Black *Aspergillus* species: implications for ochratoxin A in Australian grapes and wine

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Statement of originality

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Abstract

Ochratoxin A (OA), a nephrotoxin and potential carcinogen, has been found in many foods, including grapes and grape products. Limits of 2 µg/kg in wine and 10 µg/kg in dried vine fruit have been introduced by the European Union. This study presents information on the ecology of ochratoxin A production by black *Aspergillus* spp. in Australian vineyards, and the passage of the toxin throughout winemaking.

Aspergillus niger and *A. carbonarius* were isolated from vineyard soils in 17 of 17, and four of 17 Australian viticultural regions, respectively. *A. aculeatus* was isolated infrequently. All thirty-two isolates of *A. carbonarius* and three of 100 isolates of *A. niger* produced OA. Of Australian *A. niger* isolates analysed for restriction fragment length polymorphisms within the internal transcribed spacer region of 5.8S ribosomal DNA, 61 of 113 isolates, including the three toxigenic isolates, were of type N pattern, and 52 were type T. A selection of these *A. carbonarius* and *A. niger* aggregate isolates, as well as imported isolates, were compared using enterobacterial repetitive intergenic consensus (ERIC)-PCR, amplified fragment length polymorphisms (AFLP) and microsatellite markers. ERIC and AFLP clearly differentiated *A. niger* from *A. carbonarius*. AFLP further divided *A. niger* into types N and T. Six polymorphic microsatellite markers, developed specifically for *A. niger*, also differentiated strains into N and T types. There was no clear relationship between genotypic distribution and ochratoxigenicity, substrate or geographic origin.

The survival of *A. carbonarius* spores on filter membranes was examined at water activities (a_w) 0.4-1.0, and at 1 °C, 15 °C, 25 °C and 37 °C. Survival generally increased at lower temperatures. The lowest water activity, 0.4, best supported the survival of spores, but 0.6-0.9 a_w was often deleterious. Complex interactions between temperature and water activity were observed. Viability of *A. carbonarius* spores on filter membranes decreased *ca* 10^5 fold upon exposure to sunlight, equivalent to 10 mWh of cumulative ultraviolet irradiation at 290-400 nm. Growth and toxin production were examined for five isolates of *A. carbonarius* and two of *A. niger* on solid medium simulating juice at early veraison, within the range 0.98-0.92 a_w, and at 15 °C, 25 °C, 30 °C and 35 °C. Maximum growth for *A. carbonarius* and *A. niger* occurred at *ca* 0.965 a_w / 30 °C and *ca* 0.98 a_w / 35 °C, respectively. The optimum temperature for OA production was 15 °C and little was produced above 25 °C. The optimum a_w for toxin production was 0.95 for *A. niger* and 0.95-0.98 for *A.*

carbonarius. Toxin was produced in young colonies, however, levels were reduced as colonies aged.

Black *Aspergillus* spp. were more commonly isolated from the surface than from the pulp of berries, and increased with berry maturity, or damage. *A. niger* was isolated more frequently than *A. carbonarius* and *A. aculeatus.* Populations of *A. carbonarius* inoculated onto bunches of Chardonnay and Shiraz decreased from pre-bunch closure to early veraison. Populations from veraison to harvest were variable, and increased in bunches with tight clustering and splitting. In a trial with Semillon bunches, omitting fungicide sprays after flowering did not increase the development of Aspergillus rot. Inoculation of bunches with *A. carbonarius* spore suspension did not necessarily result in Aspergillus bunch rot. *In vitro* trials suggested that the severity of rot was mediated primarily by the degree of berry damage, followed by the extent of spore coverage. No clear trends regarding cultivar susceptibility were observed. For Semillon bunches inoculated with *A. carbonarius* spores with and without berry puncture, increased susceptibility to rot and OA formation was associated with berry damage, in particular at greater than 12.3 °Brix (20 d before harvest). OA contamination of bunches was related to the number of mouldy berries per bunch, with shrivelled, severely mouldy berries the primary source of OA.

Puncture-inoculation of white grapes (Chardonnay and Semillon) and red grapes (Shiraz) on the vine with *A. carbonarius* resulted in berries containing OA. Inoculated grapes displayed greater total soluble solids due to berry shrivelling, and greater titratable acidity due to production of citric acid by the fungus. Samples taken throughout vinification of these grapes were analysed for OA. Pressing resulted in the greatest reduction in OA (68-85% decrease in concentration, compared with that of crushed grapes). Additional reductions occurred at racking from grape and gross lees, and after storage. OA was removed by binding to marc, grape and gross lees. Pectolytic enzyme treatment of white must, bentonite juice fining, recovery of juice or wine from lees, and static or rotary style fermentation of red must, had no effect on OA contamination. Bentonite in white wine (containing 56 mg/L grape-derived proteins) and yeast hulls in red wine were effective fining agents for removing OA.

Findings from these studies may contribute to the improvement of strategies to minimise OA in Australian wine and dried vine fruit.

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Publications arising from this project

Esteban, A., Leong, S.L., Tran-Dinh, N., 2005. Isolation and characterization of six polymorphic microsatellite loci in *Aspergillus niger*. Molecular Ecology Notes 375-377.

Leong, S.L., 2006. Wine and fungi - implications of vineyard infections. In: Dijksterhuis, J., Samson, R.A. (Eds), New challenges in Food Mycology. Marcel Dekker Inc., New York, in press.

Leong, S.L., Hocking, A.D., Pitt, J.I., Kazi, B.A., Emmett, R.W., Scott, E.S., 2006. Australian research on ochratoxigenic fungi and ochratoxin A. International Journal of Food Microbiology, in press.

Leong, S.L., Hocking, A.D., Pitt, J.I., Kazi, B.A., Emmett, R.W., Scott, E.S., 2006. Black *Aspergillus* spp. in Australian vineyards: from soil to ochratoxin A in wine. In: Hocking, A.D., Pitt, J.I., Samson, R.A., Thrane, U. (Eds.), Advances in Food Mycology. Springer, New York, 153-171.

Leong, S.L., Hocking, A.D., Scott, E.S., 2006. Survival and growth of *Aspergillus carbonarius* on wine grapes before harvest. International Journal of Food Microbiology, in press.

1 Introduction

1.1 Rationale for the project

Fungi classified in *Aspergillus* section *Nigri* (the black aspergilli) are ubiquitous saprophytes in soils around the world, particularly in tropical and subtropical regions (Klich and Pitt, 1988; Pitt and Hocking, 1997). Several species in this section are common in vineyards and are often associated with bunch rot of grapes (Amerine et al., 1980). Within this group, *A. carbonarius* and *A. niger* have been shown to produce the mycotoxin, ochratoxin A (OA) (Abarca et al., 1994; Téren et al., 1996). OA is a demonstrated nephrotoxin, which may also be carcinogenic, teratogenic, immunogenic and genotoxic. It has been classified by the International Agency for Research on Cancer (IARC) as Group 2B, a "possible human carcinogen" (Castegnaro and Wild, 1995).

OA has been detected in grapes and grape products including juice, wine, dried vine fruit and wine vinegars (Zimmerli and Dick, 1996; MacDonald et al., 1999; Majerus et al., 2000; Markarki et al., 2001; Da Rocha Rosa et al., 2002; Sage et al., 2002). A survey of 600 Australian wines showed that OA was present only at low concentrations. Fifteen percent of samples had more than 0.05 µg/L and 85% of these were less than 0.2 µg/L. The maximum found was 0.61 µg/L (Hocking et al., 2003). The European Union has introduced limits for OA in dried vine fruits and wine of 10 µg/kg (ppb) and 2 µg/kg, respectively (European Commission, 2005).

It is now widely accepted that OA contamination of wine and other grape products is a result of the growth of toxigenic *A. carbonarius* and *A. niger* in grapes [reviewed by Abarca et al. (2004)]. Toxigenic isolates of *A. carbonarius* have been obtained from Australian grapes grown for dried vine fruit production (Heenan et al., 1998; Leong et al., 2004). However, the occurrence of such fungi in the wine grape vineyards of Australian viticultural regions has not yet been examined. Little is known about the ecology of the black aspergilli in Australian vineyards, in particular, the effects of environmental factors on their survival, infection, growth and ability to produce OA in grapes. The passage of OA from grapes into wine is also not well understood. This project was undertaken to investigate these factors.

1.2 Toxicity of ochratoxin A

OA was first identified as a secreted toxic metabolite of *Aspergillus ochraceus* (van der Merwe et al., 1965), a species belonging to section *Circumdati.* Other members of this section, as well as sections *Aspergillus*, *Flavi, Fumigati* and *Nigri*, have been reported to produce OA [reviewed by Abarca et al. (2001); Varga et al. (2001b)]. *Penicillium verrucosum* and its close relative, *P. nordicum*, are the only species in the genus *Penicillium* in which a significant proportion of strains consistently produce OA (Pitt, 1987; Larsen et al., 2001; Castellà et al., 2002), although production by other species has been reported [reviewed by Varga et al. (2001b)].

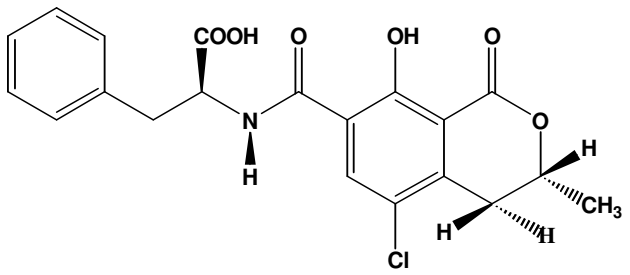


Figure 1.1: Ochratoxin A

OA contains an isocoumarin ring linked to L-phenylalanine through an amide bond (Fig. 1.1), and is nephrotoxic to humans and animals, as well as displaying alternative modes of toxicity [reviewed by Marquardt et al. (1990); Kuiper-Goodman (1996); Creppy (1999); Petzinger and Ziegler (2000); Pfohl-Leszkowicz et al. (2002); Pitt and Tomaska (2002); O'Brien and Dietrich (2005)]. The nephrotoxicity of OA to birds and mammals, with the exception of ruminants, is well documented (Pfohl-Leszkowicz et al., 2002). In particular, OA plays a major role in the etiology of porcine nephropathy (Krogh et al., 1973). In humans, OA binds to serum albumen in blood, where it has a long half-life of elimination (840 h) (Petzinger and Ziegler, 2000). It has been detected in blood, breast milk and kidneys. OA has been linked to the chronic nephropathic condition Balkan Endemic Nephropathy (BEN) in Bulgaria and the former Yugoslavia, but its role in the etiology is still not clear (Pfohl-Leszkowicz et al., 2002). Other possible etiological agents of BEN have been raised, such as the presence or absence of metals, other elements and minerals, infection with bacteria or viruses, or the synergistic action of fumonisins (Jurjevic et al., 1999) or citrinin (Vrabcheva et al., 2000) with OA.

OA damages cells of the immune system (is immunotoxic), as demonstrated in animals and in human lymphocyte cell culture (Pfohl-Leszkowicz et al., 2002), and is also thought to damage DNA (genotoxic). The genotoxicity of OA is a matter of some controversy, as various standard tests for genotoxicity using prokaryotes were negative or only slightly positive. However, genotoxicity has since been demonstrated in animal and human cell culture, and also observed *in vivo* in the DNA of mice injected or fed with OA (Pfohl-Leszkowicz et al., 2002).

OA is carcinogenic in rats and mice, causing the development of renal cancers. The carcinogenicity of OA in humans has not been proven, although there is a correlation between BEN and urinary tract tumours, leading some to suggest that the two should be considered a single syndrome with the same etiology (Pfohl-Leszkowicz et al., 2002). A role for OA in testicular cancer has also been hypothesised (Schwartz, 2002). Based on this information, IARC has classified OA as a group 2B carcinogen, meaning that it is *possibly* carcinogenic for humans, but not yet *probably* carcinogenic (group 2A) (Castegnaro and Wild, 1995). Clarification of the genotoxicity of OA to human cells may lead to a re-classification into group 2A.

The Provisional Tolerable Daily Intake (PTDI) for OA has been proposed within the range 1.2-14 ng/kg body weight, the latter figure being calculated based on nephrotoxicity data [reviewed by Pitt and Tomaska (2002)]. The European Union Scientific Committee for Food continues to support a PTDI of 5 ng/kg body weight (European Commission, 2002), and this value was used by Pitt and Tomaska (2002) to assess the extent of exposure to OA for Australian consumers. However, Petzinger and Ziegler (2000) argued for a lower PTDI based on the unfavourable toxicokinetics of OA in humans, particularly its persistence in human blood. Ingestion of contaminated food may not be the sole means of exposure to OA; inhalation of dust (Di Paolo et al., 1994; Richard et al., 1999; Iavicoli et al., 2002) or fungal spores (Skaug et al., 2000) containing OA may represent alternative means of exposure.

Several compounds have been tested for their ability to reduce the various toxic effects of OA. Groups of compounds tested include phenylalanine and analogues such as aspartame, which prevent OA binding to plasma proteins; antioxidants such as vitamin C and vitamin E; and adsorbents such as cholestyramine, which enhance faecal excretion of OA by binding to it in the gastrointestinal tract before it enters the bloodstream [reviewed by Creppy (1999); Varga et al. (2001a)]. Extracts from grape berries and leaves were shown to moderate the haematological, hepatic and renal effects of OA in mice, possibly due to the vitamins present in grapes (Jeswal, 1998).

1.3 Occurrence of ochratoxin A

The presence of OA has been reported in a number of foods, including cereals (for human and animal consumption) and cereal products such as bread, biscuits, pasta and muesli [reviewed by Creppy (1999); Cholmakov-Bodechtel et al. (2000a); Varga et al. (2001b); Pitt and Tomaska (2002)]. OA has also been detected in nuts, beans, oil seeds, soy products, coffee, wine, beer, pork, poultry, dried fruits, cocoa powder and chocolate, liquorice, fruit juices and vinegars, ciders, sauces, mustards, ketchup, chillis and spices (Patel et al., 1996; Zimmerli and Dick, 1996; MAFF, 1997; Ueno, 1998; Burdaspal and Legarda, 1999; MAFF, 1999; Bresch et al., 2000; Engel, 2000; Majerus et al., 2000; Filali et al., 2001).

Though OA may be found occasionally in a wide variety of foods, the relative contribution by each food type to OA in the diet is often insignificant, given the amounts of that food consumed. The primary sources of OA in an Australian diet are wine, vine fruits and coffee, and their relative contributions to OA exposure are shown in Table 1.1.

Table 1.1: Estimates of ochratoxin A exposure for Australian consumers (taken from Pitt and Tomaska (2002))

Food	Mean exposure[a] (µg/kg)	Intake for Australian population (g/d)[b]	Mean OA intake (ng/d)[c]	Proportion of PDTI (%)
POPULATION (age 2 years and over)				
Wine	0.1	43.5	4	1.3
Vine fruits	2.5	4	10	3.3
Coffee	1	3.4	3	1.0
Total			*17*[d]	5.6
CONSUMERS				
Wine	0.1	321	32	10.7
Vine fruits	2.5	8.8	22	7.3
Coffee	1	7.1	7	2.3
Total			*61*	20.3
95TH PERCENTILE CONSUMER				
Wine	0.1	775	78	26
Vine fruits	2.5	30	75	25
Coffee	1	18.2	18	6
Total			*171*	57

[a] data for exposure taken from MAFF (1997) in the UK. Cereals, an important source of OA consumption in Europe have been omitted here in the absence of evidence for the occurrence of OA in Australian cereals. Intake data for food consumption taken from Australian Total Diet Survey (ANZFA, 1996). A PDTI of 5 ng/kg body weight has been used, equivalent to 300 ng/d for a 60 kg individual. The estimate assumes that all food consumed contains OA at the mean concentration, an overestimate of intake

[b] mean intake of wine, vine fruits and coffee for the total population age 2 years and over, mean intake for consumers of those foods, and intake for an individual consuming amounts greater than 95% of other consumers

[c] multiplication of columns two and three gives the mean daily intake of OA by individuals

[d] figures summed are in italics

Exposure to OA in European countries potentially represents a greater risk than in Australia, due to OA contamination of cereals and cereal products, and the greater consumption of cereal products in Europe. Cereal-based foods contributed 40-50% of the total dietary intake of OA by German adults. These were followed in importance by coffee (14.5%) and beer (9.8%) (Cholmakov-Bodechtel et al., 2000b). The contribution of wine and champagne was negligible for the median consumer (1.4%), but increased to more than 11.3% for the top 10% of consumers, a significant source of exposure to OA. Exposure of children to OA was primarily through fruit juices, especially red grape juice (15.4%) and sweets such as chocolate, cereal-based bars and biscuits (9.9%). The mean daily exposure for adults in Germany was 39.9 ng; this figure is lower than the Australian estimate in Table 1.1, because the German figures

were calculated using the median of OA concentration which is typically lower than the mean concentration, as the concentrations of OA in food surveys are typically skewed towards lower values. An individual on the 90th percentile of the bell-shaped curve for consumption of foods containing OA in the German study was exposed to higher levels of OA than an individual on the 95th percentile of a similar bell-shaped curve in Australia (247.9 ng/d *cf* 171 ng/d). Recently, Sizoo and Van Egmond (2005) estimated from a study of actual diets in the Netherlands that the daily OA intake was 72 ng/d, an amount below the tolerable daily intake proposed by Pitt and Tomaska (2002). Nevertheless, OA continues to be of great concern to the European Union, which has introduced maximum levels for OA of 5 µg/kg in raw cereals, 3 µg/kg in cereal products, 5 µg/kg in coffee beans, 10 µg/kg in instant coffee, 10 µg/kg in dried vine fruits, 2 µg/kg in wine and grape juice, and 0.5 µg/kg in baby foods (European Commission, 2002, 2005). Countries exporting products such as wine and dried vine fruits to Europe must invest in strategies to ensure that their products consistently meet the above limits.

Several surveys of OA in wine have been published, and these are summarised in Fig. 1.2 and Table 1.2. Wines from most of the major viticultural regions of the world were represented in these surveys, but the number of red wines assayed far exceeded that of white and rosé wines. The range of OA concentrations reported varied among surveys, depending on the extraction and assay technique (differing limits of detection) and source of the wine. The highest concentration of OA reported to date in wine is 15.25 µg/L, in mistelle from Spain (Bellí et al., 2004a), followed by 7.63 µg/L, in a commercial red wine from Italy (Visconti et al., 1999). In most of the surveys of wines produced commercially, the incidence and/or mean concentration of OA contamination was higher in red wines than in white wines (Fig. 1.2, Table 1.2). Zimmerli and Dick (1996) postulated that this could be an effect of viticultural region, with red grapes more commonly grown in warm climates than white grapes. However, Otteneder and Majerus (2000) demonstrated that red wines from cool regions (Germany and France) contained higher levels of OA than white wines from the same regions. They suggested that the primary source of difference was in processing, i.e. white grapes are pressed off skins immediately, whereas crushed red grapes may be put aside for several days before fermentation.

OA was detected most frequently in wines from Mediterranean countries and northern Africa (Fig. 1.2), following a trend for increased prevalence in wines from southern (warmer) regions compared with northern regions in the Northern hemisphere (Majerus and Otteneder, 1996; Zimmerli and Dick, 1996; Ospital et al., 1998; Otteneder and Majerus, 2000; Markarki et al., 2001; Pietri et al., 2001). Occurrence of OA in wines from the so-called "new world" (USA, Canada, South America, South Africa, Australia, New Zealand) was low. In wines from Australia and South Africa, no obvious differences were observed between OA in red and white wines, and wines from warmer areas did not show increased contamination rates (Stander and Steyn, 2002; Hocking et al., 2003; Leong et al., 2005a).

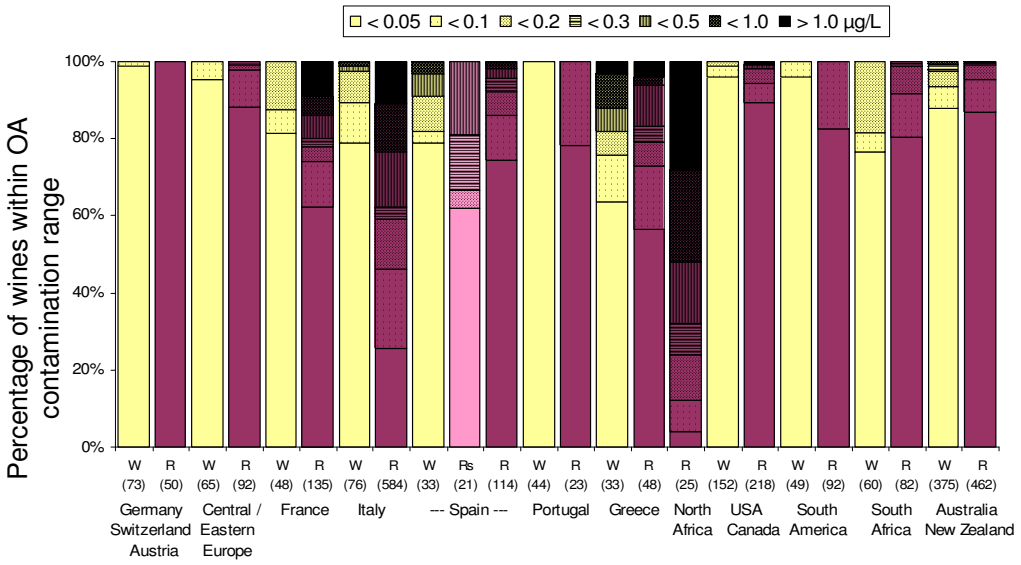


Figure 1.2: Incidence and degree of ochratoxin A contamination in wines produced in viticultural regions worldwide. White wines (W), red wines (R), rosé (Rs); the cumulative number of wines tested for each region from various surveys is given in brackets. Dessert and fortified wines were not included. Data presented here were calculated from Majerus and Otteneder (1996), Zimmerli and Dick (1996), MAFF (1997), Ospital et al. (1998), MAFF (1999), Tateo et al. (1999), Visconti et al. (1999), Castellari et al. (2000), Festas et al. (2000), Tateo et al. (2000), Filali et al. (2001), Markarki et al. (2001), Pietri et al. (2001), Soleas et al. (2001), Tateo and Bononi (2001), Eder et al. (2002), Stander and Steyn (2002), Hocking et al. (2003), Micheli et al. (2003), Siantar et al. (2003), Soufleros et al. (2003), Tateo and Bononi (2003), Blesa et al. (2004), Ng et al. (2004), Ratola et al. (2004), Rosa et al. (2004), Czerwiecki et al. (2005) and Leong et al. (2005a)

