken to investigate the fungal species present on the surface of grape berries from Portuguese vineyards in four winemaking regions. Emphasis was given to *Aspergillus* and *Penicillium* species due to their relevance for mycotoxin production. From the 3517 fungal strains detected 27 genera were identified. The region of origin markedly influenced the spoilage fungal population to which berries are exposed. The main differences found were in the incidence of *A. niger* aggregate, *Botryis cinerea* and *Penicillium* species (*P. brevicompactum*, *P. citrinum*, *P. glabrum/spinulosum*, *P. expansum*, *P. implicatum* and *P. thomii*). In more humid climates, *Botrytis* seems to be the main pathogen and spoiling agent, and the incidence of black *Aspergillus* is minimal. The most important mycotoxin-producing species found was *A. carbonarius*, which is an ochratoxin A producer. The present study provides a detailed description of the fungi found on the berry surface of Portuguese grapes and shows the *Aspergillus* and *Penicillium* species, which vary significantly by geographic origin. This is of crucial importance to understand fungal hazards for grapes and wine and to the knowledge of field ecology of the species.

Introduction

The concern about filamentous fungi in the vineyard has traditionally been linked to spoilage of grapes due to fungal growth. The main fungus responsible for grape rot is *Botrytis cinerea*, a pathogen that damages the berries and has a detrimental effect on the organoleptic properties. Nevertheless, other saprobic fungi can cause rot in grapes and in addition produce mycotoxins. Two main genera are responsible for mycotoxin production in grapes: *Aspergillus* and *Penicillium*. The mycotoxin production is characteristic of the species and therefore by identifying the species one can predict potential mycotoxin hazards.

The threat to wines by mycotoxins, in particular ochratoxin A (OTA), instigated detailed studies of the grape mycobiota. Surveys of the fungi to which grapes are exposed in the vineyard were conducted in the main wine-producing countries in Europe such as France (Sage *et al.* 2004), Greece (Tjamos *et al.* 2004), Italy (Battilani *et al.* 2003), Portugal (Abrunhosa *et al.* 2001; Serra *et al.* 2003, 2005) and Spain (Bau *et al.* 2005), and also in countries such as Australia (Leong *et al.* 2004), Argentina and Brazil (Rocha Rosa *et al.* 2002).

The comparison of grape mycobiota from distinct wine-growing regions is difficult due to several variable factors, such as the use of different sampling methods, harvest years, grape varieties, and viticultural practices applied. In our work the goal was to assess the fungi present on the surface of healthy grapes destined for commercial winemaking at harvest time and compare the differences due to the geographic origin. To accomplish this task 11 vineyards from four distinct

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0953-7562/$ – see front matter
doi:10.1016/j.mycres.2006.05.010

Portuguese winemaking regions were studied over a three-year period.

Material and methods

Study area

Eleven vineyards were selected in four Portuguese winegrowing regions: Alentejo, Douro, Ribatejo and Vinhos Verdes. The geographical locations of these regions are indicated in Table 1. According to European Community (EC) regulation no. 822/87 revoked by EC regulation no. 1493/1999 of 17 May 1999, Portugal has two distinct winegrowing zones, CIa and CIIIb. Winegrowing zones are defined as geographic regions with distinct climatic conditions adequate for grape cultivation. Alentejo, Douro and Ribatejo have Mediterranean climates and are classified as CIIIb. The Vinhos Verdes region has a sub-Mediterranean climate and is classified as CIa. The sub-Mediterranean climate is a variant of the temperate climate with Mediterranean influence (Rivas-Martinez & Arregui 1999) and it is more humid than typical Mediterranean climates due to Atlantic influences. The maximum temperature observed in high summer in Vinhos Verdes is 38 °C and the mean annual precipitation ranges from 1235–1515 mm; in Mediterranean regions the maximum temperature observed in high summer is 42 °C and the mean annual precipitation regimes ranges from 672–739 mm.