Table 1.2: Prevalence of ochratoxin A in wines

Citation	Limit of detection (µg/L)	Wines assayed	% Samples in which OA was detected	Mean of positives (µg/L)	Mean[a] (µg/L)	Median (µg/L)	Range (source of maximum value, where known) (µg/L)	Country of origin
Majerus and Otteneder (1996)	0.01	41 white 14 rosé 89 red	34 43 45	n.r.[b] n.r. n.r.	n.r. n.r. n.r.	0.07 0.1 0.19	< 0.01-1.20 < 0.01-2.40 < 0.01-7.00 (Italy)	Australia, Chile, France, Germany, Greece, Italy, Macedonia, Moldova, Portugal, South Africa, Spain, Tunisia, USA
Zimmerli and Dick (1996)	0.003 LOQ[c] 0.005	24 white 15 rosé 79 red	27[d] 92[d] 78[d]	n.r. n.r. n.r.	0.011[e] 0.025[e] 0.039[e]	< 0.003 0.019 0.013	< 0.003-0.178 (France) < 0.003-0.123 (France) < 0.003-0.388 (Tunisia)	Argentina, France, Italy, North Africa, Portugal, South Africa, Spain, Switzerland
MAFF (1997)	0.2	10 white 10 red	0 40	n.d.[f] 1.0	0.1 0.4	< 0.2 < 0.2	< 0.2 < 0.2-1.1 (France)	France, Italy, Spain
Ospital et al. (1998)	0.01	4 white 2 rosé 20 red	25 50 60	0.016 0.110 0.083	0.008 0.058 0.052	< 0.01 0.060 0.015	< 0.01-0.016 (France) < 0.01-0.11 (France) < 0.01-0.27 (France)	France, Portugal (2), Spain (1)
Ueno (1998)	0.003	5 white 5 rosé 36 red	20 60 42	0.006 0.024 0.052	0.002 0.015 0.023	< 0.003 n.r. < 0.003	< 0.003-0.006 (Germany) < 0.003-0.037 < 0.003-0.245 (France)	Australia, Chile, France, Germany, Italy, Japan, South Africa, USA
Burdaspal and Legarda (1999)	0.003	69 white 32 rosé 91 red 12 sparkling	45 29 84 83	n.r. n.r. n.r. n.r.	0.020[g] 0.031[g] 0.054[g] 0.012[g]	< 0.003 < 0.003 n.r. n.r.	< 0.003-0.267 (Spain) < 0.003-0.161 (France) < 0.003-0.603 (Spain) < 0.003-0.037 (Spain)	France, Germany, Hungary, Italy, Portugal, Spain, USA
MAFF (1999)	0.01	50 red	56	0.13	0.08	0.02	< 0.01-0.8 (France)	Argentina, Australia, Bulgaria, Chile, Italy, France, South Africa, Spain, USA

Tateo et al. (1999)	0.012	2 red	100	0.13	0.13	0.13	0.09-0.17	Italy (bottle & cask)
Visconti et al. (1999)	0.01	9 white 8 rosé 38 red	44 88 97	0.29 0.72 1.24	0.13 0.63 1.21	< 0.01 < 0.01 < 0.01	< 0.01-0.97 < 0.01-1.15 < 0.01-7.63	Italy (commercial & home-made)
Castellari et al. (2000)[h]	0.015	2 white 9 red	100[i] 100[i]	0.10 2.34	0.10 2.34	0.10 2.22	0.10 (Italy & France) 0.03-4.90 (Italy)	Italy (8), France, Spain, USA
Festas et al. (2000)	0.02	30 Vinho Verde	0	n.d.	0.01	< 0.02	< 0.02	Portugal
Otteneder and Majerus (2000)	0.01	60 white 55 rosé 305 red	25 40 54	0.40 0.28 0.36	0.10 0.12 0.20	< 0.01 < 0.01 0.02	< 0.01-1.36 (Sth Europe) < 0.01-2.38 < 0.01-7.0 (Sth Europe)	Worldwide
Tateo et al. (2000)	0.013 LOQ 0.06	31 red	100	1.30	1.30	0.97	< 0.065-3.80	Italy (casks)
Filali et al. (2001)	0.01	7 white 3 rosé 20 red	100 100 100	0.073 0.22 0.91	0.073 0.22 0.91	0.048 0.090 0.79	0.028-0.18 0.04-0.54 0.04-3.24	Morocco
Markarki et al. (2001)	0.002	31 red	100	n.r.	n.r.		0.002-3.40 (France)	France, Greece, Italy, Morocco, Spain-Portugal
Pietri et al. (2001)	0.001	96 red 15 white dessert	85 60	0.490 1.226	0.419 0.736	0.090 0.008	< 0.001-3.177 < 0.001-3.856	Italy
Soleas et al. (2001)	0.05	362 white 580 red	4 17	n.r. n.r.	n.r. n.r.	< 0.05 < 0.05	< 0.05-0.10 < 0.05-0.20 (Australia)	Argentina, Australia, Canada, Chile, Central Europe, France, Greece, Germany, Italy, New Zealand, Portugal, Spain, USA
Tateo and Bononi (2001)	0.013 LOQ 0.06	2 white 18 red	100 100	0.07 0.65	0.07 0.65	n.r. 0.36	< 0.06-0.10 (France) < 0.06-4.83 (Italy)	Italy (bottle), France (1 white)
Eder et al. (2002)	0.010	25 white 17 sweet white 32 red	0 0 3	n.d. n.d. 0.020	0.005 0.005 0.005	< 0.01 < 0.01 < 0.01	< 0.01 < 0.01 < 0.01-0.02	Austria

Table 1.2 (cont.)

Citation	Limit of detection	Wines assayed	% Positives	Mean of positives	Mean[a] (µg/L)	Median (µg/L)	Range (source of maximum) (µg/L)	Country of origin
Lopez de Cerain et al. (2002)	0.05	12 white 28 red	58 46	0.186 0.156	0.119 0.086	n.r. < 0.05	< 0.05-0.208 < 0.05-0.316	Spain (from experimental vineyards)
Stander and Steyn (2002)	0.01	15 white (inexp.)[j] 16 red (inexp.) 5 dessert (inexp.) 27 white (exp.)[j] 49 red (exp.) 3 dessert (exp.)	40 63 80 52 18 100	n.r. n.r. n.r. n.r. n.r. n.r.	n.r. n.r. n.r. n.r. n.r. n.r.	< 0.01 0.014 0.130 n.r. n.r. 1.732	< 0.01-0.195 < 0.01-0.217 < 0.01-0.270 < 0.01-0.1 < 0.01-0.126 < 0.01-2.672	South Africa
Hocking et al. (2003)	0.02 LOQ 0.05	257 white 344 red	16[c] 14[c]	0.11 0.12	0.04 0.04	< 0.05 < 0.05	< 0.05-0.5 < 0.05-0.62	Australia (bottle and cask)
Micheli et al. (2003)	0.01	6 red	100	0.145	0.145	0.145	0.06-0.26	Italy (organic wine)
Shephard et al. (2003)	0.01	8 white 7 white dessert 9 red 3 white 5 red	100 100 100 100 100	0.19 0.12 0.25 0.05 0.58	0.19 0.12 0.25 0.05 0.58	n.r. n.r. n.r. n.r. n.r.	0.04-0.33 0.06-0.18 0.07-0.39 0.01-0.08 0.23-0.91	South Africa (bottle and cask) Italy
Siantar et al. (2003)	0.01	27 white 1 rosé 54 red	26 0 33	0.010 n.d. 0.112	0.006 0.005 0.041	< 0.01 n.r. < 0.01	< 0.01-0.08 n.r. < 0.01-1.68	USA
Soufleros et al. (2003)	0.02	17 white 1 rosé 17 red	65 0 65	0.612 n.d. 0.627	0.399 0.010 0.409	0.08 < 0.02 0.14	< 0.02-3.20 < 0.02 < 0.02-2.51	Greece (dry and sweet wines)

Stefanaki et al. (2003)	0.05	118 white 20 rosé 104 red 18 dessert 8 retsina	53 65 68 83 75	0.45 0.25 0.49 0.69 0.78	0.25 0.17 0.34 0.58 0.59	0.06 0.08 0.09 0.33 0.27	< 0.05-1.72 < 0.05-1.16 < 0.05-2.69 < 0.05-2.82 < 0.05-1.75	Greece
Tateo and Bononi, (2003)	0.013 LOQ 0.06	4 white 76 red	0 95	n.d. 0.27	0.007 0.26	< 0.013 0.12	< 0.013 (Italy) < 0.013-2.90 (Italy)	Italy, Chile (1), France(1)
Bellí et al. (2004a)	0.05	50 white 130 red 10 sparkling	8 18 40	0.40 0.60 0.44	0.06 0.13 0.19	< 0.05 < 0.05 < 0.05	< 0.05-1.13 < 0.05-4.24 < 0.05-0.71	Spain
Blesa et al. (2004)	0.01	24 white 21 rosé 61 red	17 57 34	0.41 0.20 0.17	0.07 0.12 0.06	< 0.01 ~ 0.03 < 0.01	< 0.01-0.76 < 0.01-0.46 < 0.01-0.53	Spain
Ng et al. (2004)	white 0.004 red 0.008	43 white (Can.)[k] 36 red (Can.) 53 white (imp.)[k] 48 red (imp.)	23 14 42 54	0.038 0.125 0.281 0.383	0.010 0.021 0.118 0.209	< 0.004 < 0.008 < 0.004 n.r.	< 0.004-0.156 < 0.008-0.393 < 0.004-3.720 (Greece) < 0.008-2.320 (Italy)	Canada Algeria (1), Cyprus (1), France, Greece, Italy, Spain, Turkey (2), USA
Ratola et al. (2004)	0.084	189 Port, 85 Vinho verde, 66 other	20 total	all but two < 0.5	n.r.	< 0.084	< 0.084-2.1	Portugal
Rosa et al. (2004)	0.021	15 white (S. Am.)[l] 5 rosé (S. Am.) 22 red (S. Am.) 18 white (Europe) 20 red (Europe)	13 20 32 22 45	0.028 0.035 0.039 0.024 0.034	0.013 0.015 0.020 0.013 0.021	< 0.021 < 0.021 < 0.021 < 0.021 < 0.021	< 0.021-0.028 (Brazil) < 0.021-0.035 (Brazil) < 0.021-0.071 (Chile) < 0.021-0.028 (Portugal) < 0.021-0.057 (Portugal)	Argentina, Brazil, Chile France, Italy, Portugal, Spain
Berente et al. (2005)	0.024	5 white 5 red 57, type not stated	0 0 0	n.d. n.d. n.d.	n.d. n.d. n.d.	< 0.024 < 0.024 < 0.024	< 0.024 < 0.024 < 0.024	Hungary

Table 1.2 (cont.)

Citation	Limit of detection	Wines assayed	% Positives	Mean of positives	Mean[a] (µg/L)	Median (µg/L)	Range (source of maximum) (µg/L)	Country of origin
Brera et al. (2005)	0.01	21 white (Italy)	19	n.r.	n.r.	< 0.01	< 0.01-0.21	Hungary, Italy
		9 rosé (Italy)	56	n.r.	n.r	n.r.	< 0.01-1.04	
		159 red (Italy)	84	n.r.	n.r.	n.r.	< 0.01-4.00	
		19 dessert (Italy)	63	n.r.	n.r.	n.r.	< 0.01-1.64	
		30 white (Hng.)[m]	0	n.d.	n.d.	< 0.01	< 0.01	
		2 rosé (Hng.)	0	n.d.	n.d.	< 0.01	< 0.01	
		27 red (Hng.)	0	n.d.	n.d.	< 0.01	< 0.01	
Czerwiecki et al. (2005)	0.0005	53 red	92	n.r.	0.039[g]	0.478	< 0.0005-6.710 (France)	Argentina, Bulgaria, Croatia, France, Germany, Greece, Hungary, Italy, Romania, Spain
Lin et al. (2005)	0.2	10 red	50	0.3	0.2	n.r.	< 0.2-0.5	Italy, Taiwan (1 of 2 tested was positive)

[a] mean OA concentrations calculated assuming any results below the limit of detection contained OA at half the limit of detection, unless otherwise noted
[b] n.r.: not reported
[c] LOQ: limit of quantification
[d] positive samples contained OA above the limit of quantification
[e] mean as reported, samples below limits of quantification and detection were defined as having the limiting values
[f] n.d.: not detected
[g] mean as reported, values attributed to samples below limits of detection unknown
[h] results from reference method (pre-extraction with chloroform followed by clean-up with Ochraprep columns)
[i] OA positive samples chosen for this study
[j] (inexp.) refers to wines of low to medium price purchased locally; (exp.) refers to higher priced, export-quality wines
[k] Canadian (Can.) and imported (imp.) wines
[l] South American (S. Am.) wines
[m] Hungarian (Hng.) wines

Australian wines have been included in international surveys for the presence of OA (Table 1.3). Hocking et al. (2003) completed an extensive survey of Australian wines, testing 257 white and 344 red wines, including dessert and sparkling wines. Less expensive wines, such as those sold in plastic-lined cardboard boxes (casks) were also included in the survey. OA was detected at greater than 0.05 µg/L in 16% of white wines and 14% of red wines, but 85% of these positive samples were below 0.2 µg/L. The highest concentration observed was 0.62 µg/L, in a bottle of red "lambrusco"-style wine. No single grape variety showed markedly higher OA contamination than any other, though the incidence of contamination in Shiraz and Shiraz blends was slightly greater than in Cabernet Sauvignon and Cabernet blends. Among the whites, Riesling and Riesling blends showed a slightly higher contamination rate than Chardonnay, Semillon or Sauvignon Blanc. Generally, unnamed blends and styles such as "dry red", "lambrusco", or "sweet white" had a higher incidence of contamination. These blends were often lower quality bottled wines or those packaged in casks. Cask wines were more likely to contain OA than bottled wines (38% and 15% positive, respectively); this trend was also noted for wines in Italy and South Africa (Tateo et al., 2000; Tateo and Bononi, 2001; Stander and Steyn, 2002; Tateo and Bononi, 2003).

Table 1.3: Prevalence of ochratoxin A (µg/L) in Australian wines

Wine	Maximum OA	Number of samples in the concentration range				Citation
		< 0.05	0.05 - < 0.1	0.1 - < 0.2	≥ 0.2	
Red	0.22				1	Majerus and Otteneder (1996)
Red	n.r.[a]	3				Ueno (1998)
Red	0.05	4	1			MAFF (1999)
Red	0.2	36	8		1	Soleas et al. (2001)
White	n.r.	10	1			
Red	0.62	295	29	16	4	Hocking et al. (2003)
White	0.50	216	19	13	9	
Red	n.r.	57				Australian Wine and Brandy Corporation[b]
White	0.17	75	1	2		

[a] not reported
[b] unpublished data. From wines exported in 2001-2004

Most of the results summarised in Table 1.2 were from surveys of still red and white table wines, with the exception of Hocking et al. (2003), where dessert and sparkling wines were included in the overall analysis. In three of four surveys, OA contamination of dessert wines was greater than that of white and red wines in the same surveys (Pietri et al., 2001; Stander and Steyn, 2002; Shephard et al., 2003; Stefanaki et al., 2003), whereas, in sparkling wines, OA was present at levels similar to those of still wines in the same surveys (Burdaspal and Legarda, 1999; Bellí et al., 2004a). OA contamination in aperitifs, port wines and sherries was typically low (Table 1.4); however, the mean contamination of wines such as marsala, malaga, moscatel, fondillón and mistelle, was 2-10 fold higher than the mean contamination reported for standard table wines in the same surveys (Table 1.2).

OA contamination of grape juice (Table 1.5) occurred over the same range as that of wine and, as observed in several wine surveys, contamination of red juice was often worse than that of white juice. Majerus et al. (2000) postulated that higher OA concentrations were found in red juice due to the practice of enzymatic treatment of the crude juice and berries at increased temperatures over an extended time to improve colour yield; however, data to differentiate between OA production by the mould, or OA partitioning from the pulp into the juice are not available. Certain studies surveyed both wine and grape juice for OA contamination (Table 1.5 *cf* Table 1.2), which was often greater in red juice than in red wine. Binding of OA to yeast cells during and after fermentation (Bejaoui et al., 2004) may have reduced its concentration in wine. Zimmerli and Dick (1996) observed greater OA contamination in juice blends from warmer climates in France and southern Italy. Roset (2003), likewise, noted that OA in juice increased in conditions of high rainfall and warmth prior to harvest, in particular from vineyards near the coast and from late-harvested grape cultivars. OA was also detected in fresh juice from wine grapes (Table 1.5) and, in southern France, appeared to be associated with isolation of ochratoxigenic fungi from those vineyards (Sage et al., 2002, 2004). However, consistent relationships between the presence of visible mould or ochratoxigenic fungi and OA in grapes, were not demonstrated in studies of grapes from Italy, Spain and Switzerland (Zimmerli and Dick, 1996; Bellí et al., 2004a; Battilani et al., 2005b).

Table 1.4: Prevalence of ochratoxin A in aperitifs, fortified and other special wines

Samples	Positive samples	Mean[a] (µg/kg)	Range (µg/kg)	Country of origin
Aperitifs				
Vermouth (Zimmerli and Dick, 1996)	0/2	< 0.003	< 0.003	Italy
Burdaspal and Legarda (1999)	35/47	0.04[b]	< 0.003-0.254	France, Germany, Portugal, Spain
Port wines and sherries				
Port (Zimmerli and Dick, 1996)	6 tested	0.011[c]	< 0.003-0.017	Portugal
Sherry (Zimmerli and Dick, 1996)	2/2	0.042	0.029-0.054	Spain
Port (Festas et al., 2000)	3/34[d]	0.01	< 0.02-0.08	Portugal
Port (Tateo et al., 2000)	4/6	0.085	< 0.013-0.34	not stated
Fortified (Stander and Steyn, 2002)	3/7	median 0.021	< 0.01-0.168	South Africa
Marsala, Malaga, Moscatel, Fondillón, Mistelle				
Zimmerli and Dick (1996)	5/5	0.250	0.044-0.451	Italy, Spain
Burdaspal and Legarda (1999)	15/16	1.048[b]	< 0.003-2.540	Italy, Spain
Tateo et al. (2000)	0/2	< 0.033	< 0.033	not stated
Bellí et al. (2004a)	9/20	2.03	< 0.05-15.25	Spain, including sherry and vermouth
Blesa et al. (2004)	8/13	0.133	< 0.01-0.40	Spain
Ice wine				
Eder et al. (2002)	0/5	< 0.01	< 0.01	Austria

[a] mean OA concentrations calculated assuming any results below the limit of detection contained OA at half limit of detection, unless otherwise noted
[b] mean as reported, values attributed to samples below limits of detection unknown
[c] mean as reported, samples below limits of quantification and detection were defined as having the limiting values
[d] the three positive samples had been adulterated in an unspecified manner

Table 1.5: Prevalence of ochratoxin A in commercial grape juice and juice from crushed grapes at harvest

Citation	Samples	Positive samples	Mean[a] (µg/L)	Range (µg/L)	Country of origin
Majerus and Otteneder (1996)	White juice	1/6	0.016	< 0.01-0.73	purchased in Europe
	Red juice	12/14	median 1.8	< 0.01-4.7	
Zimmerli and Dick (1996)	White juice	1/3 brands	0.003[b]	< 0.003-0.005	France, Italy, Switzerland
	Red juice	7/8 brands	0.188[b]	< 0.003-0.337	
	Juice from red and white grapes (including visibly mouldy fruit)	0/17	not detected	< 0.003	Italy, Switzerland
Ueno (1998)	White juice	0/3	not detected	< 0.003	purchased in Japan
	Red juice	2/6	0.018	< 0.003-0.006	
Burdaspal and Legarda (1999)	Grape + other juices	10/10	0.044[c]	0.015-0.102	France, Spain
	Juice from red and white grapes	8 tested	0.046[c]	< 0.003-0.176	Spain
MAFF (1999)	White juice	10/11	0.28	< 0.01-0.45	not known
	Red juice	9/9	0.74	0.03-2.05	
Majerus et al. (2000)	White juice	21/27	median 0.09	< 0.01-1.3	purchased in Europe
	Red juice	56/64	median 0.27	< 0.01-5.3	
Sage et al. (2002)	Juice from red wine grapes	8/11	not reported	< 0.010-0.461	France
Abrunhosa et al. (2003)	Juice from wine grapes	2/11	not reported	all < 0.01	Portugal
Roset (2003)	Juice from grapes (for commercial juice production prior to processing)	not reported	not calculated	< 0.02-0.1	Austria
				< 0.02	Germany
				0.8-8.0	Greece
				< 0.02-4.5	France
				< 0.02-8.5	Italy
				< 0.02-6.0	Spain
Abdulkadar et al. (2004)	Grape juice	0/5	not detected	< 0.15	purchased in Qatar
Bellí et al. (2004a)	Grape juice	0/10	not detected	< 0.05	Spain
	Juice from white wine grapes	2/10	0.05	< 0.05-0.18	
	Juice from red wine grapes	0/10	not detected	< 0.05	

Table 1.5 (cont.)

Citation	Samples	Positive samples	Mean[a] (µg/L)	Range (µg/L)	Country of origin
Bellí et al. (2004c)	Juice from white (12) and red (28) wine grape samples	6/40	not reported	< 0.07-0.81	Spain
Ng et al. (2004)	White juice	4/25	0.009	< 0.008-0.071	Canada, USA
	Red juice	5/45	0.010	< 0.008-0.104	
Rosa et al. (2004)	Red juice	14/48	0.019 (of positives 0.038)	< 0.021-0.100	Brazil
Sage et al. (2004)	Juice from white and red wine grapes	11/37	0.02	< 0.01-0.43	France
Berente et al. (2005)	Juice from white and red wine grapes	0/10	not detected	< 0.024	Hungary
Czerwiecki et al. (2005)	White grape juice	3/3	0.044	0.0029-0.064	Poland

[a] mean OA concentrations calculated assuming any results below the limit of detection contained OA at the half limit of detection, unless otherwise noted
[b] mean of brands
[c] mean as reported, values attributed to samples below limits of detection unknown

OA contamination of wine vinegar was fairly common, particularly in balsamic vinegars. Majerus et al. (2000) reported that 50% of 38 wine vinegars contained OA above 0.01 µg/L (median 0.1 µg/L, maximum 1.9 µg/L, similar to wines in the same survey), but 83% of 29 balsamic vinegars were positive for OA, and at higher levels (median 0.65 µg/L, maximum 4.35 µg/L). Similarly, a survey of 15 wine vinegars by Markarki (2001) demonstrated that the highest concentrations were found in three balsamic vinegars (0.156, 0.102 and 0.252 µg/L), whereas the remaining 12 samples fell between 0.008- 0.046 µg/L. These concentrations were slightly lower than those reported for wines in the same survey. Grape must is concentrated up to 50 fold during the production of balsamic vinegar (http://lebaccanti.com/travel-incentives-tuscany.php?id=35, accessed 05/04/05), which may explain the higher OA contamination in this product.

OA was detected frequently in dried vine fruits (Table 1.6) and, although the mean contamination was below that of the proposed EU limit of 10 µg/kg (European Commission, 2002), the maximum contamination reported in most surveys exceeded the limit by up to five fold. No single type of dried vine fruit displayed the greatest OA contamination in all the surveys. The mean contamination observed for dried vine fruit (Table 1.6) appeared to be closer to the 10 µg/kg limit than the corresponding mean concentration for wine (Table 1.2) and its 2 µg/kg limit (European Commission, 2005). For example, even in this limited number of surveys, three sets of samples [currants and sultanas reported by MacDonald et al. (1999); currants reported by MAFF (1999)] displayed mean contamination greater than 40% (arbitrarily assigned) of the limit. In contrast, mean contamination of only four sets of wine samples from a far greater number of analyses achieved concentrations greater than 40% of the limit (Table 1.2; Visconti et al., 1999; Castellari et al., 2000; Tateo et al., 2000; Filali et al., 2001). Consistently meeting the EU limit will probably be a greater challenge for dried vine fruit producers than for wine producers.

OA in dried vine fruit may be reduced during processing. Automated laser sorters reject misshapen and darkened berries during processing of Australian dried vine fruit (Australian Dried Fruits Association Inc., 1998). In some cases, berry darkening occurs due to the action of the browning enzyme, polyphenol oxidase (Grncarevic and Hawker, 1971). Browning is often caused by slow drying, as in the case of "natural" sultanas dried without emulsion. However, cell damage associated with berry splitting may also trigger browning, and rots caused by ochratoxigenic fungi have been observed to result in berry discoloration (Clarke et al., 2003). In seasons when berry splitting was common, dark berries contained OA at greater concentrations than light berries (Leong et al., 2005a). Discarded berries are sometimes added to cow feed, and might have posed a potential risk for OA contamination in dairy products, were it not for cleavage of the molecule by rumen microbes into non-toxic products (Engel, 2000); dairy products typically do not contain OA (264 samples below 0.01µg/kg), thus this practice appears to be safe.