Harvest years

The vineyards were sampled at harvest time in three consecutive years: 2001, 2002 and 2003. The year 2001 was hotter in all regions and rainier in South Portugal. In 2002, the air temperatures were colder and the precipitation values much higher than the average for this time of the year. In 2003, the temperature was higher and precipitation values lower than the average. The Portuguese Meteorological Institute provided the meteorological data and as normal values the month average of 30-year studies was considered.

Grape samples

Wine companies provided the grapes grown for commercial winemaking. The samples comprised 10 bunches of grapes collected as described in Serra et al. (2003), across two diagonal transects. The location of the fields selected and the grape varieties studied are listed in Table 1. Alvarinho and Loureiro are white varieties and the remaining are red varieties typically cultivated in the winemaking regions. Each of the 11 selected fields was sampled once a year (total of three samples) close to harvest time between late August and late September, the exact date defined by the companies according to their needs. We were allowed to gather samples in the field a few days before harvest. Field number 2 was not sampled in 2002 due to sudden adverse weather conditions that forced immediate harvest. Therefore a total of 32 samples were taken: six in Alentejo, nine in Douro, nine in Ribatejo and eight in Vinhos Verdes.

A questionnaire was designed to assess the phytosanitary treatments performed. Fungicides and/or insecticides are applied in the vineyards at several stages of the berry development starting in March–April and ending in July–August. The last applications of fungicides in Alentejo, Douro and Ribatejo usually occurred in middle July, while the last applications of fungicides in Vinhos Verdes usually occurred in middle August. This is due to the fact that Vinhos Verdes is a region very susceptible to grey rot due to frequent rain periods close to harvest time.

Mycological analysis of grapes

The mycobiota of grapes was determined as described in Serra et al. (2003): a total of 50 berries of each sample were plated in Dichloran Rose Bengal Chloramphenicol medium (DRBC, Oxoid, Basingstoke, Hampshire, England) without surface disinfection and incubated at 25 °C in the dark for one week (five berries per bunch in each plate). The spore-producing filamentous fungi detected were identified to genus level and *Penicillium* and *Aspergillus* strains were isolated and identified to species level. The monoverticillate *Penicillium* species

Table 1 – Geographical coordinates, grape varieties and winegrowing zone of the vineyards (fields) studied in the four winemaking regions of the Portuguese mainland

Field	Region	Zone	Location		Grape variety
			Latitude	Longitude	
1	Vinhos Verdes	CIa	42° 06′ 49″ N	8° 15′ 36″ W	Alvarinho
2	Vinhos Verdes	CIa	41° 50′ 50″ N	8° 25′ 14″ W	Loureiro
3	Vinhos Verdes	CIa	41° 50′ 50″ N	8° 25′ 14″ W	Vinhão
4	Douro	CIIIb	41° 09′ 30″ N	7° 47′ 02″ W	Tinta Barroca
5	Douro	CIIIb	41° 11′ 35″ N	7° 32′ 51″ W	Touriga Franca
6	Douro	CIIIb	41° 09′ 30″ N	7° 14′ 51″ W	Tinta Barroca
7	Ribatejo	CIIIb	39° 12′ 34″ N	8° 37′ 46″ W	Periquita
8	Ribatejo	CIIIb	39° 12′ 34″ N	8° 37′ 46″ W	Tinta Miúda
9	Ribatejo	CIIIb	39° 12′ 34″ N	8° 37′ 46″ W	Cabernet Sauvignon
10	Alentejo	CIIIb	38° 33′ 38″ N	7° 54′ 30″ W	Aragonês[a]
11	Alentejo	CIIIb	38° 25′ 27″ N	7° 32′ 04″ W	Periquita

a The variety Trincadeira was collected in 2003 instead of Aragonês at this field.

P. glabrum and *P. spinulosum* are treated as an aggregate, as they are very similar species and form an interface (Pitt 1985). The use of this aggregate is further justified by their relevance in terms of similar mycotoxin production and ecophysiological requirements (Pitt *et al.* 1990). The black bisseriate *Aspergillus* strains morphologically identified as *A. niger* were treated as the *A. niger* aggregate. Representative strains of the filamentous fungal genera detected and all the isolated *Aspergillus* strains were preserved in 10 % glycerol at −80 °C. The incidence (number of colonized berries) of each fungal taxon in grape samples was recorded in a relational database where all the information concerning grape samples was deposited. The data were exported to other applications for analysis.