Table 1.6: Prevalence of ochratoxin A in dried vine fruits

Citation, LOD / LOQ[a]	Samples	Positive samples	Mean[b] (µg/kg)	Maximum (µg/kg)	Country of origin
Saxena and Mehrotra (1990), qualitative assessment	Raisins	1/20	n.r.[c]	n.r.	India
Abdel-Sater and Saber (1999), LOD not reported	Raisins	0/20	n.d.[c]	n.d.	Egypt
MacDonald et al. (1999), LOD > 0.2 µg/kg	Currants	19/20	9.2	53.6	Greece
	Raisins	17/20	2.8	20.0	Australia, Chile, USA, mixed origin
	Sultanas	17/20	4.9	18.1	Greece, Turkey, mixed origin
MAFF (1999), LOD > 0.1 µg/kg	Currants	96/100	5.0	40.8	Australia, Greece, mixed origin
	Raisins	98/101	3.4	29.8	USA, Chile, South Africa, mixed origin
	Sultanas	92/100	2.9	25.1	Australia, Greece, Turkey
Möller and Nyberg (2003), LOD > 0.1 µg/kg	Currants	16/17[d]	1.3[d]	10.2[d]	not stated
	Raisins	80/101[d]	2.0[d]	34.6[d]	
Stefanaki et al. (2003), LOD > 0.5 µg/kg	Currants	43/54	2.8	13.8	Greece
	Sultanas	17/27	2.1	13.2	Greece
Abdulkadar et al. (2004), LOD > 0.15 µg/kg	Raisins	2/7	0.4	1.2	Qatar
Lombaert et al. (2004), LOQ > 0.1 µg/kg	Currants	2/2	2.8	4.9	Australia, Chile, Greece, Hong Kong, Iran, Israel, Mexico, South Africa, Turkey, United Arab Emirates, USA, unknown origin
	Raisins	67/85	1.8	26.6	
	Sultanas	39/66	1.9	26.0	
Magnoli et al. (2004), LOD > 1 µg/kg	Black	21/31	1.7	14.0	Argentina
	White	16/19	3.2	7.5	

[a] LOD: limit of detection; LOQ: limit of quantification
[b] mean OA concentrations calculated assuming any results below the limit of detection contained OA at half the limit of detection, unless otherwise noted
[c] n.r.: not reported; n.d.: not detected
[d] number of samples included subsamples from the same retail packages. OA results reported for extraction with sodium bicarbonate and methanol

1.4 Source of ochratoxin A

The source of OA in foods is well understood for certain foods only [reviewed by Pitt and Tomaska (2002)]. The occurrence of OA in cereals and cereal products is known to be primarily due to growth of *P. verrucosum* during storage in cool, temperate regions such as northern and central Europe and Canada. When OA-contaminated grain is fed to non-ruminant animals, the toxin accumulates in the flesh. *P. verrucosum* is rarely isolated from Australian grains. The probable source of OA in coffee is *A. ochraceus*, with some potential contribution from toxigenic strains of *Aspergillus* section *Nigri.* More recently, the source of OA in cured and fermented meats has been associated with the growth of *P. nordicum*, a close relative of *P. verrucosum* (Larsen et al., 2001; Castellà et al., 2002). Whereas it is generally accepted that OA is produced as a secondary metabolite during fungal infection of foods, Mantle (2000) and Trucksess and Maragos (2001) postulated an alternative mechanism for the presence of OA in crops, namely, that OA produced by toxigenic fungi in soil could be taken up into the plant. However, the natural occurrence of OA in soils at concentrations of concern is yet to be demonstrated.

The source of OA in grapes and grape products has been elucidated over the past 15 years. *Aspergillus niger* and other species within section *Nigri*, such as *A. aculeatus* and *A. carbonarius*, are common saprophytes often isolated from soils (Klich and Pitt, 1988), and it has long been known that bunch rots caused by black *Aspergillus* spp. sporadically occur in vineyards situated in warm to temperate regions (Amerine et al., 1980; Hewitt, 1988; Snowdon, 1990; Emmett et al., 1992). *A. niger* is used widely in food processing, and has been awarded a Generally Regarded As Safe (GRAS) status by the US Food and Drug Administration (Pitt and Hocking, 1997); thus it was surprising when Abarca et al. (1994) reported production of OA by two strains of *A. niger* from feed. In the following year, Horie (1995) reported OA production by a second species, *A. carbonarius*, also used in industrial enzyme production (Kiss and Kiss, 2000; Okolo et al., 2001), but more importantly, known to cause grape rots (Gupta, 1956).

Following the first reports of OA in wine (Majerus and Otteneder, 1996; Zimmerli and Dick, 1996), Heenan et al. (1998) isolated ochratoxigenic black *Aspergillus* spp.

during dried grape processing, suggesting that such species may indeed be the source of OA in grapes. Since then, black *Aspergillus* spp. which produce ochratoxins have frequently been isolated from grapes in France (Sage et al., 2002, 2004; Bejaoui et al., 2005), Italy (Battilani et al., 2003b), Spain (Cabañes et al., 2002; Abarca et al., 2003; Bellí et al., 2004c; Bau et al., 2005a), Portugal (Serra et al., 2003, 2005a), Greece (Tjamos et al., 2004, 2005), Israel (Guzev et al., 2005), South America (Da Rocha Rosa et al., 2002; Magnoli et al., 2003, 2004) and Australia (Leong et al., 2004), as well as from other substrates such as coffee and feed (Table 1.7). Isolates of *A. carbonarius* from a range of substrates were frequently toxigenic (up to 100% of isolates), whereas a relatively small proportion of *A. niger* strains produced OA. Reports of toxin production by a few *A. aculeatus* or *A. japonicus* isolates are yet to be confirmed. Toxigenic isolates of *A. ochraceus* have only occasionally been isolated from grapes (Da Rocha Rosa et al., 2002; Battilani et al., 2003b; Serra et al., 2003; Bellí et al., 2004c; Bau et al., 2005a). It can safely be concluded that *A. carbonarius* is the primary source of OA contamination of grapes.

The role of toxin production in the ecology of ochratoxigenic fungi has not been elucidated. Størmer and Høiby (1996) suggested that OA may confer a competitive advantage to the fungi by sequestering iron in the environment, thus making it unavailable to competing organisms. Alternatively, OA in the sclerotia of *A. carbonarius* may represent a chemical defence system against fungivorous insects (Wicklow et al., 1996).

Table 1.7: Surveys for ochratoxin A production by black *Aspergillus* spp.

Citation	Species	Positive strains	Growth medium	Detection method	Range of OA production (ppb, µg/L, µg/kg)	Source of isolates (source of OA-producing isolates, where known, shown in **bold**)
Abarca et al. (1994)	*A. niger*	2/19	corn, YES[a] broth	screening by TLC[b], confirmation by HPLC[b]	210-590	**poultry mixed feed**, corn, **soya beans**, peas
Ono et al. (1995)	*A. niger* aggregate	5/27	rice	screening by TLC and HPLC	9-239	Institute for Fermentation, Osaka culture collection, including strains used for food and beverage fermentations
Horie (1995)	*A. carbonarius*	1/1	malt-yeast broth, rice	TLC, HPLC	400-6780	**culture collection**
	A. niger aggregate	0/5			not detected	
Téren et al. (1996)	*A. niger* aggregate	3/100	YES agar/broth	screening by ELISA[b] and TLC; confirmation by TLC and HPLC of concentrated extracts	20-100	**culture collections**, including type strains, clinical isolates, isolates from rotted onion bulbs, Ugandan coffee beans, soil from various countries
	A. japonicus	0/45			not detected	culture collections, isolates from various countries
	A. carbonarius	5/12			60-150	**culture collections**, **isolates various countries**
Wicklow et al. (1996)	*A. carbonarius*	1/1	corn	HPLC and ^{13}C-NMR[b]	not reported	**culture collection, NRRL 369**
Téren et al. (1997)	*A. niger* aggregate	some strains positive	YES broth	TLC, ELISA	not reported	**green coffee beans**
Nakajima et al. (1997)	not stated	2/30	rice	not stated	0.08-0.56	**green coffee beans, Yemen**

Heenan et al. (1998)	*A. niger*	2/115 (0/114[d])	CCA[a], YES agar	reverse fluorescence on CCA; TLC of CCA and YES	not detected	**washwater from dried sultana processing line containing fruit from Australia and the Middle East**
	A. carbonarius	30/33 (34/34[d])			not reported	
Taniwaki et al. (1999)	*A. niger*	2/173	YES agar	TLC	not reported	**coffee, Brazil**
	A. japonicus	0/4			not detected	
	A. carbonarius	28/51			not reported	
Accensi et al. (2001)	*A. niger*	6/92[e]	YES broth	screening by TLC, confirmation by HPLC	26-1124	**animal feed**, **raw materials**, soil, animals/humans, **culture collections**
Joosten et al. (2001)	*A. carbonarius*	9/10	CCA, coffee cherries	reverse fluorescence on CCA; coffee cherries by HPLC	3-4810	**coffee cherries, Thailand; tomatoes, air**, apples
Urbano et al. (2001)	*A. niger*	87/344	YES agar	TLC	not reported	**raw coffee, Brazil**
Cabañes et al. (2002)	*A. niger*	0/1	CYA[a], YES agar	HPLC	not detected	**wine grapes, Spain**
	A. carbonarius	18/18			0.22-1.51	
Da Rocha Rosa et al. (2002)	*A. niger*	16/53	CYA, YES broth	TLC and HPLC	26,000-96,000	**Malbec and Chardonnay, Brazil**
	A. carbonarius	8/32			18,000-234,000	
	A. niger	8/48			32,000-77,000	**Malbec and Chardonnay, Argentina**
Dalcero et al. (2002)	*A. niger* aggregate	41/82	YES broth	HPLC	13-25	**poultry feed, pig feed, rabbit feed, Argentina**
	A. japonicus	4/10			13-14	
	A. aculeatus	0/2			not detected	
Sage et al. (2002)	*A. niger* aggregate	0/73	CYA, YES agar	HPLC	not detected	**wine grapes, southern France**
	A. carbonarius	14/15			100-87,500	
Abarca et al. (2003)	*A. niger*	1/168	CYA, YES agar	HPLC	not reported	**dried grapes, Spain**
	A. carbonarius	88/91			not reported	

Table 1.7 (cont.)

Citation	Species	Positive strains	Growth medium	Detection method	Range of OA (µg/kg)	Source (source of OA-producing isolates shown in **bold**)
Abrunhosa et al. (2003)	*A. niger*	13/202	CYA	HPLC	not reported	**wine grapes, Portugal**
	A. carbonarius	32/33			not reported	
Battilani et al. (2003b)	Biseriate i.e. *A. niger*	~ 14/270	Czapek Yeast Sucrose broth	HPLC	not reported	**wine grapes, Italy**
	Uniseriate i.e. *A. japonicus*	~ 3/108			not reported	
	A. carbonarius	~ 52/86			not reported	
Magnoli et al. (2003)	*A. niger* aggregate	25/63	YES broth	HPLC	2-25	**wine grapes, Argentina**
Serra et al. (2003)	*A. niger* aggregate	12/294	CYA	HPLC	not reported	**wine grapes, Portugal**
	A. japonicus	0/1			not detected	
	A. carbonarius	38/39			not reported	
Taniwaki et al. (2003)	*A. niger*	16/549	YES agar	TLC	not reported	**coffee, Brazil**
	A. carbonarius	42/54			not reported	
Accensi et al. (2004)	*A. niger*	3/52	CYA, YES agar	HPLC	11,600-20,530	Cereals, legumes, mixed feeds, **Spain**
Bellí et al. (2004c)	*Aspergillus* section *Nigri* (not separated into species)	18/386	CYA	HPLC	0.02-2.82	**wine grapes, Spain**
Leong et al. (2004)	*A. niger*	0/470	CCA	reverse fluorescence on CCA; some isolates also tested by TLC and/or HPLC	not detected	**fresh and dried grapes, Sunraysia, Australia,**
	A. aculeatus	0/200			not detected	
	A. carbonarius	245/245			not reported	

Magnoli et al. (2004)	*A. niger* var. *niger* and var. *awamori*	41/188	YES broth	HPLC	2-61	**dried grapes, Argentina**
	A. carbonarius	19/23			2-5203	
Sage et al. (2004)	*A. niger*	17/29	CYA, YES agar	HPLC	not detected	**wine grapes (veraison and harvest), France**
	A. carbonarius	10/10			0.01-1.90	
Suárez-Quiroz et al. (2004)	*A. niger*	2/2	coffee bean medium, rice medium	HPLC	2.2-114	**coffee cherries, Mexico**
Tjamos et al. (2004)	*A. niger*	123/159	Czapek Dox agar	ELISA	0.25- > 25	**raisins and wine grapes, Greece**
	A. carbonarius	58/60				
Battilani et al. (2005b)	*A. niger*	61/761	CYA	HPLC	< 10- > 100	**wine grapes, Italy**
	A. carbonarius	295/328			< 10- > 100	
	A. japonicus	0/640			not detected	
Bau et al. (2005a)	*A. niger*	0/474	CYA, YES agar	HPLC	not detected	**wine grapes, Spain**
	A. carbonarius	101/101			not reported	
	A. japonicus var. *aculeatus*	0/5			not detected	
Bellí et al. (2005a)	*A. niger*	14/392	CYA	HPLC	not reported	**wine grapes, Spain**
	A. carbonarius	104/135			not reported	
	A. japonicus	0/117			not detected	
Serra et al. (2005a)	*A. niger*	23/571	CYA	HPLC	137	**wine grapes, Portugal**
	A. carbonarius	68/68			1129	

[a] YES: yeast extract sucrose; CCA: coconut cream agar; CYA: Czapek yeast extract agar

[b] TLC: thin layer chromatography; HPLC: high performance liquid chromatography; ELISA: enzyme-linked immunosorbent assay; NMR: nuclear magnetic resonance spectroscopy

[d] these isolates were re-tested: one *A. niger* isolate was re-identified as *A. carbonarius*. The other *A. niger* isolate did not show OA production when assayed by HPLC. *A. carbonarius* isolates that were negative by CCA fluorescence and TLC were positive by the more sensitive HPLC method

[e] of the six positive isolates, five were isolates previously described as positive in other papers

1.5 Fungal infection of grapes

The production of OA in grapes requires the presence of the toxigenic fungus in the vineyard environment and the transfer of the fungus from that environment to the berry, followed by conditions suitable for infection, growth and toxin production. The interior of a grape berry is sterile, comprising fleshy mesocarp tissue (pulp) and seeds. The berry exocarp (skin) is the primary barrier to fungal infection, consisting of a waxy cuticle, epidermal and sub-epidermal cells. The microbiota on the skin comprises yeasts and bacteria which exist at a low metabolic rate on small amounts of nutrients that leak from the berry, and dormant fungal and bacterial spores (McGechan, 1978). When the skin is damaged, nutrients are no longer limiting, and the microbial population increases dramatically. Skin damage can be caused by many factors, including disease (black spot, downy mildew, powdery mildew), pests (bunch mites, mealy bug, light brown apple moth) and the vineyard environment (wind injury, sunburn, hail damage, bird damage). Some of these factors cause localised hardening of the skin, which may increase the susceptibility of the fruit to splitting.

Berry splitting occurs when the influx of moisture into a berry is significantly greater than the efflux. Water taken into a vine from the roots is typically lost through evaporation from the leaves and, to a lesser extent, from the berries. In conditions of high atmospheric humidity, evaporation from the leaves may cease, thus berries become the natural sink for excess water in the vine, particularly when they accumulate solutes with increasing maturity. As evaporative loss from a berry is slow, it must expand to allow for the extra volume. The mesocarp of the berry is highly extensible; however, the epidermis and sub-epidermis have a limited capacity to extend. At critical turgor pressure, a berry skin will have extended to its limit, and will split (Considine and Kriedemann, 1972). Skin extensibility is affected by the number and shape of epidermal and sub-epidermal cells, as determined by cultivar. Temperature also has an inherent effect on skin extensibility, as, at higher temperatures, the internal pressure increases in a manner which cannot solely be attributed to the concomitant volume increase (Lang and During, 1990). In addition, broader factors which affect the fine balance of water in the vine may influence splitting, e.g. bunch architecture or canopy management, which result in localised areas of high humidity and thus low evaporative rates. Chief among such factors that

cause splitting is summer rain while the grapes are ripening, as this simultaneously increases moisture uptake and decreases evaporative losses (Considine and Kriedemann, 1972).

When the berry skin stretches due to a slow increase in turgor pressure, cracking may occur, but if the increase is rapid, splitting is likely to occur. The distinction is important because in cracking, only the cuticle and epidermis are breached, and these breaches are sealed off with suberin; whereas, in splitting, damage occurs throughout the cuticle, epidermis, sub-epidermis and outer cells of the mesocarp, and this damage is not sealed off. Thus in split berries, the nutrient rich contents of the mesocarp cells are available for the growth of microorganisms (Swift et al., 1974). Apart from the induction of anti-fungal pathogenesis-related proteins and phytoalexins in the berry (Jacobs et al., 1999; Jeandet et al., 2002), no further mechanisms hinder infection throughout the entire berry.

A positive correlation has been demonstrated between splitting, especially at the pedicel end of the berry, and bunch rots of Rhine Riesling grapes in Western Australia (Barbetti, 1980). In addition, a positive correlation was found between rots and bunch weight. It can be postulated that tight, heavy bunches are more susceptible to splitting due to decreased evaporation and expanding berries pressing against each other. Barbetti (1980) also noted that the predominant fungi associated with bunch rots were different from those isolated from bunches at flowering. This suggests that "bunch-rotting" fungi, which may have been present on the bunch at low levels, possessed a competitive advantage when the berries split and nutrients became available.

Moulds that compete effectively in this situation include *Aspergillus* spp. (in particular the black aspergilli), *Rhizopus* spp., *Penicillium* spp. and *Cladosporium* spp. (Emmett et al., 1992). These moulds are generally thought to be wound and/or secondary invaders; however, Hewitt (1988) stated that *A. niger* in a drop of water may infect mature fruit directly through the skin at 20-32 °C. The incidence of these moulds in vineyards varies according to climate, with *Aspergillus* spp. favoured by hot, dry conditions, and *Penicillium* spp. favoured by cool, humid conditions (Amerine et al., 1980).

Aspergillus rot (Fig. 1.3), caused by *A. niger* and the related species, *A. aculeatus* (Jarvis and Traquair, 1984) and *A. carbonarius* (Gupta, 1956), results in berries becoming watery with a foul odour. A coating of black/brown spores develops and after complete decay, only shells of berries remain (Emmett et al., 1992). Control of Aspergillus bunch rot primarily lies in the prevention of splitting. Many fungicides are applied at flowering, hence, have little effect on the rapid increase in fungal population that occurs when berries split some months later (Barbetti, 1980). Pyrimethanil, or a combination of fludioxonil and cyprodinil, were effective in reducing Aspergillus rots and OA in grapes, particularly when applied nearing veraison (Lataste et al., 2004; Tjamos et al., 2004; Battilani et al., 2005b), the stage marked by the colour change and softening of berry skins, and a steady increase in berry sugars. Application of *Candida guillermondi* as an antagonistic biocontrol agent has also shown some success in reducing Aspergillus rots in Israel (Zahavi et al., 2000). In addition to causing bunch rots, *A. niger* has been implicated in causing grape-vine cankers, infecting the vines through wounds (Michailides et al., 2002).

Figure 1.3: Infection of Semillon berries by *Aspergillus carbonarius*

Apart from OA, black *Aspergillus* spp. may produce other toxins such as ceramide, produced by *A. niger* (Amerine et al., 1980), and naphthopyrones, produced by *A. niger* and *A. carbonarius* (Ghosal et al., 1979; Priestap, 1984). However, these toxins are not listed among those of concern to human and animal health (CAST, 2003), and thus their occurrence in grapes and grape products has not been examined. Other mycotoxins potentially present in grapes and wine include citrinin, patulin and trichothecenes produced by fungi such as *Penicillium expansum*, *Fusarium* spp. and *Trichothecium roseum* (Ough and Corison, 1980; Schwenk et al., 1989; Abrunhosa et al., 2001; Serra et al., 2005a).

1.6 Effect of processing on ochratoxin A

Most of the raw commodities in which OA is produced, such as cereals, coffee cherries and grapes [reviewed by Pitt and Tomaska (2002)] undergo further processing prior to consumption. Fresh table grapes are the exception, and even these undergo careful inspection for the removal of visibly mouldy berries. Thus, the concentration of OA in the final product is a function of the concentration in the raw commodity and the effect of inspection and/or processing. OA, the food matrix, and the process interact in three ways. First, OA may be located in a specific part of the commodity that is removed during processing, for example, the husks of grains (Alldrick, 1996) or coffee beans (Blanc et al., 1998). Battilani et al. (2001) reported greater concentrations of OA in the grape skins compared to pulp in six of eight samples. Second, OA may preferentially partition or bind to certain matrices based on its chemical characteristics, as affected by the pH and aqueous or organic nature of the matrix. For example, OA bound to spent grains in beer production rather than dissolving in the aqueous matrix (Baxter et al., 2001), and it bound to yeasts in beer and wine fermentation (Bejaoui et al., 2004). Third, OA may undergo some thermal degradation during baking and coffee roasting (Subirade, 1996; Viani, 1996), although a substantial amount remained and was concentrated during boiling in the production of the grape beverage, Pekmez (Arici et al., 2004). Baxter et al. (2001) suggested that a proportion of OA may be degraded by yeast during beer fermentation; however, Bejaoui et al. (2004) and Lataste et al. (2004) did not find evidence of degradation during wine fermentation. Many strategies to reduce OA during food processing [reviewed by Scott (1996)], such as gamma irradiation and degradation by enzymes (Deberghes et al., 1995; Stander et al., 2000), do not seem immediately applicable to wine production, as they may affect the sensory properties of wine.

In the production of wine, grapes undergo multiple stages during which solids and liquids are separated. In white vinification, grapes are crushed, then pressed to remove the skins and seeds. The juice may be treated with a pectinase to enhance precipitation of grape solids before fermentation commences. In red vinification, grapes are crushed then fermented in the presence of skins and seeds to extract colour and tannins. This mixture is later pressed to remove the skins and seeds. Both white and red wines undergo successive clarification stages to remove precipitated yeasts and

other solids. Malolactic fermentation, in which malic acid is converted into lactic acid by lactic acid bacteria, may also occur after fermentation. Fernandes et al. (2003, 2005) reported reduction of OA whenever solids and liquids were separated, thus these authors proposed that the partitioning or binding of OA to certain substrates was the primary means of removal during vinification.

1.7 Detection of ochratoxin A

Numerous methods exist for the detection of OA, both in foods and, in culture media during screening for toxigenic isolates [reviewed by Varga et al. (2001b)]. The principal methods in current use include thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) (Table 1.7). For quantification of OA in grapes and wine, sample purification using immuno-affinity columns followed by quantification by HPLC is most widely used [reviewed by Bellí et al. (2002), see also Saez et al. (2004); Serra et al. (2004)]. For the rapid screening of fungal isolates, production of OA can be detected *in situ* on white coconut cream agar (CCA), by observing the natural blue fluorescence of the toxin in acid/neutral media under long-wavelength ultra-violet (UV) light, followed by a change to violet fluorescence under basic conditions (Heenan et al., 1998). A second screening method involves growing the fungi on plates, and extraction of OA from agar plugs into an organic solvent, which is then filtered and injected directly into the HPLC apparatus for quantification (Bragulat et al., 2001). Recently, a lateral flow device based on an immuno-assay for OA has been developed for detection of the toxin extracted from agar plugs (Danks et al., 2003).

1.8 Objectives

Mycotoxin contamination of food is often a multi-stage process. Mycotoxins occur in crops when a mycotoxigenic strain is in the environment, comes in contact with the crop, either in the field or during storage, and is able to infect and grow in that crop in conditions that allow mycotoxin production. The mycotoxin must then be stable throughout any subsequent processing, in order to present a potential health risk in the final product. Consumers now have a greater focus on food integrity (Winemakers' Federation of Australia, 2000), and the presence of toxins in foods must be handled with sensitivity. It is likely that other governments will follow the EU in introducing limits for the allowable amount of OA in foods. Research on the ecology of the black aspergilli and OA production in Australian vineyards is needed to ensure that the wine industry can develop appropriate management strategies in the field and during winemaking to minimise contamination with OA.

To this end, the project has four primary objectives, each examining one aspect of the biology of black *Aspergillus* spp. in grapes and OA in wine. First, to determine the occurrence of toxigenic black *Aspergillus* spp., in particular *A. carbonarius*, in the primary viticultural regions of Australia and to characterise molecular relationships among these strains using PCR-based techniques. Second, to test the effect of ultra-violet radiation, temperature and water activity on the survival of *A. carbonarius* spores *in vitro*, and to assess the effect of temperature and water activity, manipulated in a simulated grape juice medium, on growth and toxin production. Such studies may be useful to predict the potential response of *A. carbonarius* to various environmental conditions in vineyards. Third, to examine the incidence and infection processes of *A. carbonarius* on both white and red grape cultivars *in vitro* and in vineyards, with a particular focus on survival of spores on berry surfaces in the vineyard, and infection in the presence of berry damage. Last, to monitor the stability and partitioning of OA during white and red vinification based on practices widely used in Australia.

2 General Materials and Methods

2.1 *Enumeration and identification of black* Aspergillus *spp. on various substrates*

Samples, including soil, dried rachides, fungal suspensions dried onto membrane filters, and grapes, were first homogenised in a manner appropriate to the sample (shaking, mixing in a stomacher, mixing in a blender). Further details are given in the relevant chapters. Homogenates were serially diluted in sterile peptone solution (0.1% w/v; Amyl Media, Dandenong, Vic, Australia). Appropriate dilutions (0.1 mL) were plated in duplicate onto Dichloran Rose Bengal Chloramphenicol agar (DRBC; Pitt and Hocking (1997); Appendix A). Plates were incubated in the dark at 25 °C for 3 d, after which colonies of black *Aspergillus* spp. were enumerated. Colonies were presumptively identified as *A. aculeatus*, *A. carbonarius* or *A. niger*, based on spore size, colour, depth of the colony and tendency for the conidial chains to split into columns around the vesicles when viewed under a stereomicroscope (M5-72033, x750; Wild Heerbrugg, Switzerland). *A. aculeatus* colonies were typically a light brown in colour with a powdery appearance to the sporulating surface, and a tendency for conidial chains to split into columns around the vesicles. *A. niger* colonies were various shades of brown and had the smallest spores of the three species. *A. carbonarius* colonies were dark charcoal brown (almost black) with large, glistening spores.

When the identity of an isolate was unclear, the isolate was subcultured by a three-point inoculation onto Czapek Yeast Agar (CYA; Pitt and Hocking (1997); Appendix A) and grown in the dark at 25 °C for 7 d. Aspergilla (vesicles plus conidiogenous structures) and conidia, removed from the colony with a fine inoculating wire, were mounted in lactic acid, and identification was confirmed by examination of slides using a compound microscope (Axioskop, x800; Zeiss, Göttingen, Germany). Isolates with rough conidia (ellipsoidal or spherical) and which displayed only phialides surrounding the vesicle (uniseriate) were identified as *A. aculeatus.* Isolates with rough conidia approximately 3-5 µm in diameter, with both phialides and metulae surrounding the vesicle (biseriate) were identified as *A. niger.* Isolates with tuberculate (highly roughened) conidia approximately 6-8 µm in diameter, with both

phialides and metulae surrounding the vesicle (biseriate) were identified as *A. carbonarius* (Klich and Pitt, 1988).

2.2 Storage of fungal isolates

For regular use, isolates were stored on CYA slants in the dark at 25 °C with subculturing as necessary. For long-term storage, isolates were grown on CYA plates as described in section 2.1. Spores were collected on the tip of an inoculating wire, and were introduced into an Eppendorf tube containing 1 mL sterile glycerol solution (25% w/w). The suspension was mixed by vortexing, snap frozen in 95% ethanol containing dry ice, and stored at -80 °C. Selected isolates were freeze-dried and accessioned into the FRR culture collection (Food Science Australia, North Ryde, NSW, Australia). Cultures were retrieved from storage by inoculation onto CYA. Freeze-dried cultures were rehydrated with sterile reverse osmosis (RO) water prior to inoculation.

2.3 Preparation of spore suspensions

Isolates were inoculated at three points onto CYA plates and incubated in the dark at 25 °C for 7-14 d. Spores were harvested by flooding the plates with sterile RO water containing 0.05% (w/v) Tween-80® (Merck, Kilsyth, Vic, Australia) as a wetting agent, after which spores were scraped from the surface of the colonies with a sterile spatula. The resulting suspension was shaken in a bottle containing sterile glass beads (2-3 mm diameter, Crown Scientific, Moorebank, NSW, Australia) to break up the spore chains. The concentration of spores was determined using a haemocytometer, after which the suspension was further diluted in sterile Tween-80® solution to achieve the desired concentration.

2.4 Assessment of ochratoxin A production on agar plates

2.4.1 Sampling and extraction

OA production on agar plates was assessed by the method of Bragulat et al. (2001) with the following modifications. Agar plugs (5 mm diameter) were taken from the edge and the centre of the colony, and midway between the edge and the centre. Plugs were stored in 4 mL borosilicate glass vials (Alltech, Deerfield, IL, USA) at -20 °C until extraction. The three plugs were vortexed with 1 mL methanol, allowed to stand for 1 h at room temperature (*ca* 22 °C), mixed again, and the extract filtered through

Millex®-HV filters (0.45 µM, 13 mm or 25 mm diameter; Millipore, Billerica, MA, USA). The extracts were kept at room temperature and analysed by HPLC the same day.

2.4.2 HPLC analysis of culture extracts

OA in culture extracts was detected by chromatography through an Ultracarb (30) C18 4.6 x 250 mm, 5 µm column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile:water:acetic acid (55:44.1:0.9, v/v), and was delivered through the heated column (40 °C) at a flow of 1 mL/min using a Shimadzu 10A VP high pressure binary gradient solvent delivery system (Kyoto, Japan). Detection of OA was achieved by post-column addition of ammonia (12.5% w/w, 0.1 mL/min) and monitoring the natural fluorescence of OA at 435 nm after excitation at 385 nm (Shimadzu, RF-10AXL). Samples were injected using a Shimadzu SIL-10Advp autosampler, and the injection volume was 3-5 µL. Samples which yielded OA concentrations that exceeded the scale of the detector were diluted in methanol and re-injected. OA in culture extracts was calculated by comparison with a calibration curve generated from OA standards (Sigma-Aldrich, St Louis, MO, USA) prepared in methanol over a range of concentrations. A typical chromatogram of OA produced by *A. carbonarius* on synthetic grapejuice medium (Appendix A) is shown in Fig. 2.1.

The limit of quantification was estimated to be 0.0018 µg/g, based on 80% of the amount yielding a small, symmetrical peak (Fig. 2.2). The limit of detection was estimated to be 20% of the limit of quantification, i.e. approximately 0.0004 µg/g, based on advice from the Analytical Chemistry section, Food Science Australia (Peter Varelis, pers. comm. 10/01/05).

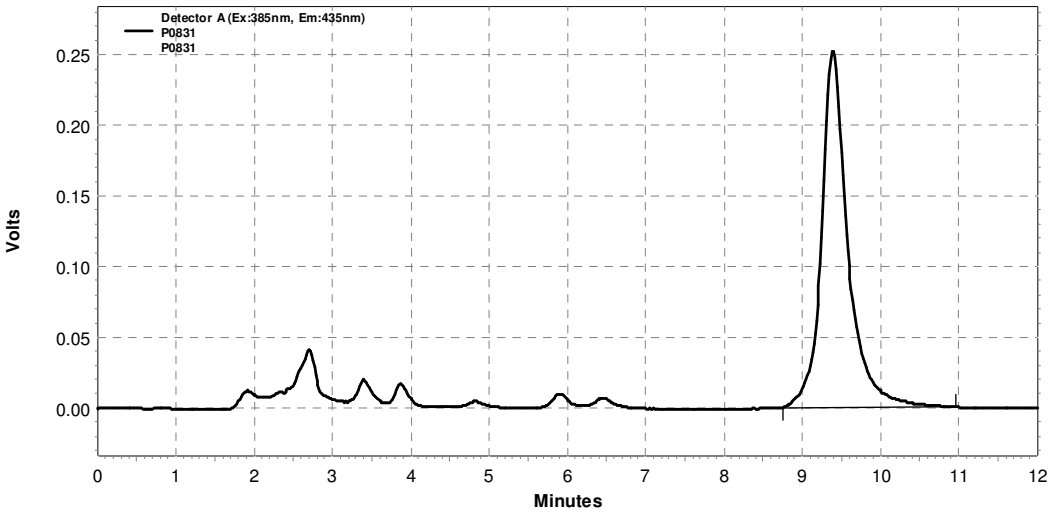


Figure 2.1: Ochratoxin A (469 ng/mL extract ≡ 2.2 µg/g medium) produced by *Aspergillus carbonarius* FRR 5690 on synthetic grape juice medium, water activity 0.965 at 15 °C after 22 d. OA eluted after 9.4 min

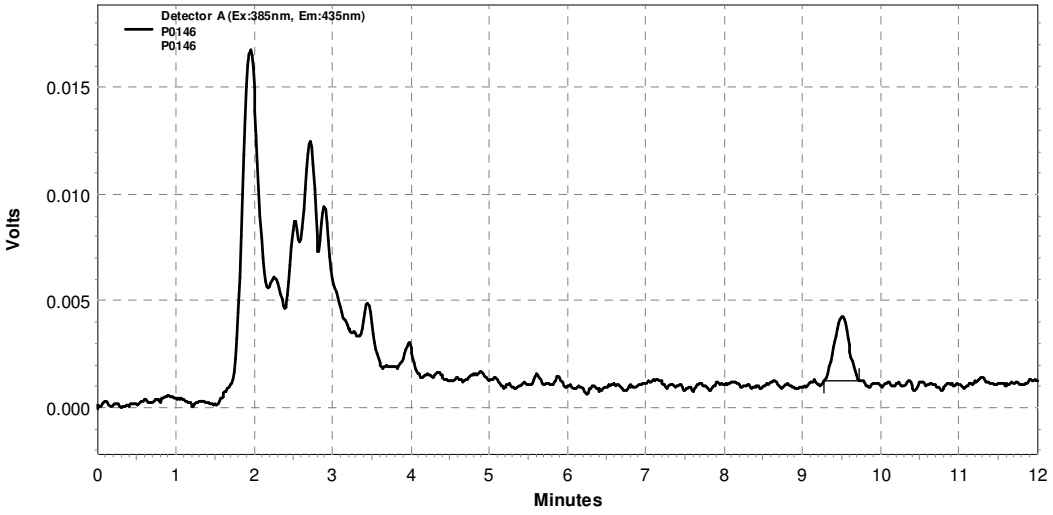


Figure 2.2: Ochratoxin A (0.49 ng/mL extract ≡ 0.0022 µg/g medium) produced by *Aspergillus niger* FRR 5695 on synthetic grape juice medium, water activity 0.95 at 25 °C after 5 d. OA eluted after 9.5 min

2.5 Assessment of ochratoxin A in grapes

2.5.1 Extraction and purification

Grapes were homogenised in a blender (Philips HR2835/AB) for 2 min, a 10 g subsample weighed into a centrifuge tube, and 5 mL Milli-Q water added. Grape samples which were likely to contain greater levels of OA due to severe infection with *A. carbonarius* were diluted up to 10 fold with RO water prior to homogenisation. Subsamples of these homogenates (15 g) were weighed into centrifuge tubes.

A solution of internal standard containing isotopically-labelled OA (250 µL; Appendix B) was added to each sample, which was mixed by vortexing and held at 1 °C overnight to equilibrate. Methanol (5 mL) and hydrochloric acid (10 N, *ca* 0.1 mL) were added, and mixed by vortexing. The mixture was centrifuged at 2500 rpm for 15 min (Orbital 420, Clements Medical Equipment Pty Ltd, Somersby, NSW, Australia). A 900 mg C18 solid phase extraction cartridge (Maxi-Clean™, Alltech, Deerfield, USA) was conditioned with 5 mL acetonitrile followed by 5 mL water, and the supernatant was passed dropwise through this cartridge under vacuum (Vacuum manifold, Alltech, Deerfield, USA). The cartridge was washed with 10 mL 10% methanol.

A 200 mg aminopropyl cartridge (4 mL Extract-Clean™, Alltech, Deerfield, USA) was conditioned with 3 mL methanol. The C18 and aminopropyl cartridges were attached in series, and the sample was eluted from the C18 cartridge onto the aminopropyl cartridge with the addition of 10 mL methanol. The sample was eluted from the aminopropyl cartridge with 10 mL 35% ethyl acetate in cyclohexane containing 0.75% formic acid. The eluate was dried under a nitrogen stream at 50 °C and resuspended in 1 mL 50% methanol containing 0.1% formic acid.

2.5.2 Liquid Chromatography-Mass Spectrometry analysis

Liquid chromatography-mass spectrometry (LC-MS) analyses were performed on a Thermo Finnigan system (Waltham, MA, USA) comprising a Surveyor quaternary pump as inlet, and injection by a Surveyor autosampler. Chromatographic separation was achieved through an Ultracarb (30) C18 2.1 x 50 mm, 5 µm column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of

methanol:water:formic acid (85:14.985:0.015, v/v), and was delivered through the heated column (30 °C) at a flow of 250 µL/min. The sample was ionised using the electrospray technique, and OA detected by a TSQ triple-stage high resolution quadrupole mass spectrometer operating in the selective reaction monitoring mode.

A set of calibration standards was prepared for a range of OA concentrations from 0-3200 ng/mL. To each 1 mL standard solution was added 250 µL of the internal standard, the same volume that was added to each test sample (section 2.5.1). The ratio of the naturally occurring isocoumarin portion of the OA molecule to the ^{13}C-labelled isocoumarin of the internal standard was plotted on the *y*-axis against the total amount of OA present on the *x*-axis. Three calibration curves were generated with the ranges 0-25 ng, 25-190 ng and 190-3200 ng. The injection volume for standards and grape samples was 5 µL. The amount of OA in the sample was calculated by comparison with the appropriate calibration curve (Fig. 2.3).

2.6 Statistical analysis

Where amenable to statistical analysis, data were examined by ANOVA (Genstat, 6th Edition, Lawes Agricultural Trust, Rothamsted, UK). In the absence of significant higher order interactions, comparison of means was conducted by Tukey's test of honestly significant difference (h.s.d.; http://faculty.vassar.edu/lowry/ch14pt2.html, accessed 01/05).

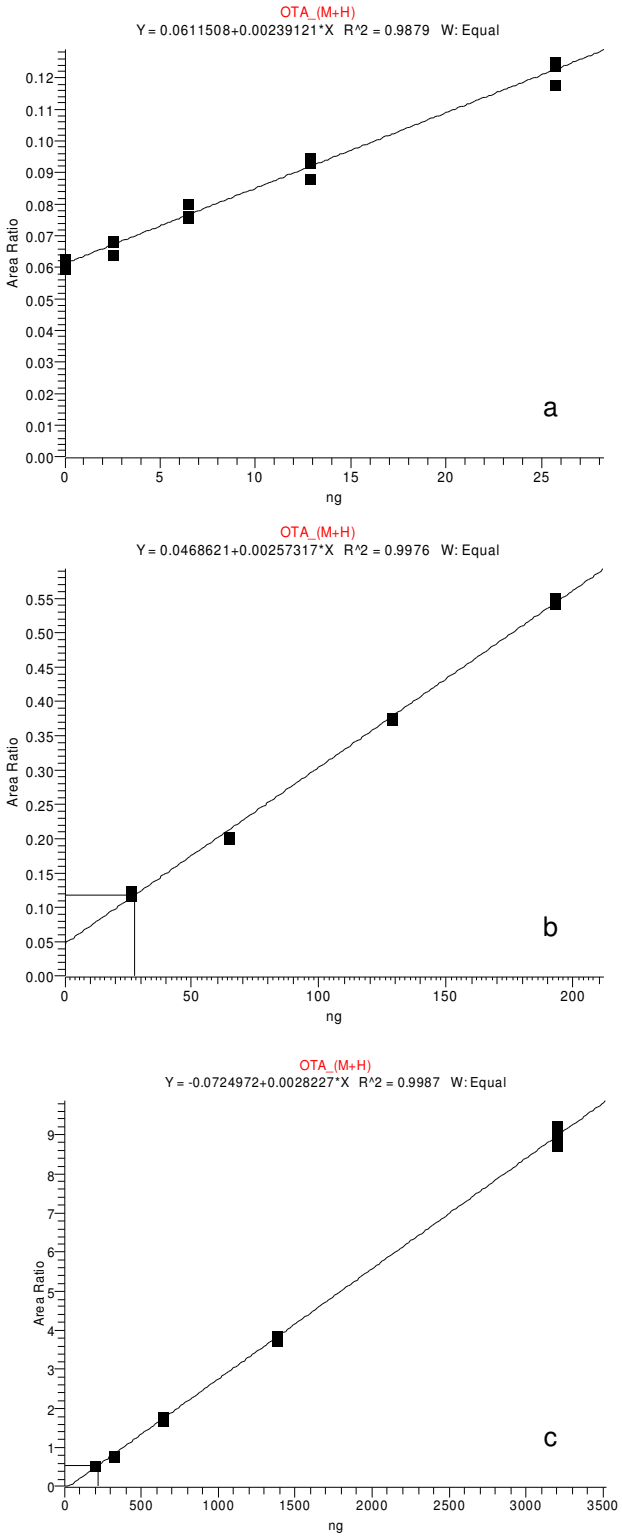


Figure 2.3: Liquid chromatography-mass spectroscopy calibration curves for ochratoxin A (*x*-axis) within the ranges (a) 0-25 ng, (b) 25-190 ng and (c) 190-3200 ng generated by three replicate injections. All data points were given equal weight. The Area Ratio on the *y*-axis refers to the relative peak areas of the unlabelled *vs* isotopically-labelled isocoumarin portion of the OA molecule

3 *Aspergillus niger* and *A. carbonarius* from Australian vineyards: isolation, toxigenicity and molecular relationships

3.1 Introduction

3.1.1 Distribution of black *Aspergillus* spp. on Australian grapes

Ochratoxigenic black *Aspergillus* spp. have been isolated from dried grapes in Sunraysia, Victoria, Australia (Heenan et al., 1998; Leong et al., 2004). Although *A. niger* was the species most commonly isolated from Australian dried grapes (Leong et al., 2004), no isolates produced OA. *A. aculeatus* and *A. carbonarius* were isolated less frequently. Toxigenicity was not demonstrated for *A. aculeatus*, whereas all isolates of *A. carbonarius* produced OA. The presence of ochratoxigenic black *Aspergillus* spp. in other Australian viticultural regions has not been demonstrated, although OA has been detected in wines from various regions (Hocking et al., 2003).

A. niger appears to be the dominant black *Aspergillus* species in vineyards in Europe and South America (Table 3.1), as well as in Australia. However, Cabañes et al. (2002) and Tjamos et al. (2004) described some vineyards where *A. carbonarius* was the dominant species. The toxigenicity of isolates is summarised in Table 1.7. Fungi were isolated directly from grapes in the European and South American studies. However, Kazi et al. (2004) demonstrated that black *Aspergillus* spp. in Australian wine grape and dried grape vineyards were most commonly isolated from soil directly beneath vines (also reported by Leong et al. (2005b)). Rachides (dried bunch stems) were another source.

Table 3.1: Isolation of *Aspergillus* section *Nigri* from wine grapes and dried grapes in Europe and South America

Country	Citation	Measure of frequency of isolation / severity of infection	*A. niger* aggregate	*A. carbonarius*	*A. aculeatus* / *A. japonicus*
France	Sage et al. (2002)	Isolation from wine grape samples	8/11	4/11	4/11
	Sage et al. (2004)	Isolation from viticultural regions	5/5	1/5	2/5
		Isolation from wine grape samples	29/60	10/60	5/60
Spain	Cabañes et al. (2002)	No. of isolates from wine grapes (single vineyard)	1	18	n.r.[a]
	Abarca et al. (2003)	Isolation from dried vine fruit samples	49/50	29/50	n.r.
	Bau et al. (2005a)	No. of isolates from wine grapes	474	101	5
		Isolation from proportion of berries	16.9%	3.6%	0.2%
	Bellí et al. (2005a)	No. of isolates	392	135	117
		Isolation from proportion of berries (2002)	3.0%	0.3%	0.8%
		Isolation from proportion of berries (2003)	3.5%	2.0%	1.2%
Italy	Battilani et al. (2003b)	No. of isolates (1999)	71	27	21
		No. of isolates (2000)	199	59	87
	Battilani et al. (2005b)	No. of isolates	761	328	640
		Proportion of berries colonised during ripening	48%	22%	31%
Portugal	Abrunhosa et al. (2003)	No. of isolates	202	33	n.r.
	Serra et al. (2003)	No. of isolates	294	39	1
		Isolation from wine grape vineyards	9/11	6/11	1/11
		Proportion of berries colonised (mean of positive vineyards)	44%	11%	n.r.
	Serra et al. (2005a)	No. of isolates	571	68	n.r.
		Isolation from wine grape regions	4/4	4/4	n.r.
		Isolation from berries	571/ 4695	68/ 4695	3/ 4695
Greece	Tjamos et al. (2004)	Proportion of infected berries (raisins, Corinthos)	40%	26%	n.r.
		(raisins, Achaia)	1%	97%	n.r.
		(Cabernet Sauvignon, Rhodes)	83%	15%	n.r.
		(Grenache Rouge, Rhodes)	7%	84%	n.r.

Table 3.1 (cont.)

Country	Citation	Frequency of isolation / severity of infection	*A. niger*	*A. carbonarius*	*A. aculeatus / japonicus*
Brazil	Da Rocha Rosa et al. (2002)	No. of isolates	53	32	n.r.
		Isolation from proportion of grape samples (Malbec)	58%	12%	n.r.
		(Chardonnay)	62%	26%	n.r.
Argentina	Da Rocha Rosa et al. (2002)	No. of isolates	48	0	n.r.
		Isolation from proportion of grape samples (Malbec)	45%	0	n.r.
		(Chardonnay)	50%	0	n.r.
	Magnoli et al. (2003)	Isolation from proportion of grape samples (surface sterilised)	60% (*niger*) 12% (*awamori*) 11% (*foetidus*)	0[b]	0[b]
		Mean proportion of infected berries (surface sterilised)	6% (*niger*) 4% (*awamori*) 4% (*foetidus*)	0[b]	0[b]
	Magnoli et al. (2004)	No. of isolates	194	23	2
		Isolation from proportion of samples[c] (surface sterilised) (black dried vine fruit)	75% (*niger*) 80% (*awamori*) 14% (*foetidus*)	45%	10%
		(white dried vine fruit)	42% (*niger*) 25% (*awamori*) 8% (*foetidus*)	16%	8% (*japonicus*) 8% (*aculeatus*)

[a] n.r. not reported
[b] all isolates of *Aspergillus* section *Nigri* identified to species level but *A. carbonarius* and *A. aculeatus/japonicus* not reported, therefore assumed to be absent
[c] highest % infection on either DRBC or Dichloran 18% Glycerol agar (DG18) reported

The three species commonly isolated from vineyards can be distinguished by morphology (see section 2.1). Isolates of *A. niger* may be further classified as type N or type T by the method of Accensi et al. (1999) based on the restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of 5.8S ribosomal DNA. To date, toxigenicity has been restricted to a few type N isolates, whereas it has not been demonstrated in type T isolates (Accensi et al., 2001). Twelve isolates from Sunraysia were previously all classified as type T (Leong et al., 2004). It is not known if this predominance of type T is representative of Australian vineyards in general. Varga et al. (1994) also noted a predominance of type T (13 of 15 isolates) from (non-vineyard) soils in the Kimberley region, Western Australia.