Statistic analysis

All statistic analyses were performed in Statistic Package for Social Sciences (SPSS®) for Windows® version 11.0. The distribution of variables was tested for normality using the Kolmogorov-Smirnov test. To evaluate whether significant differences existed between the fungal incidence in grapes from different regions, one-way analysis of variance (ANOVA) with post-hoc tests and Kruskal-Wallis test with approximation to chi-square test were used for variables with normal and non-normal distributions, respectively. Bivariate correlations with Pearson (r_P) and Spearman (r_S) correlation coefficients were used to study linear relations between parametric and non-parametric variables, respectively. The statistical analysis performed were considered significant when $P < 0.05$.

Classification tree (CT) modelling

Classification-tree (CT) models (Breiman *et al.* 1984) are a simple and robust exploratory data analysis technique that can be used in classification, regressions and summaries of data. They distil complex ecological relationships into simplified rules (decision rules) and identify the species necessary for sample classification from detailed ecological inventories (Cohen *et al.* 2005). In this work, CT modelling was performed to summarize the differences in the mycobiota of regions and find the indicator species of each origin. Modelling was performed as described in detail in Serra *et al.* (2006,in press): the incidence of all fungal taxa in grapes was used as attributes, and the region of origin was used as a class attribute. Model evaluation was performed using ten-fold cross-validation (Kohavi 1995) and model refinement was performed by attribute selection and class redefinition. The modelling was made in the WEKA platform that is open source software and can be downloaded at: (http://www.cs.waikato.ac.nz/~ml/index.html).

Results

Adequacy of sampling size

A total of 3517 strains were recorded in this study. The mean number of fungi found per grape sample was 114, and did not vary significantly between regions. This represents approximately two distinct fungal strains detected *per* grape berry.

The cumulative number of fungal taxa found in the grape subsamples was plotted to evaluate the adequacy of sampling size. It was observed that the maximum cumulative number of taxa was reached with the analysis of seven to eight grape subsamples (five berries each). The same procedure was undertaken in each of the regions, with the samples taken at the vineyards in each harvest year (50 berries each) being considered as subsamples (Fig 1). The maximum cumulative number of taxa was obtained with seven samples in Vinhos Verdes and eight samples in Douro and Ribatejo. In Alentejo the results were similar to the other regions, although the six samples taken were not sufficient to reach a 0 increment value in the species found.

Diversity of fungi found

Twenty-seven (27) fungal genera of sporulating fungi were identified from grapes (Table 2). Most of the genera were *Deuteromycota* (mitosporic fungi) with the exception of three *Zygomycota* (*Cuninghamella*, *Mucor* and *Rhizopus*) and three teleomorphic states belonging to *Ascomycota* (*Emericella*, *Eurotium* and *Neurospora*). Few fungi (five to seven taxa) were found in more than 10 % of the berries. *Alternaria*, *Botrytis* and *Cladosporium* were three of the most frequent genera in all the regions (Table 2) representing 16, 17 and 24 % of the total identified strains, respectively. According to the region considered, other frequent fungi were *Aureobasidium pullulans*, *Aspergillus niger*, *Epicoccum nigrum*, *Penicillium brevicompactum*, *P. thomii* and *Rhizopus*. The remaining taxa were detected in less than 10 % of the berries.

Aspergillus and *Penicillium* were also an important part of the mycobiota representing 15 and 24 %, respectively, of all the fungi found in the regions (Fig 2). *Aspergillus* was more frequent than *Penicillium* in all regions except Vinhos Verdes.