The first section of this chapter reports on the isolation of black *Aspergillus* spp. from soil and/or rachides from vineyards in many of the primary viticultural regions of Australia, and the ability of *A. carbonarius* and *A. niger* isolates to produce OA. In addition, RFLP analysis was used to assess the incidence of N type or T type among isolates of *A. niger*.

3.1.2 Techniques to assess molecular relationships among black *Aspergillus* spp.

Various genotypic and phenotypic methods have been employed to assess relationships among black *Aspergillus* spp., particularly within the morphologically indistinguishable *A. niger* aggregate. Methods to assess genotypic relationships have been based on both nuclear (chromosomal) and mitochondrial DNA, and have included analysis of karyotype, RFLP, sequences from ribosomal and other genes, presence of double-stranded RNA mycoviruses and random amplification of polymorphic DNA (RAPD) [reviewed by Varga et al. (2000b); Abarca et al. (2004); Varga et al. (2004a)].

This chapter reports on the development of three techniques, based on random polymorphisms within the genome, which are used to differentiate between *A. niger* and *A. carbonarius* isolates of differing toxigenicity and from various sources. Two of these techniques, enterobacterial repetitive intergenic consensus (ERIC)-PCR (Versalovic et al., 1991) and microsatellite markers, have yet to be tested for utility in

distinguishing the various black *Aspergillus* spp. Analysis of amplified fragment length polymorphisms (AFLP) has been utilised in the assessment of strains from coffee (Schmidt et al., 2004) and grapes (Perrone et al., 2005).

ERIC-PCR is closely related to RAPD, in that both techniques rely on the binding of primers to complementary sequences randomly distributed throughout the genome. The primer length for RAPD is typically 10 base pairs (bp), whereas ERIC-PCR utilises primers of 22 bp (Gillings and Holley, 1997). Although based on sequences originally characterised in eubacterial genomes, this technique has found broader application in a diverse range of fungal genera, including *Fusarium* (Edel et al., 1995; Smith-White et al., 2001), *Verticillium* (Arora et al., 1996) and *Pochonia*, a nematophagous genus (Morton et al., 2003).

AFLP analysis is based on two distinct aspects of polymorphism: first, the presence of recognition sequences at which chromosomal DNA is cut by two restriction enzymes; and second, the selective amplification of a population of fragments through complementary binding of the primers to the ligation adapters plus one or two bp into the unknown region (Vos et al., 1995; Aarts and Keijer, 1999). AFLP has previously been used in the molecular characterisation of the ochratoxigenic fungi *P. verrucosum* / *P. nordicum* (Castellà et al., 2002) and *A. ochraceus* (Schmidt et al., 2003), as well as aflatoxigenic *Aspergillus* species (Montiel et al., 2003).

Microsatellites, also known as simple sequence repeats, are tandem arrays of short DNA sequences composed of two to six bp motifs. Polymorphisms frequently occur at these loci due to slippage of DNA polymerase and subsequent misaligned reassociation of the strands. Methods of isolation for microsatellites and their utility as fungal genetic markers have been reviewed by Carter et al. (2004). One such method of isolation utilises algorithms that screen published sequences for microsatellite repeats, and has yielded six polymorphic microsatellie loci within the aflatoxigenic species *A. flavus* and *A. parasiticus* (Tran-Dinh and Carter, 2000).

Few Australian isolates of black *Aspergillus* spp. have been included in molecular studies of this group, apart from those discussed in section 3.1.1. Isolates of *A. carbonarius* and *A. niger* from Australian vineyards are included in this analysis of

over 70 strains. Isolates from European viticultural regions as well as isolates of interest from culture collections were included, as the development of these molecular techniques for black *Aspergillus* spp. was performed in collaboration with Alexandre Esteban (University of Barcelona; see section 3.3). Although toxigenicity is fairly uncommon among isolates of *A. niger* (Abarca et al., 2004), several toxigenic strains were included in the analysis in order to assess any potential association between molecular relatedness and toxigenicity. Isolates from the same substrates and/or countries were assessed for molecular relatedness.

3.2 Isolation of black Aspergillus *spp. from Australian viticultural regions*

3.2.1 Methods

3.2.1.1 Isolation

Samples of soil and rachides were sourced from many of the primary viticultural regions of Australia (Table 3.2). Collaborators were contacted between May and October (after vintage and during vine dormancy), in order to increase the likelihood of their participation, and were requested to sample soil from directly beneath vines, including any fallen vine material, from different parts of the vineyard. Samples of at least 100 g were collected in zip-lock polyethylene bags (18 cm x 17 cm, Glad Products, Padstow, NSW, Australia) and sent to the Food Science Australia laboratory in North Ryde (NSW Agriculture permit 03/3904) for analysis.

As described in section 2.1, soil samples (10 g) were added to 90 mL sterile peptone solution in a bottle and mixed by shaking. Rachides were weighed and homogenised with an appropriate volume of sterile distilled water (approximately 150 mL) in a stomacher (Bagmixer, Interscience, France). Homogenates (0.1 mL) were plated onto DRBC (five replicates), and colonies of black *Aspergillus* spp. identified and enumerated.

Isolates of *A. niger* and *A. carbonarius* representative of the various colony morphologies present in each region were stored as described in section 2.2 for further analysis.

3.2.1.2 Toxigenicity screening

Production of OA by isolates of *A. carbonarius* was tested according to the method of Heenan et al. (1998) by centrally inoculating plates of coconut cream agar (CCA; Appendix A) with spores on the point of an inoculating wire. Plates were incubated in the dark at 25 °C for 7 d. The reverse of the plates was examined under long wave UV light (Chromo-vue, Ultra-violet Products, Inc., San Gabriel, CA, USA). Colonies showing a blue fluorescence were deemed to produce OA, as compared with fluorescence by a known toxigenic strain (*A. carbonarius* FRR 5374). For isolates that fluoresced only weakly, a confirmatory test was performed. Colonies on CCA plates with the lids removed were held within an enclosed glass tank containing a solution of ammonium hydroxide (approximately 28% ammonia) for 2 h. The plates were ventilated in the fume cupboard for a few minutes before re-examining the reverse under long wave UV light. The presence of OA was detected as a strong violet fluorescence.

Production of OA by isolates of *A. niger* was tested by centrally inoculating plates of YES medium (Appendix A), as this medium was shown to be better than CYA for the production of OA by *A. niger* (Bragulat et al., 2001; Esteban et al., 2004). Plates were incubated in the dark at 25 °C for 7 d. Extraction and detection of OA by HPLC were as described in section 2.4. The identity of the OA peak in the chromatogram was confirmed by derivitization with boron trifluoride (Hunt et al., 1979). A standard solution of OA (22 ng/mL) was also derivitized for comparison.

Isolates of *A. aculeatus* were not screened for OA production because reports of OA production by *A. japonicus / A. aculeatus* (Dalcero et al., 2002; Battilani et al., 2003b) are dubious and, currently, this species is thought to be non-toxigenic (Abarca et al., 2004).

3.2.1.3 RFLP analysis of *Aspergillus niger*

3.2.1.3.1 DNA extraction

A liquid medium (approximately 15 mL) based on modified Spezielle Nahstoffarmer Agar (SNA) (Appendix A; Nirenberg (1976)) in sterile 9 mm plastic Petri dishes was inoculated with *A. niger* spores on the point of an inoculating wire. The plates were

swirled gently to distribute the spores and were incubated at 25 °C for 2-3 d, until white mycelial growth covered the surface of the medium. DNA extraction commenced before colonies sporulated heavily, to minimise the presence of pigments that could potentially interfere with PCR amplification.

DNA was extracted using a modified FastPrep protocol (BIO 101, Inc., Carlsbad, CA, USA; Smith-White et al. (2001)). The mycelial mat was transferred to a filter paper (9 cm diameter, Whatman No. 1, UK) in a Buchner funnel, and residual medium was removed under vacuum. Mycelium was scraped from the paper with a spatula and transferred to a FastPrep DNA tube. The Buchner funnel and spatula were rinsed with 10% bleach followed by RO water between samples. DNA extraction buffer (1 mL; Appendix C) was added to the mycelium in the FastPrep tube, which also contained ground garnet and a ceramic sphere to facilitate cell lysis during homogenisation. The mixture was homogenised in a FastPrep FP120 (Bio 101, Inc., Carlsbad, CA, USA) set at 5.5 for 35 s, after which the tubes were stored at -4 °C for 5 min to reduce aerosols. The tubes were centrifuged at 13,000 rpm (1K15, Sigma, Osterode am Harz, Germany) for 5 min, and the supernatant (750 µL) transferred to an Eppendorf tube containing 150 µL protein precipitating solution (Appendix C). Contents of the tube were briefly mixed by gentle vortexing, and centrifuged at 13,000 rpm for 5 min. The supernatant (700 µL) was transferred to an Eppendorf tube containing 700 mL of binding matrix (Appendix C), and mixed by inversion for 30 min. The tube was centrifuged at 10,000 rpm for 10 s and the supernatant discarded. The pellet was gently resuspended in 800 µL salt ethanol wash solution (Appendix C) using wide bore pipette tips in order to minimise shearing of chromosomal DNA. The suspension was mixed by inversion for 20 min, and centrifuged at 10,000 rpm for 10 s, after which the supernatant was discarded. The tube was centrifuged for 10 s again, and the remaining supernatant removed with a 200 µL micropipette with a fine-pointed tip. The open tube was covered with a tissue and dried overnight at room temperature. The pellet was resuspended in Tris-EDTA buffer (200 µL; Appendix C) and left to stand for 5 min, after which the tube was centrifuged at 12,000 rpm for 2 min. The supernatant (160 µL) containing the extracted DNA was transferred to an Eppendorf tube and stored at -4 °C.

3.2.1.3.2 PCR amplification and digestion of amplicons

Following the method of Accensi et al. (1999), the ITS region flanking the 5.8S rDNA unit was amplified with primers ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990). Reaction mixtures (50 µL) containing 1x PCR buffer (Qiagen, Germany), 5% glycerol, 125 µM each dNTP, 12.5 pmol of each primer, 1.25 U of *Taq* DNA polymerase (Qiagen, Germany) and 20-40 ng of DNA as template were amplified in a Hybaid PCR Express Thermal Cycler (Integrated Sciences, Willoughby, NSW, Australia) with the cycling parameters: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and then a final extension step of 72 °C for 7 min. Amplicons (10 µL) were loaded onto an agarose gel (2% w/v in TBE buffer; Appendix C) containing ethidium bromide (0.007 % v/v). After electrophoresis in TBE buffer (100V; Electro-fast® *Wide* system, ABgene, Epsom, UK; Electrophoresis constant power supply ECPS 3000/150, Pharmacia Fine Chemicals, Uppsala, Sweden), amplicons were visualised through a UV transilluminator (TFX-20.M, Vilber Lourmat, France) and the image captured via the EDAS 290 camera system and 1D Image Analysis Software (Kodak, New Haven, CT, USA). Amplicon sizes were estimated from the pGEM molecular weight marker (Promega, Madison, WI, USA).

Restriction digests of amplicons were carried out in 20 µL volumes containing 1x Buffer C (10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol), 0.1% bovine serum albumin and 5 U *Rsa*I (Promega, Madison, WI, USA). Approximately 1 µg DNA was used for each reaction (roughly equivalent to 5 µL of amplicon). Digestion was performed at 37 °C for 1 h, after which the products were electrophoresed and visualised as described above.

3.2.2 Results

3.2.2.1 Frequency of isolation from soil and rachis samples

Black *Aspergillus* spp. were isolated from vineyard soils in the 17 viticultural regions examined (Table 3.2), and were present in 33 of 37 vineyards. Soil from four vineyards in Tasmania did not yield black *Aspergillus* spp.; however, only single soil samples were examined from each of these vineyards. Isolation from rachides was less frequent than from soils. *A. niger* was the most frequently isolated species and

generally occurred in soils at a greater concentration than either *A. carbonarius* or *A. aculeatus*, being present in vineyard soil at greater than 1000 cfu/g in 11 of 17 regions. In this study, *A. carbonarius* was isolated from four regions: Hunter Valley and Riverina, New South Wales; Riverland, South Australia; and St George, Queensland. *A. carbonarius* was present in vineyard soil at greater than 1000 cfu/g in only two regions, Riverland and Queensland. This species was also frequently isolated in a follow-up study of nine table grape vineyards in Queensland (data not shown). *A. aculeatus* was isolated from six regions, including the four regions where *A. carbonarius* was present.

Representative isolates of *A. niger* and *A. carbonarius* selected from each region for further analysis are listed in Table 3.3. Also included in Table 3.3 are isolates from vineyards in Sunraysia, Victoria (Leong et al., 2004). Isolates of *A. aculeatus* were not subjected to further analysis as this species typically does not produce OA.

3.2.2.2 Frequency of toxigenicity

All 32 isolates of *A. carbonarius* displayed the blue fluorescence characteristic of OA when grown on CCA (Table 3.3). Extracts from three of 100 isolates of *A. niger* grown on YES produced peaks corresponding to the retention time of OA when analysed by HPLC. The identity of OA in each of these extracts was confirmed by noting the corresponding peak shift upon derivitization of the extracts, as shown in Fig. 3.1. Note that after derivitization, most of the original OA was derivitized and hence only a slight peak remained at the original retention time. The identity of the derivitized OA in the fungal extract is demonstrated by co-elution with the derivitized OA from the standard solution (Fig. 3.2). The three toxigenic *A. niger* isolates were accessioned into the FRR culture collection (FRR 5694, FRR 5695 and FRR 5701) as described in section 2.2. FRR 5694 was isolated from soil beneath Cabernet Sauvignon vines in Margaret River, Western Australia, and FRR 5695 and FRR 5701 were from a single sample of rachides (variety unknown) from Coonawarra, South Australia. Although the screening technique was not quantitative, it was noted that FRR 5694 produced more OA on YES than the other two isolates (data not shown).

Table 3.2: Isolation of *Aspergillus niger*, *A. carbonarius* and *A. aculeatus* from soils, rachides and berries from Australian vineyards

State	Region	Presence of black *Aspergillus* species					
		No. in which black *Aspergillus* spp. present[a] / No. tested		No. of samples within concentration range for black *Aspergillus* spp. (cfu/g)			
		Vineyards	Soil samples (unless otherwise indicated)	20-100	100-1000	10^3-10^4	> 10^4
New South Wales	Hunter Valley	4/4	16/16 2/3 r[b]	1 *n*[c] 2 *a*[c]	3 *n* 1 *c*[c] 1 *n*	12 *n*	1 *n*
	Mudgee	2/2	6/7 0/1 r 0/3 b[b]	1 *n* 1 *a*	3 *n*	1 *n*	1 *n*
	Riverina	2/2	12/12 3/4 b	1 *n* 2 *a* 3 *n*[d] 1 *c*[d]	1 *n* 2 *a*	7 *n*	3 *n*
Victoria	Alpine Valley	1/1	12/12	1 *n*	6 *n*	5 *n*	
	Yarra Valley	1/1	2/12	2 *n*			
South Australia	Adelaide Hills	1/1	7/12 0/6 r	4 *n*	3 *n*		
	Barossa Valley	2/2	12/16 9/10 r	2 *n*	6 *n* 5 *n*	4 *n* 4 *n*	
	Clare Valley	1/1	8/8 2/4 r	2 *n*	4 *n*	1 *n* 2 *n*	1 *n*
	Coonawarra	2/2	4/13 1/14 r	3*n*	1 *n*	 1 *n*	
	Langhorne Creek	1/1	6/12 0/6 r	6 *n*			
	McLaren Vale	1/1	13/13 0/6 r	3 *n* 1 *a*	2 *n*	8 *n*	
	Padthaway	1/1	9/12 0/6 r	4 *n*	4 *n*	1 *n*	
	Riverland	1/1	12/12 5/7 r	3 *c* 1 *n* 1 *c*	1 *n* 2 *c* 5 *a* 1 *n*	8 *n* 2 *c* 2 *a* 3 *n*	3 *n*
Western Australia	Margaret River	3/3	13/15 0/6 r	10 *n*			3 *n*
	Pemberton	1/1	3/12 0/6 r	3 *n*			
Tasmania		3/7	2/7 1/7 r	2 *n* 1 *n*			
Queensland		6/6 (1/6 *c*)	9/14	3 *n* 1 *a*	3 *n*	3 *n* 1 *c* 1 *a*	1 *c* 1 *a*

[a] limit of detection 20 cfu/g soil

[b] r: isolated from rachides; b: isolated from berries (berry samples only obtained from Mudgee and the Riverina)

[c] *n*: no. of samples from which *A. niger* was isolated; *a*: *A. aculeatus*; *c*: *A. carbonarius*

[d] detected at approximately 10 cfu/g berries

Table 3.3: Isolates of black *Aspergillus* spp. examined and ability to produce ochratoxin A

Species	Isolate	Accession no.	Source	Location	OA production	N/T profile[b]	Inclusion in molecular study, section 3.3
A. carbonarius	CHV1	FRR[a] 5682	Soil beneath Semillon vines	Hunter Valley, NSW[c]	+	-	yes
A. carbonarius	CHV2	FRR 5683	Soil beneath Semillon vines	Hunter Valley, NSW	+	-	yes
A. carbonarius	CHV1a	FRR 5699	Shiraz grapes	Hunter Valley, NSW	+	-	yes
A. carbonarius	CHV1b	FRR 5700	Shiraz grapes	Hunter Valley, NSW	+	-	yes
A. carbonarius	CRv1	FRR 5690	Semillon grapes	Riverina, NSW	+	-	yes
A. carbonarius	CRv2	FRR 5702	Semillon grapes	Riverina, NSW	+	-	yes
A. carbonarius	CRL2	FRR 5703	Soil beneath Chardonnay vines	Riverland, SA[c]	+	-	yes
A. carbonarius	CRL3	FRR 5704	Soil beneath Chardonnay vines	Riverland, SA	+	-	yes
A. carbonarius	CRL4	FRR 5705	Soil beneath Shiraz vines	Riverland, SA	+	-	yes
A. carbonarius	CRL5	FRR 5706	Soil beneath Shiraz vines	Riverland, SA	+	-	yes
A. carbonarius	CRL6	FRR 5707	Soil beneath Chenin Blanc vines	Riverland, SA	+	-	yes
A. carbonarius	CRL7	FRR 5708	Soil beneath Chenin Blanc vines	Riverland, SA	+	-	yes
A. carbonarius	CRL10	FRR 5709	Soil beneath Chenin Blanc vines	Riverland, SA	+	-	yes
A. carbonarius	CRL11	FRR 5710	Soil beneath Chenin Blanc vines	Riverland, SA	+	-	yes
A. carbonarius	CRL12	FRR 5711	Soil beneath Colombard vines	Riverland, SA	+	-	yes
A. carbonarius	CRL13	FRR 5691	Soil beneath Chardonnay vines	Riverland, SA	+	-	yes
A. carbonarius	CRL15	FRR 5712	Soil beneath Chardonnay vines	Riverland, SA	+	-	yes
A. carbonarius	CRL16	FRR 5713	Soil beneath Chardonnay vines	Riverland, SA	+	-	yes
A. carbonarius	CRL17	FRR 5714	Chardonnay rachides	Riverland, SA	+	-	yes
A. carbonarius		FRR 5374	Flame seedless grapes	Sunraysia, Vic[c]	+	-	yes
A. carbonarius		FRR 5573	Sultana grapes	Sunraysia, Vic	+	-	yes
A. carbonarius		FRR 5574	Sultana grapes	Sunraysia, Vic	+	-	yes
A. carbonarius	PP1-3	FRR 5693	Waltham raisins	Sunraysia, Vic	+	-	yes

A. carbonarius	RR2-4	FRR 5696	Waltham raisins	Sunraysia, Vic	+	-	yes
A. carbonarius	K3-7	FRR 5697	Sultana grapes	Sunraysia, Vic	+	-	yes
A. carbonarius	SS5-2	FRR 5698	Semillon grapes	Sunraysia, Vic	+	-	yes
A. carbonarius	Q1-9	FRR 5719	Sultana grapes	Sunraysia, Vic	+	-	yes
A. carbonarius	CQL1	FRR 5715	Soil beneath Shiraz vines	Queensland	+	-	yes
A. carbonarius	CQL2	FRR 5716	Soil beneath Shiraz vines	Queensland	+	-	yes
A. carbonarius	CQL4	FRR 5717	Soil beneath Shiraz vines	Queensland	+	-	yes
A. carbonarius	CQL5	FRR 5718	Soil beneath Shiraz vines	Queensland	+	-	yes
A. carbonarius	CQL6	FRR 5692	Soil beneath Shiraz vines	Queensland	+	-	yes
A. niger	NHV1	FRR 5720	Soil beneath Semillon vines	Hunter Valley, NSW	-	T	yes
A. niger	NHV2		Chardonnay grapes	Hunter Valley, NSW	-	T	
A. niger	NHV3		Shiraz grapes	Hunter Valley, NSW	-	T	
A. niger	NHV5		Vineyard soil	Hunter Valley, NSW	-	N	
A. niger	NHV7		Vineyard soil	Hunter Valley, NSW	-	T	
A. niger	NHV10		Vineyard soil	Hunter Valley, NSW	-	T	
A. niger	NHV11		Rachides	Hunter Valley, NSW	-	T	
A. niger	NHV13		Shiraz grapes	Hunter Valley, NSW	-	N	
A. niger	NMu2		Soil beneath Cabernet Sauvignon vines	Mudgee, NSW	-	N	
A. niger	NMu4		Soil beneath Chardonnay vines	Mudgee, NSW	-	N	
A. niger	NMu5		Soil beneath Merlot vines	Mudgee, NSW	-	T	
A. niger	NMu6		Soil beneath Merlot vines	Mudgee, NSW	-	T	
A. niger	NMu7		Soil beneath Merlot vines	Mudgee, NSW	-	N	
A. niger	NMu8		Soil beneath Shiraz vines	Mudgee, NSW	-	T	
A. niger	NMu9		Vineyard soil	Mudgee, NSW	-	N	
A. niger	NMu10		Soil beneath Chardonnay vines	Mudgee, NSW	-	T	
A. niger	NRv1		Soil beneath Cabernet Sauvignon vines	Riverina, NSW	-	T	
A. niger	NRv2		Soil beneath Semillon vines	Riverina, NSW	-	N	

Table 3.3 (cont.)