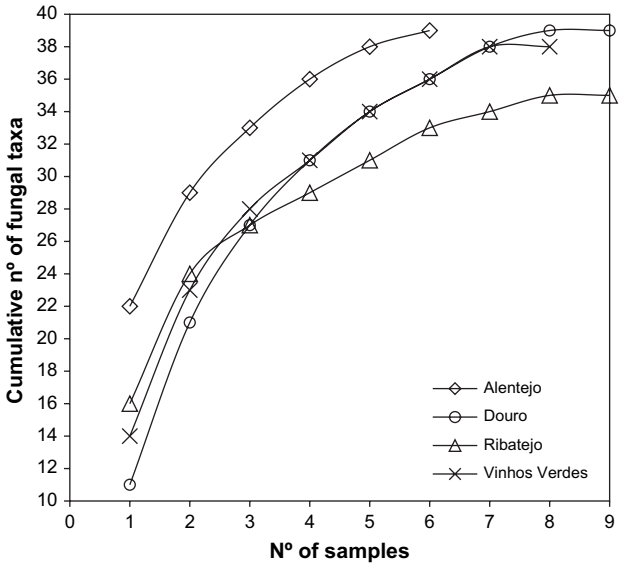

Fig 1 – Cumulative number of fungal taxa isolated from grape bunches of ten representative Portuguese vineyards at harvest time (above) and cumulative number of fungal taxa isolated from the vineyards at each harvest year in the four Portuguese winemaking regions (below).

Table 2 – Number and percentage of colonized berries with the fungal taxa identified in each of the four Portuguese winemaking regions studied

Fungal taxa	Colonized berries							
	Alentejo		Douro		Ribatejo		Vinhos verdes	
	No.	%	No.	%	No.	%	No.	%
Acremoniella sp.	0	0	0	0	0	0	1	0
Acremonium sp.	0	0	0	0	0	0	1	0
Alternaria sp.	124	41	145	32	130	29	173	43
Aspergillus								
A. carbonarius	26	9	9	2	30	7	1	0
A. flavipes	0	0	1	0	0	0	0	0
A. flavus	2	1	5	1	1	0	6	2
A. fumigatus	1	0	0	0	1	0	1	0
A. ibericus	2	1	5	1	0	0	0	0
A. japonicus	0	0	0	0	2	0	0	0
A. niger	99	33	196	44	107	24	11	3
A. ostianus	0	0	0	0	0	0	1	0
A. terreus	2	1	0	0	1	0	0	0
A. ustus	0	0	0	0	1	0	0	0
A. versicolor	1	0	0	0	0	0	0	0
A. wentii	0	0	0	0	3	1	1	0
Aureobasidium pullulans	38	13	39	9	45	10	44	11
Aureobasidium sp.	4	1	0	0	0	0	0	0
Botrytis cinerea	50	17	104	23	166	37	264	66
Chrysonilia sp.	0	0	1	0	2	0	1	0
Cladosporium sp.	193	64	164	36	277	62	224	56
Cunninghamella sp.	1	0	1	0	0	0	0	0
Curvularia sp.	0	0	0	0	1	0	1	0
Drechslera sp.	1	0	2	0	0	0	0	0
Emericella sp.	5	2	0	0	0	0	0	0
Epicoccum nigrum	41	14	15	3	40	9	15	4
Eurotium amstelodami	1	0	2	0	0	0	0	0
Fusarium sp.	0	0	0	0	2	0	1	0
Gliocladium sp.	0	0	0	0	0	0	1	0
Mucor sp.	2	1	4	1	1	0	4	1
Neurospora sp.	0	0	0	0	0	0	1	0
Neurospora tetrasperma	0	0	0	0	0	0	1	0
Nigrospora sp.	1	0	0	0	0	0	0	0
Penicillium								
P. aurantiogriseum	0	0	0	0	0	0	3	1
P. bilaii	0	0	2	0	0	0	0	0
P. brevicompactum	47	16	29	6	54	12	12	3
P. citrinum	3	1	19	4	1	0	1	0
P. corylophilum	1	0	2	0	0	0	0	0
P. crustosum	1	0	6	1	1	0	4	1
P. echinulatum	0	0	1	0	0	0	0	0
P. expansum	1	0	0	0	0	0	8	2
P. funiculosum	0	0	1	0	1	0		0
P. glabrum	6	2	8	2	2	0	5	1
P. implicatum	4	1	0	0	0	0	0	0
P. janczewskii	0	0	1	0	0	0	0	0
P. miczynskii	0	0	0	0	0	0	1	0
P. minioluteum	0	0	1	0	0	0	0	0
P. novae-zelandiae	0	0	1	0	0	0	1	0
P. oxalicum	4	1	1	0	2	0	3	1
P. purpurogenum	2	1	3	1	1	0	0	0
P. raistrickii	0	0	0	0	1	0	0	0
P. restrictum	1	0	0	0	0	0	0	0
P. roqueforti	0	0	8	2	0	0	0	0
P. sclerotiorum	0	0	2	0	2	0	0	0
P. simplicissimum	3	1	8	2	1	0	0	0
P. spinulosum	4	1	24	5	13	3	1	0
P. thomii	3	1	66	15	5	1	57	14
P. variabile	0	0	0	0	1	0	1	0
P. waksmannii	1	0	0	0	0	0	0	0

Table 2 – (*continued*)

Fungal taxa	Colonized berries							
	Alentejo		Douro		Ribatejo		Vinhos verdes	
	No.	%	No.	%	No.	%	No.	%
Phoma sp.	0	0	0	0	1	0	0	0
Pithomyces chartarum	1	0	1	0	0	0	2	1
Rhizopus sp.	3	1	29	6	49	11	49	12
Stemphylium sp.	1	0	1	0	0	0	0	0
Trichoderma sp.	5	2	2	0	5	1	13	3
Trichotecium roseum	0	0	2	0	2	0	36	9
Ulocladium sp.	14	5	1	0	3	1	1	0
Total number of strains identified	699		912		955		951	
Number of berries plated	300		450		450		400	

Nevertheless, the species richness of *Penicillium* was higher than *Aspergillus* in all regions. The most frequent *Aspergillus* strains were black *Aspergillus*, namely *A. niger* aggregate followed by *A. carbonarius*. Black *Aspergillus* alone counted for close to 85 % of the *Aspergillus* strains identified. The most frequent *Penicillium* species were *P. brevicompactum*, *P. thomii* and *P. glabrum/spinulosum* which together accounted for approximately 71 % of the strains identified in the genus.

Comparison between regions

Significant differences were detected in the incidence of *Aspergillus*, *Botrytis* and *Ulocladium*, in grape berries from distinct regions, in the *Aspergillus*, *A. niger* aggregate and in six *Penicillium* species, *P. brevicompactum*, *P. citrinum*, *P. glabrum/spinulosum*, *P. expansum*, *P. implicatum* and *P. thomii*. The number of colonized berries with these taxa is indicated in the boxplots of Fig 3.

CT models and indicator species

The best performance of the CT models was achieved by establishing three origin classes: Douro, Vinhos Verdes and South (Alentejo and Ribatejo together). The accuracy of the CT model with ten-fold cross-validation was 82 %. The species selected as indicators of the region of origin were *A. niger* and *P. thomii*. The following decision rules were generated: (1) where *A. niger* colonizes less than 8 % of grape samples ten observations occurred in the training dataset and eight were from Vinhos Verdes; (2) where *A. niger* colonizes more than 8 % of the samples and *P. thomii* colonizes less than 4 %, 15 observations occurred in the training data set and 14 were from South; (3) where *A. niger* colonizes more than 8 % of the samples and *P. thomii* colonizes more than 4 %, seven observations occurred in the training data set and all were from Douro.