Species	Isolate	Accession no.	Source	Location	OA production	N/T profile[b]	Molecular study
A. niger	NRv3		Soil beneath Semillon vines	Riverina, NSW	-	T	
A. niger	NRv4		Soil beneath Tyrian vines	Riverina, NSW	-	N	
A. niger	NRv6		Soil beneath Chardonnay vines	Riverina, NSW	-	N	
A. niger	NRv7		Soil beneath Chardonnay vines	Riverina, NSW	-	N	
A. niger	NRv8		Soil beneath Chardonnay vines	Riverina, NSW	-	N	
A. niger	NAV1		Soil beneath Sauvignon Blanc vines	Alpine Valley, Vic	-	N	
A. niger	NAV2		Soil beneath Sauvignon Blanc vines	Alpine Valley, Vic	-	N	
A. niger	NAV3		Soil beneath Sauvignon Blanc vines	Alpine Valley, Vic	-	T	
A. niger	NAV4		Soil beneath Sauvignon Blanc vines	Alpine Valley, Vic	-	N	
A. niger	FRR 5375		Sultana grapes	Sunraysia, Vic	-	T	
A. niger	FRR 5575		Sultana grapes	Sunraysia, Vic	-	N	
A. niger	FRR 5576		Merbein Seedless grapes	Sunraysia, Vic	-	T	
A. niger	AA2-2		Sultana grapes	Sunraysia, Vic	-	T	
A. niger	AD2-1		Sultana grapes	Sunraysia, Vic	-	T	
A. niger	D6-6		Sultana grapes	Sunraysia, Vic	-	T	
A. niger	DD4-4		Sultana grapes	Sunraysia, Vic	-	T	
A. niger	RR2-12		Waltham raisins	Sunraysia, Vic	-	T	
A. niger	S3-9		Currants	Sunraysia, Vic	-	T	
A. niger	T1-6		Pearlettes (table grapes)	Sunraysia, Vic	-	T	
A. niger	V1-3		Sultana grapes	Sunraysia, Vic	-	T	
A. niger	X1-5		Currants	Sunraysia, Vic	-	T	

A. niger	Z3-2		Sultana grapes	Sunraysia, Vic	-	T	
A. niger	NYV1	FRR 5721	Soil beneath Chardonnay vines	Yarra Valley, Vic	-	N	yes
A. niger	NYV2		Soil beneath Chardonnay vines	Yarra Valley, Vic	-	T	
A. niger	NAH1		Soil beneath Pinot Noir vines	Adelaide Hills, SA	-	T	
A. niger	NAH2		Soil beneath Verdelho vines	Adelaide Hills, SA	-	T	
A. niger	NAH3		Soil beneath Verdelho vines	Adelaide Hills, SA	-	T	
A. niger	NAH4		Soil beneath Cabernet Sauvignon vines	Adelaide Hills, SA	-	T	
A. niger	NBa1		Vineyard soil	Barossa Valley, SA	-	N	
A. niger	NBa2		Vineyard soil	Barossa Valley, SA	-	N	
A. niger	NBa3		Vineyard soil	Barossa Valley, SA	-	N	
A. niger	NBa7		Vineyard soil	Barossa Valley, SA	-	N	
A. niger	NBa8		Rachides	Barossa Valley, SA	-	N	
A. niger	NCL1		Soil beneath Cabernet Sauvignon vines	Clare Valley, SA	-	N	
A. niger	NCL3		Soil beneath Shiraz vines	Clare Valley, SA	-	N	
A. niger	NCL4		Soil beneath Shiraz vines	Clare Valley, SA	-	N	
A. niger	NCL5		Soil beneath Shiraz vines	Clare Valley, SA	-	N	
A. niger	NCL6		Soil beneath Petit Verdot vines	Clare Valley, SA	-	T	
A. niger	NCL7		Soil beneath Grenache vines	Clare Valley, SA	-	N	
A. niger	NCL8		Soil beneath Cabernet Sauvignon vines	Clare Valley, SA	-	N	
A. niger	NCL9		Soil beneath Cabernet Sauvignon vines	Clare Valley, SA	-	N	
A. niger	NCL10		Soil beneath Cabernet Sauvignon vines	Clare Valley, SA	-	N	
A. niger	NCL12		Soil beneath Semillon vines	Clare Valley, SA	-	N	
A. niger	NCn1		Vineyard soil	Coonawarra, SA	-	N	
A. niger	NCn2		Vineyard soil	Coonawarra, SA	-	N	

Table 3.3 (cont.)

Species	Isolate	Accession no.	Source	Location	OA production	N/T profile[b]	Molecular study
A. niger	NCn5		Vineyard soil	Coonawarra, SA	-	N	
A. niger	NCn6		Vineyard soil	Coonawarra, SA	-	N	
A. niger	NCn7	FRR 5701	Rachides	Coonawarra, SA	+	N	
A. niger	NCn9	FRR 5695	Rachides	Coonawarra, SA	+	N	yes
A. niger	NLC1		Soil beneath Merlot vines	Langhorne Creek, SA	-	N	
A. niger	NLC2		Soil beneath Shiraz vines	Langhorne Creek, SA	-	T	
A. niger	NLC3		Soil beneath Shiraz vines	Langhorne Creek, SA	-	N	
A. niger	NLC4		Soil beneath Shiraz vines	Langhorne Creek, SA	-	T	
A. niger	NLC5		Soil beneath Cabernet Sauvignon vines	Langhorne Creek, SA	-	N	
A. niger	NLC7		Soil beneath Shiraz vines	Langhorne Creek, SA	-	T	
A. niger	NMV1		Vineyard soil	McLaren Vale, SA	-	N	
A. niger	NMV2		Vineyard soil	McLaren Vale, SA	-	T	
A. niger	NMV3		Vineyard soil	McLaren Vale, SA	-	T	
A. niger	NMV5		Vineyard soil	McLaren Vale, SA	-	N	
A. niger	NMV7		Vineyard soil	McLaren Vale, SA	-	T	
A. niger	NMV9		Vineyard soil	McLaren Vale, SA	-	T	
A. niger	NPd1		Soil beneath Shiraz vines	Padthaway, SA	-	T	
A. niger	NPd2		Soil beneath Semillon vines	Padthaway, SA	-	N	
A. niger	NPd3		Soil beneath Chardonnay vines	Padthaway, SA	-	T	
A. niger	NPd4		Soil beneath Riesling vines	Padthaway, SA	-	T	
A. niger	NPd5		Soil beneath Shiraz vines	Padthaway, SA	-	N	
A. niger	NPd6		Soil beneath Cabernet Sauvignon vines	Padthaway, SA	-	T	
A. niger	NRL1		Soil beneath Chardonnay vines	Riverland, SA	-	T	

A. niger	NRL10		Chardonnay rachides	Riverland, SA	-	T	
A. niger	NRL11		Chardonnay rachides	Riverland, SA	-	N	
A. niger	NRL12		Shiraz rachides	Riverland, SA	-	N	
A. niger	NRL2		Soil beneath Shiraz vines	Riverland, SA	-	T	
A. niger	NRL3		Soil beneath Chenin Blanc vines	Riverland, SA	-	T	
A. niger	NRL4		Soil beneath Chenin Blanc vines	Riverland, SA	-	N	
A. niger	NRL7		Soil beneath Chardonnay vines	Riverland, SA	-	N	
A. niger	NRL8		Soil beneath Shiraz vines	Riverland, SA	-	T	
A. niger	NRL9		Chenin Blanc rachides	Riverland, SA	-	N	
A. niger	NMR1		Soil beneath Cabernet Sauvignon vines	Margaret River, WA	-	N	
A. niger	NMR3		Soil beneath Cabernet Sauvignon vines	Margaret River, WA	-	N	
A. niger	NMR4	FRR 5694	Soil beneath Cabernet Sauvignon vines	Margaret River, WA	+	N	yes
A. niger	NMR5		Soil beneath Shiraz vines	Margaret River, WA	-	N	
A. niger	NMR6		Soil beneath Chenin Blanc vines	Margaret River, WA	-	N	
A. niger	NMR7		Soil beneath Chenin Blanc vines	Margaret River, WA	-	T	
A. niger	NMR8		Soil beneath Chenin Blanc vines	Margaret River, WA	-	T	
A. niger	NPm1		Soil beneath Cabernet Sauvignon vines	Pemberton, WA	-	N	
A. niger	NPm2		Soil beneath Chardonnay vines	Pemberton, WA	-	T	
A. niger	NPm3		Soil beneath Chardonnay vines	Pemberton, WA	-	N	
A. niger	NTa1		Soil beneath Pinot and Merlot vines	Tasmania	-	T	
A. niger	NTa2	FRR 5722	Rachides	Tasmania	-	N	yes
A. niger	NQL1		Soil beneath Chardonnay vines	Queensland	-	N	
A. niger	NQL2		Soil beneath Chardonnay vines	Queensland	-	N	
A. niger	NQL3		Soil beneath Chardonnay vines	Queensland	-	N	

Table 3.3 (cont.)

Species	Isolate	Accession no.	Source	Location	OA production	N/T profile[b]	Molecular study
A. niger	NQL4		Soil beneath Shiraz vines	Queensland	-	N	
A. niger	NQL5		Soil beneath Merlot vines	Queensland	-	T	
A. niger	NQL6		Soil beneath Shiraz vines	Queensland	-	N	
Additional strains							
A. carbonarius		A-941[a]	Grapes	Spain	+	-	yes
A. carbonarius		A-1477	Grapes	Jerez, Spain	+	-	yes
A. carbonarius		A-1500	Grapes	Vendrell, Spain	+	-	yes
A. carbonarius		A-1040	Raisins	Spain	+	-	yes
A. carbonarius		A-1070	Raisins	Spain	+	-	yes
A. carbonarius		CBS 110.49[a]	Air	Indonesia	-	-	yes
A. carbonarius		M325	Apples	supplied by H.M.L.J Joosten	+	-	yes
A. carbonarius		CBS 127.49	Coffee	Unknown	+	-	yes
A. carbonarius		NRRL67[a]	Soil	Brazil	+	-	yes
A. carbonarius		A-642	Soil	Portugal	+	-	yes
A. niger		FRR 2522	Peanuts	Kingaroy, Australia	-	N	yes
A. niger		FRR 333	Rice	Murrumbidgee Irrigation Area, Australia	-	N	yes
A. niger		A-943	Grapes	Portugal	+	N	yes
A. niger		A-947	Grapes	Spain	-	T	yes
A. niger	w-148[a]	A-1743	Grapes	Spain	-	N	yes
A. niger		A-1241	Grapes	Turis, Spain	+	N	yes
A. niger		A-942	Raisins	Greece	+	N	yes
A. niger	A-75	FRR 5361	Feed	Spain	+	N	yes
A. niger		A-220	Feed	Spain	+	N	yes

A. niger		A-615	Feed	Spain	-	T	yes
A. niger	A-136	FRR 5353	Soy	Canada	+	N	yes
A. niger		A-655	Wheat	Spain	-	N	yes
A. niger		A-487	Wheat	Spain	-	T	yes
A. niger	CECT 2088	CBS 120.49	Unknown	USA	-	N	yes
A. niger		A-946	Coffee	Portugal	-	N	yes
A. niger		CBS 121.55	Otomycosis	Switzerland	-	N	yes
A. niger		A-656	Soil	Spain	-	T	yes
A. niger		CBS 554.65	Tannin-gallic acid ferment	USA	-	N	yes
A. awamori[d]		CBS 139.52	Kuro-koji	Japan	+	N	yes
A. foetidus[d]		CBS 618.78	Unknown	Germany	+	N	yes
A. tubingensis[d]		CBS 134.48	Unknown	Unknown	-	T	yes
A. aculeatus		A-1122	Grapes	Murcia, Spain	-	-	yes
A. aculeatus		A-1325	Grapes	Vendrell, Spain	-	-	yes
A. aculeatus		A-1355	Grapes	Penedès, Spain	-	-	yes
A. aculeatus		A-1356	Grapes	Penedès, Spain	-	-	yes

[a] isolates accessioned into the FRR culture collection, Food Science Australia, North Ryde, NSW, Australia. A and w: isolates from the collection of the Autonomous University of Barcelona, Veterinary Faculty, Bellaterra, Barcelona, Spain. CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands. CECT: Coleccion Espanola de Cultivos Tipo, Burjassot, Valencia, Spain. NRRL: Agricultural Research Service Culture Collection, Peoria, IL, USA

[b] RFLP profile for *A. niger* strains only (Accensi et al., 1999)

[c] NSW: New South Wales; SA: South Australia; Vic: Victoria; WA: Western Australia

[d] these species are closely related to *A. niger* and are often morphologically indistinguishable. They are usually described as members of the *A. niger* aggregate

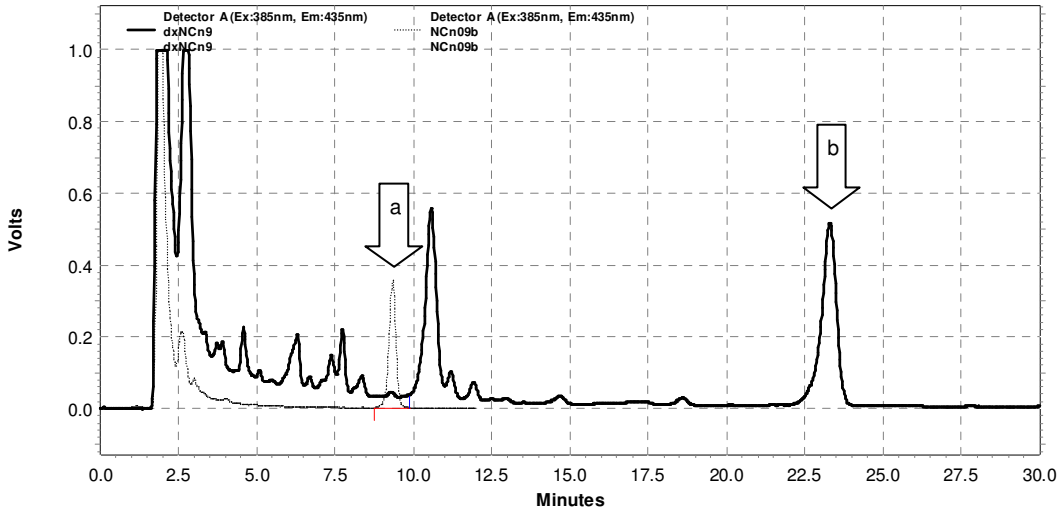


Figure 3.1: Overlaid chromatograms of ochratoxin A produced by *Aspergillus niger* FRR 5695 in (a) underivitized (retention time 9.3 min) and (b) derivitized (retention time 23.3 min) forms. The dotted trace shows the fungal extract prior to derivitization and the bold trace, the fungal extract after derivitization

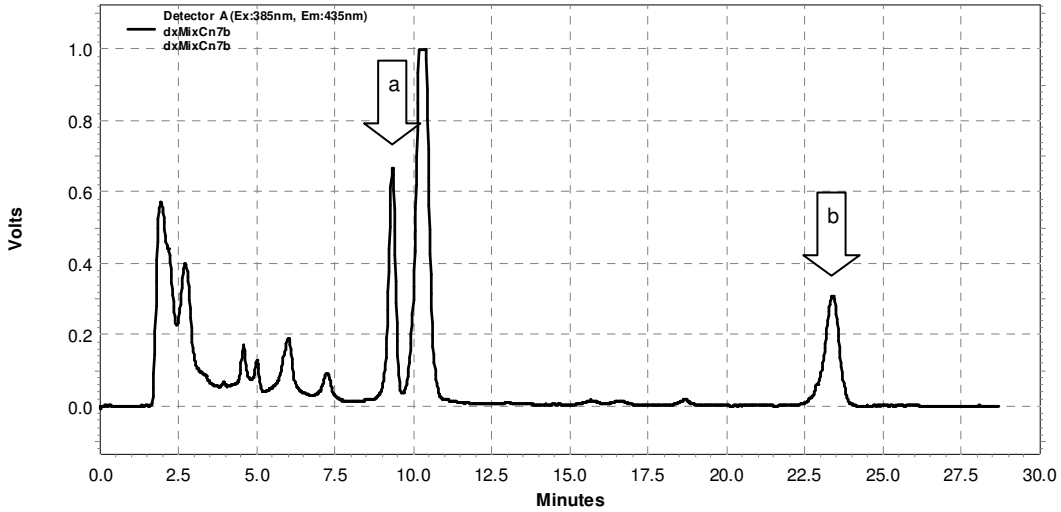


Figure 3.2: Chromatogram of a mixed sample consisting of standard ochratoxin A solution (22 ng/mL), derivitized OA standard, and derivitized extract from *Aspergillus niger* FRR 5701. Peak 'a' comprises OA from the standard, retention time 9.3 min; peak 'b' demonstrates the co-elution of derivitized OA in both the standard and fungal extract, retention time 23.4 min

3.2.2.3 Strain typing of *Aspergillus niger*

RFLP analysis of 113 isolates of *A. niger* from 18 Australian viticultural regions separated the isolates into the same two groups described by Accensi et al. (1999). Briefly, type N fragments possess the restriction site for *Rsa*I and the cleaved fragment of type N strains is smaller (519 bp) than the uncleaved fragment of type T strains (595 bp) (Fig. 3.3). Sixty one isolates, including the three toxigenic isolates, showed the type N pattern, and 52 isolates showed the type T pattern (Table 3.3).

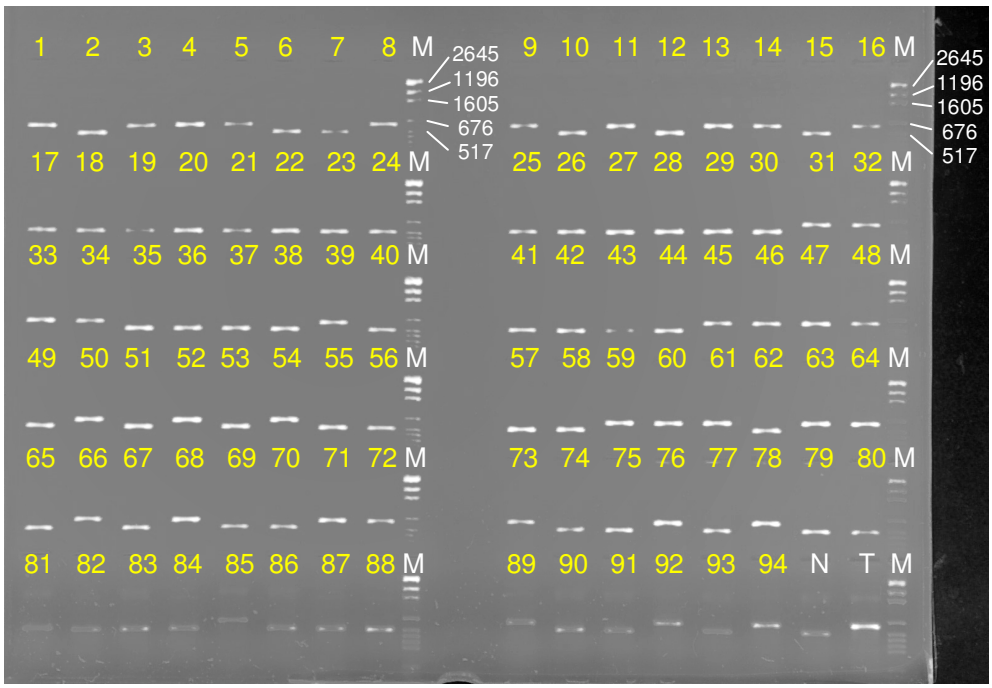


Figure 3.3: RFLP analysis of ribosomal DNA from a selection of *Aspergillus niger* isolates from Australian vineyards; differentiation of type N from type T strains. M denotes the pGEM molecular weight marker. In the final two lanes (bottom right of gel) are isolates of known RFLP type, FRR 5361 type N and FRR 3252 type T. Details of isolates are given in Table 3.3. Loaded in ascending numerical order on the gel are isolates NHV3, NHV5, NHV7, NHV10, NHV11, NHV13, NMu4, NMu5, NMu6, NMu7, NMu8, NMu9, NMu10, NRv1, NRv2, NRv3, NRv4, NRv6, NRv7, NRv8, NCn5, NCn6, NCn7 (FRR 5701), NCn9 (FRR 5695), NBa1, NBa2, NBa3, NBa7, NBa8, NMV1, NMV2, NMV3, NMV7, NMV9, NCL1, NCL3, NCL4, NCL5, NCL6, NCL7, NCL8, NCL9, NCL10, NCL12, NAH1, NAH2, NAH3, NAH4, NLC1, NLC2, NCL3, NLC4, NLC5, NLC7, NMR3, NMR4 (FRR 5694), NMR5, NMR6, NMR7, NMR8, NPd1, NPd2, NPd3, NPd4, NPd5, NPd6, NPm1, NPm2, NPm3, NTa2 (FRR 5722), NRL1, NRL2, NRL3, NRL4, NRL7, NRL8, NRL9, NRL10, NRL11, NRL12, NQL1, NQL2, NQL3, NQL4, NQL5, NQL6, NMV5, NYV1, NYV2 (FRR 5721), NAV1, NAV2, NAV3, NAV4. Isolate 94 was FRR 3911 *A. niger* var. *awamori* from soil in Western Australia

3.3 Techniques to assess molecular relationships among isolates of Aspergillus niger and A. carbonarius

The experiments reported in section 3.3 were planned, executed and analysed in collaboration with Alexandre Esteban, a PhD candidate from the Autonomous University of Barcelona on a visiting scholarship to Food Science Australia. The findings are jointly reported in the PhD theses of both Su-lin Leong and Alexandre Esteban.

3.3.1 Methods

3.3.1.1 Strain selection and DNA extraction

All the isolates of *A. carbonarius* from Australian vineyards (all toxigenic) described in section 3.2 plus one toxigenic *A. niger* isolate from each of Margaret River, Western Australia (FRR 5694) and Coonawarra, South Australia (FRR 5695) were included in this study of the molecular relationships among black *Aspergillus* spp. Three non-toxigenic *A. niger* isolates, from the Hunter Valley, New South Wales (FRR 5720), the Yarra Valley, Victoria (FRR 5721) and Tasmania (FRR 5722), were also included, with FRR 5720 representing a type T strain. The remainder of the isolates examined in this study comprised black *Aspergillus* isolates from a variety of sources and locations, several of which have been characterised for OA production by Esteban et al. (2004). Isolates from grapes in Europe were included in the study for comparison with Australian isolates. Table 3.3 gives details of the isolates used in this molecular study. DNA was extracted from the isolates as described in section 3.2.1.3.1.

3.3.1.2 ERIC-PCR

PCR amplification was performed in 20 µL reaction volumes containing 1x PCR buffer (Qiagen, Germany), 5% glycerol, 250 µM each dNTP, 20 pmol of each primer (ERIC2F: 5'-AAGTAAGTGACTGGGGTGAGCG-3' and ERIC1R: 5'-ATGTAAGCTCCTGGGGATTCAC-3'; Gillings and Holley (1997); Proligo, Boulder, CO, USA), 1 U of *Taq* DNA polymerase (Qiagen, Germany) and 20-40 ng of DNA as template. Amplification was performed in a Hybaid PCR Express Thermal Cycler (Integrated Sciences, Willoughby, NSW, Australia) with the cycling parameters: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 68 °C for

8 min, and then a final extension step of 68 °C for 8 min. Electrophoresis and visualisation of amplicons were described in section 3.2.1.3.2.

Each set of comigrating bands was designated as a locus and each isolate was scored visually for the presence (1) or absence (0) of a band at that locus (Appendix D, Table D.1). A distance matrix based on pairwise comparisons was obtained using the RAPDistance program version 1.04 (Armstrong et al. (1994); http://www.anu.edu.au/BoZo/software/, accessed 09/08/04).

3.3.1.3 AFLP

All primers and adapters used in AFLP analysis were obtained from Proligo (Lismore, NSW, Australia). This method was based on a protocol developed for black *Aspergillus* spp. as part of the EU project, Risk Assessment and Integrated Ochratoxin A Management in Grapes and Wine (Wine Ochra-Risk, QLK-CT-2001-01761) (G. Mulè, Istituto di Scienze delle Produzioni Alimentari (ISPA), CNR, Italy, pers. comm. 05/05/04).

Approximately 400-800 ng of genomic DNA was digested with the restriction endonucleases *Eco*RI (13 U) and *Mse*I (7 U) (New England BioLabs, Inc., Beverly, MA, USA) in 40 µL 1x One-Phor-All buffer (Amersham Biosciences UK Ltd, Buckinghamshire, UK) at 37 °C for 2.5 h. The restriction fragments were ligated to the double-stranded restriction site-specific adapters EcoRI (4 pmol) and MseI (38 pmol) using T4-ligase (0.75 Weiss units) in 0.2x T4 ligase buffer (Promega, Wisconsin, MI, USA), total volume 7.5 µL. Pre-amplification was performed using the combination of primers EcoRI-A (5'-GACTGCGTACCAATTCA-3') + MseI-C (5'-GATGAGTCCTGAGTA AC-3'), or EcoRI (5'-ACTGCGTACCAATTC-3') + MseI-C. The pre-amplification mixture (25 µL) contained 1x PCR buffer (Qiagen, Germany), 200 µM each dNTP, 25 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Qiagen, Germany) and 20-40 ng digested DNA as template. Amplification was performed in a Hybaid PCR Express Thermal Cycler (Integrated Sciences, Willoughby, NSW, Australia) for 20 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min.