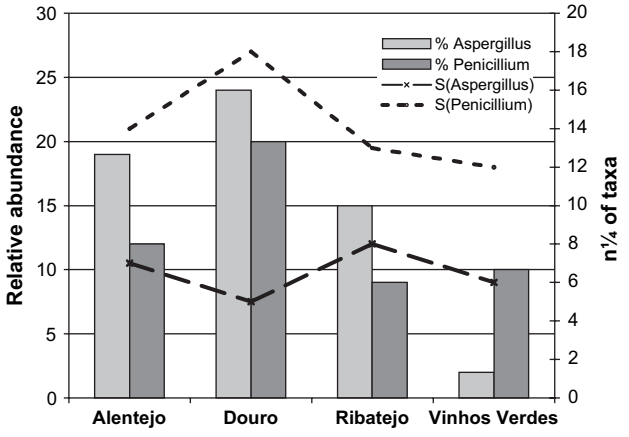

Fig 2 – Relative abundance (percent of total number of fungal strains identified in each region) and species richness (S) of *Aspergillus* and *Penicillium* in the four Portuguese winemaking regions.

A. niger incidence in samples was significantly negatively correlated with the incidence of *A. flavus*, *Alternaria*, *Botrytis* and *Cladosporium* ($r_S = -0.390$, $P < 0.05$; $r_P = -0.496$, $P < 0.01$; $r_P = -0.432$, $P < 0.05$; $r_P = -0.506$, $P < 0.01$, respectively) and significantly positively correlated with *A. carbonarius* ($r_S = 0.580$, $P < 0.001$). The incidence of *P. thomii* was significantly positively correlated with *Botrytis* and *Trichotecium roseum* ($r_S = 0.397$, $P < 0.05$; $r_S = 0.378$, $P < 0.05$, respectively).

Discussion

In this study we used plating methods without surface disinfection to detect the sporulating fungi colonizing the grape surface, as well those infecting the berry tissue. Most of the fungi found are ubiquitously distributed, such as the field fungi *Alternaria*, *Cladosporium* and *Epicoccum*, which occur commonly in the air, plant surfaces, debris and soil. Fungal species capable of causing rot in grapes (e.g. *Aspergillus niger*, *Botrytis cinerea*, *Penicillium expansum*, *Rhizopus*) were also common inhabitants of the berries surface. The geographical origin of grapes did not significantly influence the incidence of field fungi, but had a significant effect in the spoilage population, namely in *A. niger*, *B. cinerea* and *Penicillium* species. *Aspergillus* and *Penicillium* are a common component of the grapes mycobiota in all the regions. These genera are ubiquitous saprophytes whose conidia are easily distributed in the atmosphere.

A. niger aggregate was selected as an indicator species of the origin of grapes in CT models, with the samples having low *A. niger* incidence (less than 8 %) originating from Vinhos

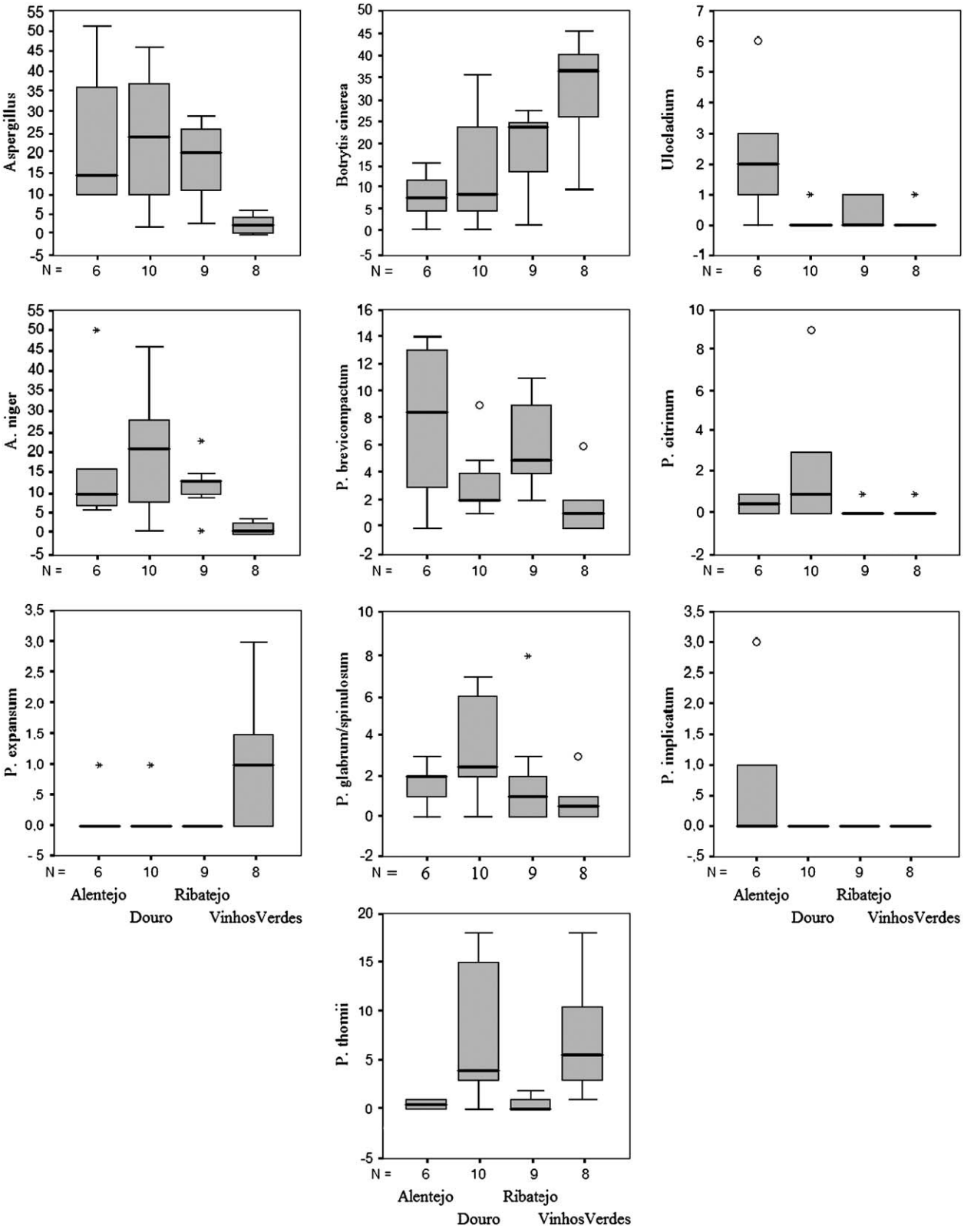

Fig 3 – Boxplots with the distribution of the incidence of fungal taxa (expressed in number of colonized berries) that differed significantly its incidence in the samples of the regions (o, outliers; *, extreme values; number of berries analysed per sample = 50).

Verdes, as opposed to grapes grown in other regions. The climate of Vinhos Verdes is unique in that it is cooler, has more rainfall, and is more humid than the other regions due to Atlantic influences. *A. niger* was significantly correlated with several species. The strongest negative correlation coefficient found was with *B. cinerea*, a species that dominates the mycobiota of the Vinhos Verdes grapes and is a very common cause of spoilage at harvest time. The incidence of *B. cinerea* in Vinhos Verdes samples (Fig 3) can be up to 90 %, which reveals the potential for spoilage when favourable conditions for berry infection occur. Ostensibly *A. niger* was significantly negatively correlated with the field fungi *Alternaria* and *Cladosporium* and with *A. flavus*. It is known that *A. flavus* and *A. niger* compete in the field (Pitt & Hocking 1997) and our results seem to corroborate this observation. The strongest positive correlation coefficient was found between *A. niger* and *A. carbonarius*. Although *A. niger* is usually much more frequent in grapes than *A. carbonarius*, and reached 100 % colonization in samples from regions with Mediterranean climate (Fig 3), both black *Aspergillus* species have similar ecological requirements occurring in grapes grown in hot and dry climates. *Aspergillus* is reported as being most frequent in warmer regions and heat-generating substrates (Domsch *et al.* 1993), and our results agree with this.