Amplicons from the pre-amplification reaction were diluted 1:10 in sterile Milli-Q water for use as a template for the selective amplification. Three primer combinations were chosen for use with template DNA from the EcoRI-A + MseI-C pre-amplification: A [EcoRI-AC (5'-GACT GCGTACCAATTCAC-3') + MseI-CC (5'-GATGAGTCCTGAGTAACC-3')]; B [EcoRI-AT (5'-GACTGCGTACCAATTCAT-3') + MseI-CG (5'-GATGAGTCCTGAGTAACG-3')]; C [EcoRI-AC + MseI-CA (5'-GATGAGTCCTGAGTAACA-3')]. A fourth primer combination, D [EcoRI-G (5'-GACTGCGTACCAATTCG-3') + MseI-CT (5'-GATGAGTCCTGAGTAACT-3')], was chosen for use with template DNA from the Eco-RI + MseI-C pre-amplification. The EcoRI primers were labelled at the 5' end with the fluorescent dye FAM for primer sets A, C and D, and with HEX for primer set B. The reaction mixture (25 µL) contained 5 µL diluted template amplicons, 1x PCR buffer (Qiagen, Germany), 200 µM each dNTP, 20 pmol of each primer and 2.5 U of *Taq* DNA polymerase (Qiagen, Germany). The cycling parameters for amplification were: 10 cycles of 94 °C for 1 min, 65-56 °C for 1 min (decreasing every cycle from 65 °C by 1 Celsius degree) and 72 °C for 90 s; followed by 20 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min. Electrophoresis and visualisation of amplicons were described in section 3.2.1.3.2. The sizes of the multiple amplicons present for each isolate were assessed using an automated sequencer (SUPAMAC, Camperdown, NSW, Australia).

Each amplicon within the size range 50-500 bp and above the threshold height (fluorescence intensity greater than 200) was defined as an allele and a distance matrix was produced by pairwise comparison using the Dice coefficient (Dice, 1945) and ADE-4 software version 2001 (LecPCR and DistAFLP; http://pbil.univ-lyon1.fr/ADE-4/microb/, accessed 23/07/04).

To base the phylogenetic analysis on the most robust data, the entire procedure was replicated from the DNA digestion stage with selective primer sets B and D. For each isolate, only alleles (amplicons) that were detected in both replicates (Align2, ADE-4 software version 2001; http://pbil.univ-lyon1.fr/ADE-4/microb/, accessed 16/09/04) were included in the phylogenetic analysis. Data from primer sets B and D were combined for pairwise comparison.

3.3.1.4 Microsatellites

Database searches of published sequences were performed using software available through the WebANGIS interface of the Australian National Genomic Information Service (ANGIS; http://www.angis.org.au, accessed 06/04). Sequences of *A. niger* and *A. carbonarius* from GenBank (3390 in total) were screened for microsatellite motifs, using all possible dinucleotide and trinucleotide motifs, each in ten tandem repeats, as queries in BLASTN searches. Primers for PCR amplification were designed to complement sequences flanking the microsatellite loci identified in the BLASTN search, using Primer3© (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and Net primer© (Biosoft International, Palo Alto, CA, USA) software.

Polymorphism at these loci was detected by screening a subset of *A. niger* isolates (A-136, A-656, A-942, A-946, A-947, FRR 2522 and FRR 5694) comprising both toxigenic and non-toxigenic isolates and isolates of both type N and type T RFLP profiles. Isolates of *A. carbonarius* (A-941, CBS 110.49, FRR 5371 and FRR 5715) and *A. aculeatus* (A-1122) were also included in this subset, to determine if these microsatellite markers could be used to characterise species closely related to *A. niger*. PCR amplification was performed as described in section 3.2.1.3.2, with reaction volume adjusted to 20 µL. Polymorphisms were presumptively identified as slight differences in amplicon size when visualised on an agarose gel. Amplicons were selected for sequencing (SUPAMAC, Camperdown, NSW, Australia) if presumptive polymorphisms were observed, or if amplified from isolates of different species, RFLP type or toxigenic ability. Sequences were aligned using ClustalW (accessed through WebAngis).

Details of the six loci utilised in this study are listed in Table 3.4. The reverse primer from each polymorphic locus was 5'-labelled with a fluorophor (PE Biosystems, Foster City, CA, USA) for sizing with an automated sequencer and PIG-tailed (Brownstein et al., 1996) in order to standardise the adenylation of the 3' end of the complementary strand.

To achieve clear and reproducible bands and to minimise non-specific annealing of the primers, PCR conditions were optimised by increasing the annealing temperature in 1 Celsius degree increments from 54 °C to 62 °C, using the subset of isolates described above. The optimal cycling parameters were: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 54 °C for 1 min (ACNM2, 5, 6, 7) or 61 °C for 1 min (ACNM1, 3) and 72 °C for 1 min, and then a final extension step of 72 °C for 10 min. Amplification with combinations of forward and reverse primer concentrations at 50 nM, 200 nM and 800 nM was also tested, but did not improve clarity of bands; hence, the standard concentration of 250 nM for both primers was retained.

Based on trends observed during the screening of the subset of isolates, PCR amplifications using ACNM1, 2, 3, 5 and 7 were performed for all *A. niger* isolates. Amplification using ACNM6 was only performed for *A. niger* type N isolates, as no amplicons were observed for type T isolates during screening. The presence of amplicons within the appropriate size range was assessed following electrophoresis on an agarose gel, as described in section 3.2.1.3.2, after which the products were diluted and the size of amplicons determined using an automated sequencer (SUPAMAC, Camperdown, NSW, Australia). Amplicons from ACNM1, 2 and 3 were combined into a single sample for analysis, as were amplicons from ACNM 5, 6 and 7. The amplicons differed in size and were labelled with different fluorophores, thus the fluorescence peaks detected by the sequencer were unlikely to overlap and interfere with the signal.

Each amplicon of unique size was designated an allele and the absence of an amplicon was defined as a null allele. Pairwise population distances were calculated using the Microsat2 program (http://hpgl.stanford.edu/projects/microsat/, accessed 24/09/04) based on the proportion of shared alleles. Bootstrap analysis was based on 200 resampled data sets.

3.3.1.5 Construction of dendrograms

Distance matrices derived by the various techniques were analysed using the neighbour-joining algorithm in Phylip (Felsenstein (1989); accessed via WebANGIS, 2004-05; also available from http://evolution.genetics.washington.edu/phylip.html) and dendrograms drawn using TreeExplorer 2.12 (K. Tamura;

http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html, accessed 22/07/1999). Where possible, isolates of *A. aculeatus* were used as the outgroup, as this uniseriate species is distinct from *A. carbonarius* and *A. niger*, which are both biseriate. Bootstrap analyses for the ERIC-PCR and AFLP dendrograms were based on 1000 resampled data sets generated using PAUP* (D.L. Swofford; Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4, Sinauer Associates, Sunderland, MA, USA).

3.3.2 Results

3.3.2.1 ERIC-PCR

The dendrogram was constructed based on the presence or absence of bands at 16 distinct loci (Fig. 3.4; Appendix D, Table D.1). *A. carbonarius* and *A. niger* formed two clusters when *A. aculeatus* isolates were used as the outgroup (Fig. 3.5). Three toxigenic isolates of *A. niger* (two from Spain and one from Australia) fell outside the main *A. niger* cluster. ERIC-PCR did not differentiate among the *A. carbonarius* isolates. Differences among isolates of *A. niger* were detected; however, this differentiation did not seem to be reliable. For example, a toxigenic strain, A-136, was not differentiated from a non-toxigenic strain, A-946. Toxigenic and non-toxigenic isolates, and N and T RFLP types within the *A. niger* aggregate were mixed in the same clusters by this technique. Isolates of *A. niger* from Australia clustered together, with the inclusion of a single Spanish isolate. No other association between origin or substrate and molecular relatedness was observed. Bootstrap analysis provided little support for the ERIC-PCR dendrogram.

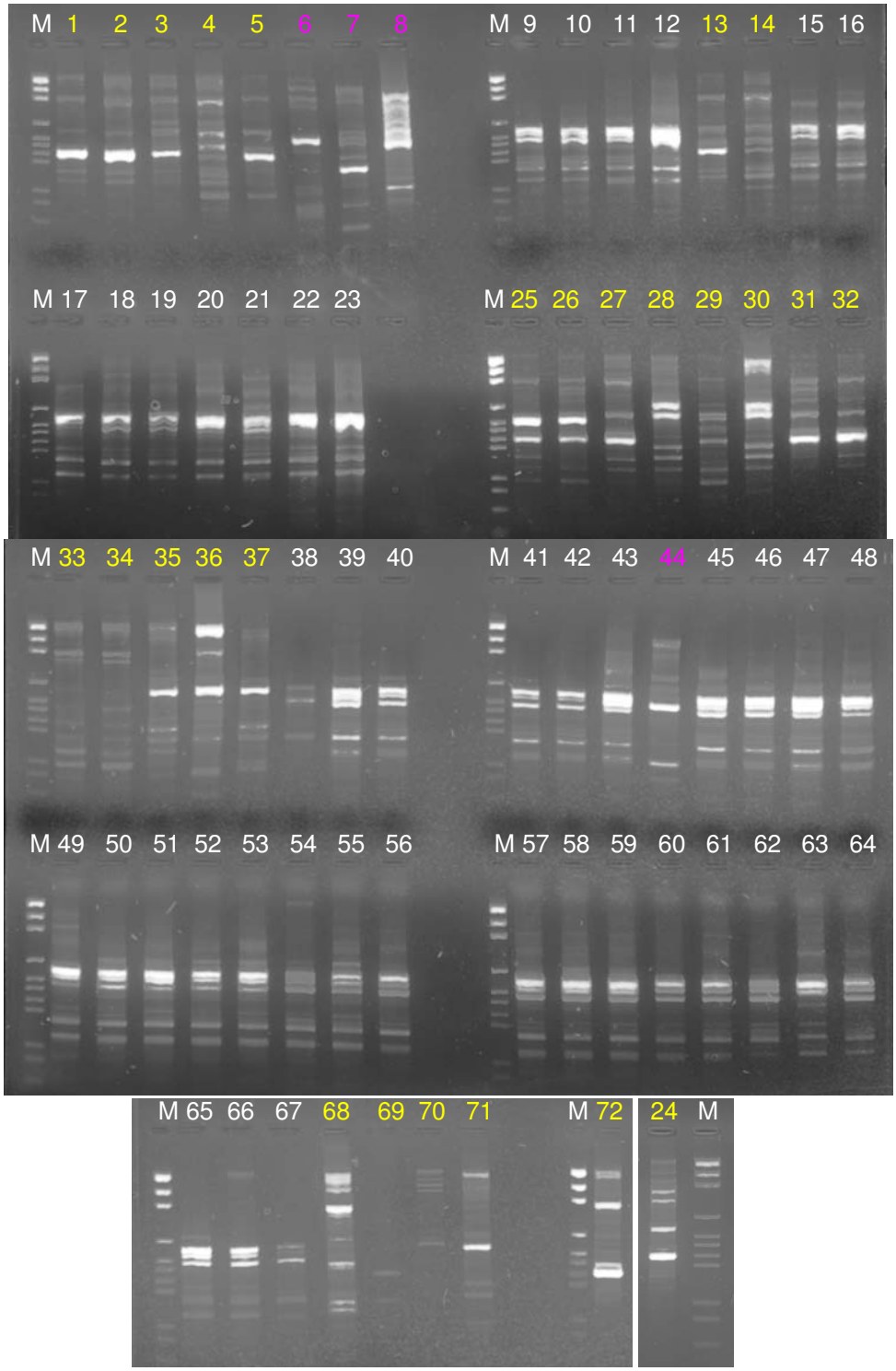


Figure 3.4: Amplification of DNA from black *Aspergillus* spp. in PCR with ERIC primers. Lanes containing amplicons from *A. niger* isolates are numbered in yellow, from *A. carbonarius* isolates, in white and from *A. aculeatus* isolates, in pink. Details of isolates are tabulated in Appendix D, Table D.1. M denotes the pGEM molecular weight marker

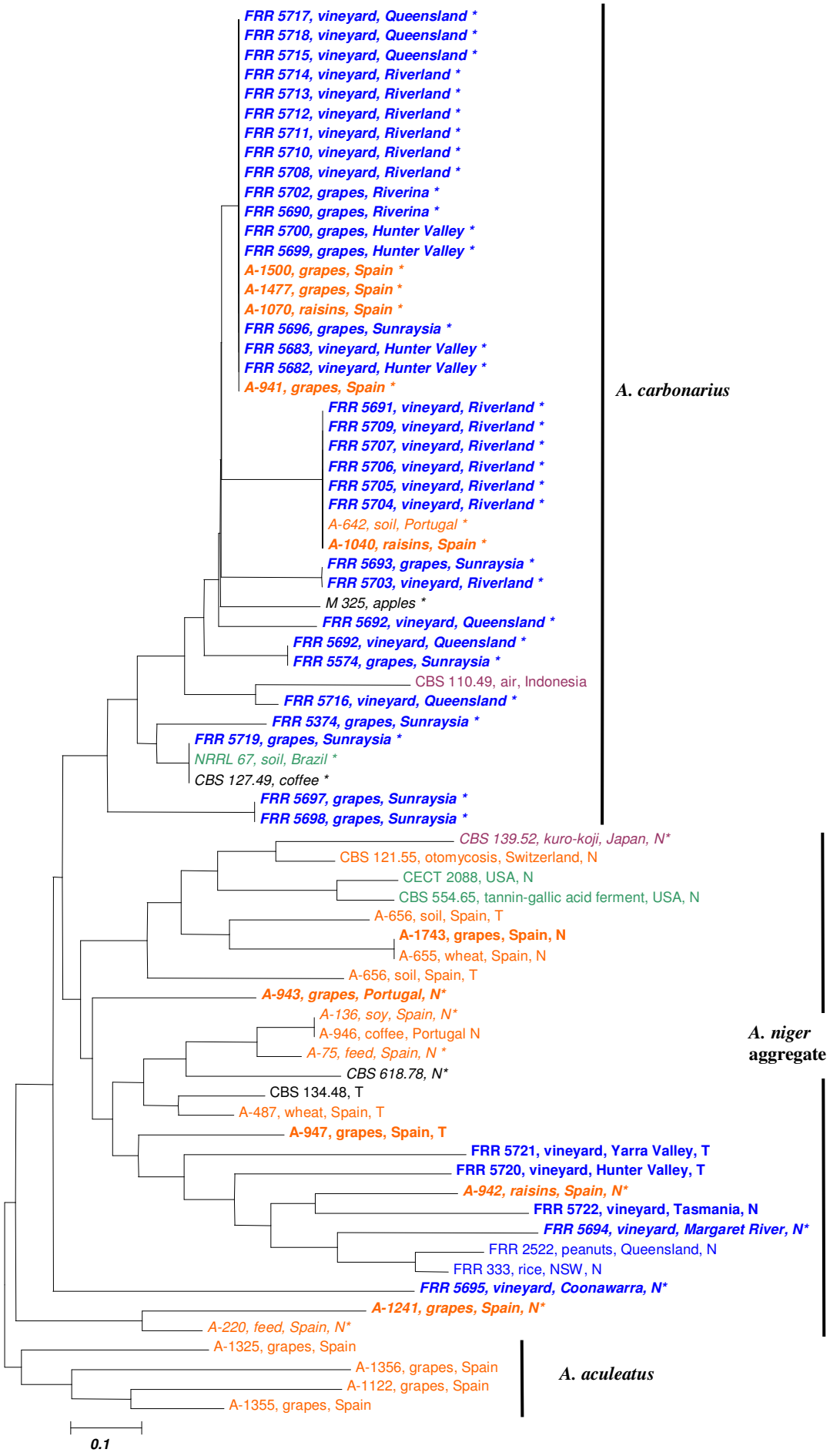


Figure 3.5: Determination of molecular relationships among isolates of *Aspergillus carbonarius*, *A. niger* aggregate and *A. aculeatus* by ERIC-PCR. Colours denote the continent of origin, if known: Australia, Europe, Asia, Americas. Toxigenic isolates are *italicised* and denoted *. Isolates associated with grapes (including those from vineyard soil, raisins, rachides) are in **bold**. The RFLP type (N/T; Accensi et al. (1999)) is given for *A. niger* isolates

3.3.2.2 AFLP

Data from selective amplification primer combinations B (EcoRI-AT + MseI-CG) and D (EcoRI-G + MseI-CT) produced dendrograms that reliably separated *A. carbonarius*, *A. niger* and *A. aculeatus* into three clusters (data not shown), hence, these combinations were selected for repetition of the analysis from DNA digestion onwards. Data were also analysed using the Jaccard coefficient of similarity (Sneath and Sokal, 1973) and the dendrograms produced showed clustering similar to those produced using the Dice coefficient (data not shown).

AFLP analyses of combined data from the primer combinations mentioned above differentiated between the three species and also separated the *A. niger* aggregate into two distinct groups corresponding to the N and T RFLP types (Fig. 3.6). The type N cluster was further subdivided into two groups, with bootstrap value 92%, each group containing a mixture of toxigenic and non-toxigenic isolates. Clustering among *A. carbonarius* isolates was not evident. Relationships between major clusters and substrate or geography were not observed, with isolates from vineyards closely related to isolates from various other sources. Similarly, isolates from Europe and Australia were interspersed in the dendrogram. Isolates of *A. carbonarius* from a single Queensland vineyard shared some similarities, appearing at the bottom of the *A. carbonarius* cluster together with isolates from Brazil and Indonesia. Isolates of *A. carbonarius* from other viticultural regions were interspersed in the middle and upper portions of the cluster.

3.3.2.3 Microsatellites

From the search of sequences in GenBank, ten microsatellite loci were identified among *A. niger* sequences. However, no loci were identified in sequences of *A. carbonarius* lodged as at June, 2004. Of these ten loci, six were polymorphic. Multiple sequence alignments showing polymorphisms within the six microsatellite loci are shown in Fig. 3.7. ACNM3 and ACNM7 contained perfect microsatellite repeats, whereas other loci contained imperfect repeats, with occasional base substitutions within the repeated motif. Differences in allele size among isolates did not always correspond exactly to the addition or deletion of dinucleotide or trinucleotide repeat units, which suggested that other polymorphisms existed within the amplified region.

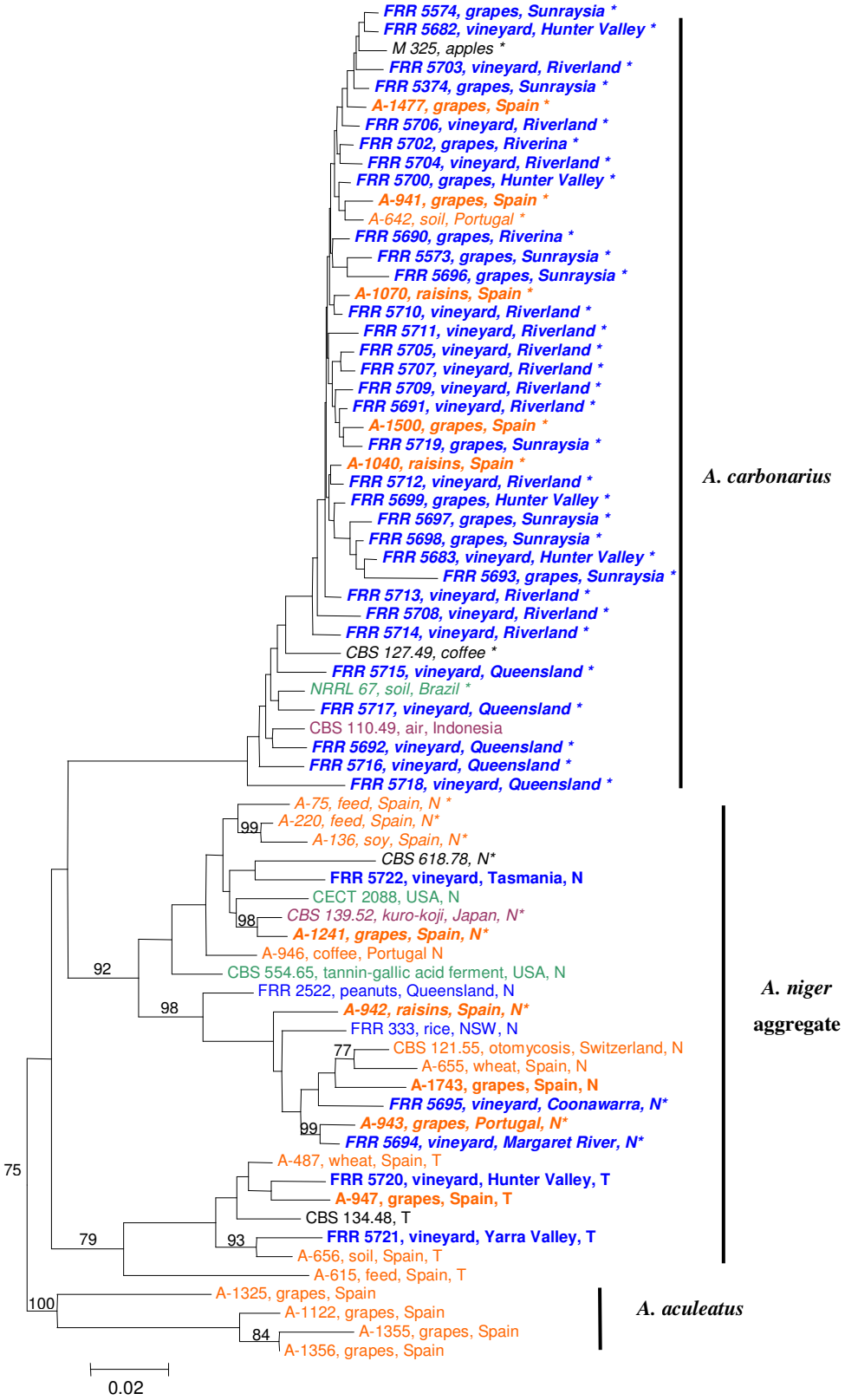


Figure 3.6: Determination of molecular relationships among isolates of *Aspergillus carbonarius*, *A. niger* aggregate and *A. aculeatus* by AFLP. Colours denote the continent of origin, if known: Australia, Europe, Asia, Americas. Toxigenic isolates are *italicised* and denoted *. Isolates associated with grapes (including those from vineyard soil, raisins, rachides) are in **bold**. The RFLP type (N/T; Accensi et al. (1999)) is given for *A. niger* isolates

Figure 3.7: Multiple sequence alignment of microsatellite loci (a) ACNM1, (b) ACNM2, (c) ACNM3, (d) ACNM5, (e) ACNM6 and (f) ACNM7 from isolates of *Aspergillus niger* and *A. carbonarius.* The microsatellite repeat units are underlined and the allele size in base pairs is given after the sequence

Figure 3.7a: ACNM1 multiple sequence alignment showing $(CA)_n$ repeat

```
                331       341       351       361       371
A. niger A-136 TAAGTCTCAACCTTGCCACACACACACACACACACACACACAGA--
A. niger A-942 TAAGTCTCAACCTTGCCACACACACACACACACACACACACACACA

          381       391       401
A-136 ------------------AAGGGAAAGAAATA (485)
A-942 CACACACACACACACAAAAAGGGAAAGAAATA (509)
```