Penicillium is described as being frequent in soils and temperate regions. Unlike *Aspergillus*, no significant differences were detected in the occurrence of *Penicillium* at the genus level between regions of distinct climates. Nevertheless differences were found at the species level (Fig 3). To discriminate the regions with Mediterranean climate the model selected *P. thomii* as an indicator species. Grape samples grown in the North region Douro were separated from those grown in South based on the higher incidence of *P. thomii* in North samples. *P. thomii* was one of the most frequently isolated species from berry surfaces together with *P. brevicompactum* and the monoverticillate species *P. glabrum/spinulosum*. According to Domsch *et al.* (1993), *P. brevicompactum* is a cosmopolitan species but never particularly frequent. However, we isolated the species frequently from grape surfaces at 100 % rate in some samples (Serra *et al.* 2006,in press). *P. glabrum* is a very frequent species of worldwide distribution with rapid growth. *P. thomii* is a fast-growing species capable of forming sclerotia. All the aforementioned species share similar ecophysiological requirements as they share the ability to grow at low water activities and low temperatures (5 °C) and are unable to grow at 37 °C (Pitt & Hocking 1997). *P. brevicompactum*, *P. glabrum/spinulosum* and *P. thomii* are associated with the berry surface from the earliest stages of its development, being the most frequent *Penicillium* species isolated at all maturation stages (Serra *et al.* 2005). The three previously mentioned species increase their numbers as maturation advances, which indicate that they may have a role in the vineyard as active decomposers. *P. brevicompactum* and *P. thomii* seem to compete on the field as one is most frequent in grape samples originating from regions where the other is infrequently isolated (Fig 3). *Penicillium* species produce a vast array of secondary metabolites and very little is known about their field interactions with other species. Ostensibly *P. thomii* was weakly positively correlated with *B. cinerea* and *T. roseum*, also abundant in the regions where *P. thomii* was most frequently isolated. *T. roseum* strains were found growing on rotten bunches over *Botrytis* mycelium (Serra *et al.* 2005) being isolated more frequently from samples where the incidence of *Botrytis* was higher.

No studies are available in the literature for comparison of the mycobiota of grapes grown in CIa regions, but detailed mycobiota studies were performed in Spanish grapes from CIIIb regions using the same methodology as our study (Bau *et al.* 2005). The most notable differences found were in the incidence of *Botrytis*, *Cladosporium* and *Penicillium*. The most frequent fungi found at harvest time in Spanish vineyards were *Alternaria* (*ca* 60 % of the berries) and *Aspergillus* (*ca* 40 %), while the incidence of *Botrytis*, *Cladosporium* and *Penicillium* was less than 2 % of the berries. However, black *Aspergillus* spp. were also the predominant species isolated from Spanish vineyards, namely *A. niger* (75 % of the isolated strains) and *A. carbonarius* (16 %).

The relevance of the species isolated for mycotoxin production has been discussed in detail elsewhere (Serra *et al.* 2005). Concerning ochratoxin A the main species found is *A. carbonarius*, although other species may occasionally produce the toxin in smaller amounts.

Conclusion

We confirmed in this study that the origin of grapes markedly influences the spoilage fungal population to which berries are exposed. Both *Aspergillus* and *Penicillium* are important components of the spoilage mycobiota of grapes but *Aspergillus* are important in hot and dry climates, where black *Aspergillus*, namely *A. carbonarius*, can lead to ochratoxin A production in grapes. In more humid climates, *Botrytis* seems to be the main pathogen and spoiling agent, and the incidence of black *Aspergillus* is minimal. These results support the theory that areas at risk of mycotoxin contamination can be defined based on the mycobiota, and contribute to the knowledge of field ecology of *Aspergillus* and *Penicillium* species in the vineyard environment.

Acknowledgments

The authors gratefully acknowledge the support of the EC, Quality of Life Program (QoL), Key Action 1 (KA1) on Food, Nutrition and Health; contract number QLK1-CT-2001-01761-Wine-Ochra Risk. R.S., A.L. and P.A. were supported from Fundação para a Ciência e Tecnologia by grants SFRH/BPD/2827/2004, SFRH/BD/8242/2002 and SFRH/BD/17579/2004, respectively. Authors also acknowledge the English revision by Stephen W. Peterson.

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