Figure 3.7b: ACNM2 multiple sequence alignment showing $(GA)_n$ repeat

```
                341       351       361       371       381
A. niger A-136 TGAATTCTCCATTGGGAACGAATCAGAGAGAGAGAGAGAGAGAGAAA
A. niger A-942 TTAATTTTCCATTGGGAAAGAATCAGAGAGAGAGAGAGAGAGAGA--
A. niger A-946 TTAATTTTCCATTGGGAAAGAATCAGAGAGATAGAGAGAGAGAGATA

         391       401       411
A-136 GATAGAAAGAGA------GGCAGAGTAA (428)
A-942 ------------------GGCAGAGTAA (418)
A-946 AAGAGAGAGAGAGAGAGAGGCAGAGTAA (446)
```

Figure 3.7c: ACNM3 multiple sequence alignment showing $(CCA)_n$ repeat

```
                        101       111       121       131
A. niger A-136          ATCAACTTTATTGTGGAGAAACCACCACCACCACCA
A. niger A-942          ATCAACTTTATTGTGGAGAAATCACCACCACCACCA
A. niger FRR 2522       ATCAACTT-ATTGTGGAGAAACCACCACCACCACCA
A. carbonarius FRR 5374 ATCAACTTTATTGTGGAGAAATCACCACCACCACCA

             141       151       161
A-136    CCACCAACAACCACGGAGGCAAGTTAA (192)
A-942    CCA---ACAACCACGGAGGCAAGTTA- (189)
FRR 2522 -------CAACCACGGAGGGAAGTTAA (184)
FRR 5374 CCA---ACAACCACGGAGGCAAGTTAA (189)
```

Figure 3.7d: ACNM5 multiple sequence alignment showing $(CAA)_n$ repeat

```
                81        91        101       111       121
A. niger A-136 GAGCGGTTGCCGGAGGGTTTCCAACAGCAA---------------
A. niger A-942 GAGCGGTTGCCGGAGGGTTTCCAACAA-----------------
A. niger A-946 GAGCGGTTGCCGGAGGGTTTCCAACAACAACAACAACAACAACAA

           131       141       151
A-136 ---------CAACAACGTATCTTGTGACTCAAACCT (164)
A-942 ---------CAACAACGTATCTTGTGACTCAAACCT (163)
A-946 CAACAACAACAACAACGTATCTTGTGACTCAAACCT (204)
```

Figure 3.7e: ACNM6 multiple sequence alignment showing $(ATC)_n$ repeat

```
                181       191       201       211       221
A. niger A-136 GACAATAACAACAACAACAACATCATCATCATCATCATCATCATC
A. niger A-942 GGCAATAACAAC--------------------ATCATCATCACC

           231       241       251
A-136 ATCATCATCATCACAAAGGAGCTGTATA (455)
A-942 ATCATCATCATCACAAAGGAGCTGTATA (431)
```

Figure 3.7f: ACNM7 multiple sequence alignment showing $(GTA)_n$ repeat

```
                        141       151       161       171
A. niger A-136          ATTCGACCACATTTGACGATGGTAGTAGTAGTAGTA
A. niger A-942          ATTCGACCACATTTGACGATGGTAGTAGTAGTAGTA
A. carbonarius FRR 5374 ATTCGACCACATTTGACGATGGTAGTAGTAGTAGTA

             181       191       201       211       221
A-136    GTAGTAGTAGTAGTAGTATAGATGTTCTGTAGAAGTATGTATAAGCC (405)
A-942    GTAGTAGTA------GTATAGATGTTCTGTAGAAGTATGTATAAGCC (399)
FRR 5374 GTAGTAGTA------GTATAGATGTTCTGTAGAAGTATGTATAAGCC (399)
```

Amplicons for five of the six polymorphic loci were detected from a majority of the 26 *A. niger* strains, and displayed 6-13 alleles (Table 3.4), with an average of 9.67 alleles per locus. Based on the initial screening results, amplification of ACNM6 was attempted only from type N *A. niger* strains; however, amplicons were detected from both toxigenic and non-toxigenic type N strains. When these microsatellite markers were tested using four isolates of *A. aculeatus* and 32 isolates of *A. carbonarius*, amplicons were detected for ACNM1 and ACNM3 from *A. aculeatus*; however, ACNM3 often displayed two bands of similar size from individual isolates. It is not clear whether the double bands resulted from non-specific amplification or if they represented two alleles within individual isolates. Amplicons from certain *A. carbonarius* isolates were detected for ACNM1, 2, 3, 5 and 7, and sequencing demonstrated the presence of microsatellites for ACNM3 and 7 (Fig. 3.7c,f). However, consistent results could not be achieved, the loci were not amplified in several isolates and ACNM3 again commonly displayed double bands for individual isolates. Hence, insufficient data precluded the inference of phylogenetic relationships among *A. carbonarius* and *A. aculeatus* isolates using these microsatellite primer sets. Allele sizes for all amplified loci are listed in Appendix D, Table D.2.

Table 3.4: PCR primer sequences, number of alleles and size range observed for microsatellite loci in *Aspergillus niger*

Locus	Repeat motif	Primer (5'-3')	Size range	No. of alleles	D[b]	H_O[c]	GenBank accession no.
ACNM1	$(CA)_{15}$	TCTCGACTCTGGCTCCTACC [aF]GTTTGCTTACTCACCGACTGGAAAA	464-509	13	0.93	0.90	AY081845
ACNM2	$(CT)_{10}$	TGCCCTTACTCTGCCTCTCT [H]GTTTCCATTATTCACCCTCCCTTCT	409-446	6	0.80	0.77	AX952973
ACNM3	$(CCA)_{15}$	TAACTTGCCTCCGTGGTTGT [R]GTTTGAGACCGGAAACATTGGAGTAG	177-215	12	0.91	0.88	BE759201
ACNM5	$(GTT)_{12}$	CGTTTTCTCGGAAGGTTTGA [R]GTTTGTGCGTGTTGGGGACTATCT	163-204	8	0.73	0.71	ANAJ5117
ACNM6	$(ATC)_{12}$	CGACAGCCGCATCATAGTT [F]GTTTCCTGCTCTTTTTGCCTTCTTT	429-458	9	0.84	0.81	AY081847
ACNM7	$(GTA)_{10}$	TGAGGGAAGGGGGTTTTATT [H]GTTTGATCTACGGGGGTGTTTGTC	378-468	10	0.89	0.86	ANI278532

[a] superscript letters indicate fluorescent labels: [F]FAM, [H]HEX, [R]ROX
[b] numerical index of discriminatory power (Hunter, 1991)
[c] observed heterozygosity (Nei, 1978)

Polymorphisms at the six microsatellite loci separated isolates of the *A. niger* aggregate into two distinct clusters which corresponded with the N and T types of the RFLP analysis (Fig. 3.8). The microsatellite dendrogram showed similar topology to the AFLP dendrogram, although bootstrap analysis of the microsatellite data showed low support. Type N isolates were subdivided into two groups that corresponded with those derived by AFLP (Fig. 3.6), apart from slight differences in the minor branching.

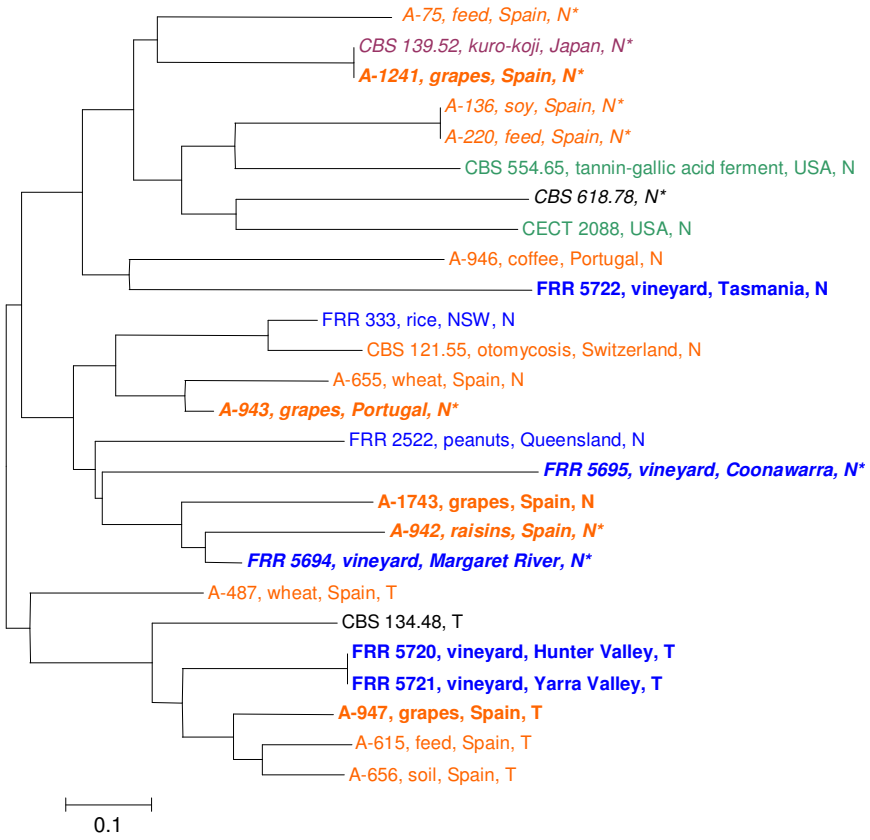


Figure 3.8: Determination of molecular relationships among isolates of the *Aspergillus niger* aggregate by analysis of six polymorphic microsatellite loci. Colours denote the continent of origin, if known: Australia, Europe, Asia, Americas. Toxigenic isolates are *italicised* and denoted *. Isolates associated with grapes (including those from vineyard soil, raisins) are in **bold**. N/T denotes the RFLP type (Accensi et al., 1999), and type T isolates were used as the outgroup

3.4 Discussion

3.4.1 Isolation and toxigenicity of black *Aspergillus* spp. from Australian viticultural regions

The dominance of *A. niger* over *A. carbonarius* and *A. aculeatus* in Australian vineyards mirrors the frequent isolation of this species from grapes in Europe and South America (discussed in section 3.1.1, Table 3.1). The dominance of *A. niger* has been attributed to its high optimum and maximum growth temperatures compared with the other two species (Battilani et al., 2003c; Bellí et al., 2004b; Leong et al., 2004; Bellí et al., 2005a) and its rapid rate of growth. This is examined in greater detail in chapter 4. *A. carbonarius* was isolated primarily from warm regions such as the Riverland and Queensland (Table 3.2), and *A. aculeatus* was often isolated from the same samples or regions. It is not clear whether these two species share some characteristic which was favoured by local conditions in those vineyards and/or regions.

The frequency of toxigenicity among *A. niger* and *A. carbonarius* isolates from Australian vineyards is similar to that among isolates from grapes in Spain (Cabañes et al., 2002; Abarca et al., 2003; Bau et al., 2005a), Italy (Battilani et al., 2005b), France (Sage et al., 2002) and Portugal (Serra et al., 2003, 2005a). Nearly all *A. carbonarius* isolates from grapes in Greece (Tjamos et al., 2004) or from dried vine fruit in Argentina (Magnoli et al., 2004) produced OA; however, toxigenicity, albeit at low concentrations, was also observed in a higher proportion of *A. niger* isolates than reported in the other countries listed above. This high frequency of toxigenicity among *A. niger* isolates and, conversely, a relatively low frequency of toxigenicity among *A. carbonarius* isolates from grapes in South America was reported by Da Rocha Rosa et al. (2002) and Magnoli et al. (2003). It is not apparent whether these variations represent differing population structures in vineyards in these countries, or if they result from misidentification of the fungi or erroneous detection of OA at low concentrations. Non-toxigenic isolates of *A. carbonarius*, tentatively designated as a new species *A. ibericus* (as mentioned by Serra et al. (2005a), with preliminary molecular characterisation by Bau et al. (2005b)), have not been reported in Australia. For *A. niger* isolates from grapes in Europe screened for OA production and subsequently classified as type N or type T based on RFLP analysis, toxigenicity

occurred in about 2% of isolates, which were solely type N (F. J. Cabañes, pers. comm. 18/11/03), as previously demonstrated extensively with strains from a variety of sources (Accensi et al., 2001). In the European study, N and T types were isolated with equal frequency from grapes. This is mirrored by the findings from Australian vineyards. Both N and T types were isolated from all regions, with the exception of the Adelaide Hills (four T types), the Barossa Valley (five N types) and Coonawarra (seven N types). Examination of additional isolates is likely to reveal the presence of the other type in these regions; for example, the presence of 12 T type strains from Sunraysia was reported by Leong et al. (2004); however, examination of a thirteenth strain revealed it to be type N.

The consistent presence of both N and T types within a single region suggested that no region is exempt from the possibility of OA contamination of grapes by a toxigenic isolate of *A. niger*. Furthermore, this species is dominant in vineyards of the world. Fortunately, toxigenicity occurs infrequently in this species. The toxigenicity of all Australian *A. carbonarius* isolates examined to date indicated that this is the primary species of concern for the minimisation of OA in Australian grapes. It is perhaps fortuitous that this species does not occur with the same frequency as *A. niger*.

3.4.2 Molecular relationships among *Aspergillus niger*, *A. carbonarius* and *A. aculeatus*

3.4.2.1 Evaluation of techniques

ERIC-PCR was the least informative technique for strain differentiation. Although the three species clustered separately, subdivisions within those clusters did not correspond with previously demonstrated differences such as N and T RFLP type for *A. niger*, neither did subdivisions correspond with toxigenicity of the strains. The species clusters were not robust, and changed if certain strains were removed from the analysis (Esteban et al., 2005b). Hence, the grouping of Australian isolates of *A. niger* into a single cluster was unlikely to be reliable and reproducible. In ERIC-PCR, a relatively small proportion of the genome was surveyed through binding of the single set of primers, resulting in 16 loci for analysis. Differences at these loci were not representative of the genetic variation among black *Aspergillus* spp. In contrast, analysis of RAPD with multiple primers surveyed a greater proportion of the genome [122 loci analysed by Megnegneau et al. (1993), 59 loci analysed by Kevei et al.

(1996), 72 loci analysed by Fungaro et al. (2004b)]; thus, more meaningful molecular data were generated.

AFLP was a fairly robust technique for the examination of strain relationships among black *Aspergillus* spp., supported by bootstrap values over 75%. Two of the four primer combinations used by researchers at ISPA, Italy were readily transferable to the collection of strains in this study, and produced data that separated the three species into distinct clusters, as well as separating the *A. niger* aggregate into N and T RFLP types. Similar clustering was reported by researchers at ISPA for isolates primarily from grapes (24 month report; http://www.ochra-wine.com, accessed 24/03/05). AFLP analysis of isolates, primarily from coffee, with selective primers EcoRI-AT + MseI-CT was also capable of robust species separation; however, no clustering within the *A. niger* aggregate, that could potentially correspond with the N / T separation, was observed, as toxigenic isolates (likely to be type N) were distributed among all the minor clusters within the aggregate (Schmidt et al., 2004). The RFLP profiles of the isolates in that study were not reported; hence, the absence of a potential type T cluster could be due to the omission of type T isolates from the analysis.

This study represents the first characterisation (Esteban et al., 2005c) and use of polymorphic microsatellite loci for the examination of molecular relationships among strains of the *A. niger* aggregate. The relatively high numerical indices of discriminatory power (Hunter, 1991) and observed heterozygosities (Nei, 1978) (Table 3.4) demonstrated a level of diversity suitable for differentiation of isolates within the same species. The amplification of these loci in certain isolates of *A. carbonarius* and *A. aculeatus*, as well as limited sequencing data for the former species, suggested that several of these microsatellite loci characterised for *A. niger* may also be present within the genome of other black *Aspergillus* spp. So-called "null" alleles in *A. carbonarius* and *A. aculeatus*, from which no amplicons were detected, may have resulted from polymorphisms in the flanking regions to which the primers anneal. Further refinements in primer design and PCR may increase the utility of these microsatellite markers for analysis of inter- as well as intra-specific variation. Low bootstrap support values for the microsatellite dendrogram likely arose due to the relatively small number of loci examined, and the frequency of null alleles for certain

loci. Nevertheless, the strong homology between the microsatellite and AFLP dendrograms suggests that the clustering generated by both these independent sources of polymorphism accurately reflects the genetic relationships among the *A. niger* aggregate isolates examined.

3.4.2.2 Significance

The various molecular techniques applied to the black *Aspergillus* spp. as a group are considered robust only if they reliably differentiate between the species that are clearly delineated by morphological techniques [reviewed by Varga et al. (2000b); Abarca et al. (2004); Varga et al. (2004a)]. AFLP analysis fell into this category. Although microsatellite markers were applicable only to the *A. niger* aggregate, similar clustering in dendrograms derived from both AFLP and microsatellite data confirmed the validity of these techniques. Given that sources of polymorphism in AFLP and microsatellite analyses are independent, molecular relationships demonstrated by both these techniques were likely to parallel the phylogenetic relationships among the strains. The separation of the *A. niger* aggregate into two groups corresponding to N and T RFLP types by both techniques strongly suggested that these are two different species. This separation into two species, often called *A. niger* and *A. tubingensis*, has been observed by RFLP analysis of ribosomal DNA, mitochondrial DNA and chromosomal DNA, the latter followed by hybridisation with probes; as well as RAPD, isoenzymes (Megnegneau et al., 1993; Fungaro et al., 2004b) and sequencing [reviewed by Abarca et al. (2004)]. Sequencing of the cytochrome *b* gene also divided the *A. niger* aggregate into two groups, however, it is not apparent if the two proposed clades, *A. niger* and *A. awamori* (Yokoyama et al., 2001) correspond with N and T types. Many of the studies in which clustering corresponded with N and T types showed further subdivisions within these types, hence the subdivision of our N type strains into two groups was not unexpected. An area for future study would be the comparison of this subdivision with those reported by means of RFLP techniques (Megnegneau et al., 1993; Varga et al., 1993, 1994; Parenicová et al., 1997).

The lack of sub-groups among isolates of *A. carbonarius* is in keeping with reports that this species displays less intra-specific variation than *A. niger* (Kevei et al., 1996; Parenicová et al., 1996). Those authors observed some differences among *A.*

carbonarius strains based on RAPD and RFLP analysis of ribosomal and mitochondrial DNA. Fungaro et al. (2004b) divided isolates of *A. carbonarius* from coffee into two clusters, with one group comprising only toxigenic strains, and the other, a mixture of toxigenic and non-toxigenic isolates. Schmidt et al. (2004) also divided *A. carbonarius* isolates from coffee into two groups by AFLP, however, both groups contained a mixture of toxigenic and non-toxigenic strains. No such clustering was observed for the *A. carbonarius* isolates in this study, which were primarily from Australian vineyards; rather, all isolates of *A. carbonarius* appeared to be closely related.

Few associations between genetic relatedness and phenotype have been demonstrated among black *Aspergillus* spp., apart from the absence of toxigenicity to date among type T *A. niger* isolates. This absence of any association between toxigenicity and molecular relatedness was observed in dendrograms based on ERIC-PCR, AFLP and microsatellites, and has also been reported by Fungaro et al. (2004b) and Schmidt et al. (2004). Clusters delimited within *A. carbonarius* or within the *A. niger* aggregate by molecular techniques could not be differentiated by phenotypic analysis of carbon utilization patterns (Kevei et al., 1996). In contrast, clusters in *P. verrucosum* based on RAPD and AFLP analysis (Castellà et al., 2002) corresponded with those derived from secondary metabolite profiles (Larsen et al., 2001), including degree of toxigenicity, thus supporting the delineation of a separate species, *P. nordicum*. Within *A. ochraceus*, certain clusters corresponded with toxigenicity (Varga et al., 2000a), whereas others did not (Fungaro et al., 2004a). Mühlencoert et al. (2004) proposed that the poor relationship observed at times between genotype and toxigenicity may be due to environmental conditions suppressing toxin production in certain strains. Development of novel conditions and additives to enhance OA production during the screening of black *Aspergillus* spp. continues to be an area for research.

No association between molecular relatedness and substrate or country of isolation was observed for black *Aspergillus* spp. in this study, as Varga et al. (2000a) also reported for *A. ochraceus.* Our findings support the belief that the black *Aspergillus* spp. are cosmopolitan in their distribution over a range of substrates and locations (Klich and Pitt, 1988).

3.4.3 Implications for viticulture and oenology

One motivation for elucidating the phylogeny of mycotoxigenic fungi using molecular techniques is to search for similarities among toxigenic isolates. If present, such similarities may potentially be exploited for the detection and/or reduction of that specific group of isolates. Given the cosmopolitan nature of the black *Aspergillus* spp. and the absence of any association between molecular relatedness and toxigenicity, such an approach currently does not show potential for this group of fungi. Based on current knowledge, it seems unlikely that management strategies can be designed to selectively identify and minimise toxigenic isolates. Rather, management will focus on reduction of the black aspergilli in vineyards overall, thus proportionally reducing any toxigenic isolates which may be present [reviewed by Leong et al. (2005a)].

Another potential application of molecular techniques in the viticultural setting is in the rapid detection of the toxigenic species in grapes and, by inference, some estimation of OA likely to be present. PCR-based techniques are more rapid than isolation, growth, identification and screening for toxin production on traditional culture media; furthermore, expertise in identification of isolates based on morphological characteristics is not required. Sensitive and specific PCR-assays for the detection of *A. carbonarius* have been developed, some of which have also been validated in artificially and naturally contaminated biological matrices (coffee beans) (Fungaro et al., 2004b; Perrone et al., 2004; Schmidt et al., 2004; reviewed by Niessen et al. (2005) for all ochratoxigenic fungi). However, a few key issues regarding the use of molecular methods as a screening tool are highlighted below [reviewed by Edwards et al. (2002); Varga et al. (2004b)].

First, the presence of the potentially toxigenic species on grapes does not necessarily indicate the presence of toxin. As these fungi are ubiquitous in vineyards, they may be present as dormant spores on berries without producing OA. It also follows that the amount of fungal DNA is not necessarily indicative of the amount of OA present. Schmidt et al. (2004) did not observe any relationship between the amount of *A. carbonarius* template DNA in naturally infected coffee beans, as estimated by fluorescence intensity of the amplicons on the gel, and OA concentration in the beans.

Second, species-specific PCR detects both toxigenic and non-toxigenic isolates, as was the case for the PCR assays reported above for *A. carbonarius.* Attempts to

identify DNA sequences that were present only in toxigenic isolates were unsuccessful (Fungaro et al., 2004b). It would be desirable to amplify toxigenic isolates of both *A. carbonarius* and *A. niger* in the same assay. One strategy to overcome this is to target genes directly involved in toxin synthesis. A correlation was demonstrated between amount of DNA for a gene involved in trichothecene synthesis in *Fusarium* spp., as quantified by real-time PCR, and the toxin deoxynivalenol in wheat (Schnerr et al., 2002). None of the genes involved in OA synthesis in black *Aspergillus* spp. have been identified, although some have been characterised for *A. ochraceus* (O'Callaghan et al., 2003) and *P. nordicum* (Färber and Geisen, 2004). These genera possibly possess different pathways for OA synthesis under different regulatory mechanisms [reviewed by Niessen et al. (2005)]. An additional problem with *Aspergillus* spp. is the likelihood that homologues of genes for toxin synthesis exist in non-toxigenic strains, although this issue does not arise in genera such as *Fusarium* [reviewed by Edwards et al. (2002)]. Furthermore, subtle differences in gene sequences among related species may hinder development of a single test for toxigenic isolates of both *A. carbonarius* and *A. niger*.

Third, although quantification of mRNA transcripts of genes involved in OA synthesis in the black aspergilli would overcome many of the barriers described above, environmental factors (temperature, water activity, pH) may differ in their influences on gene regulation. In studies of the OA polyketide synthase gene in *P. nordicum*, Geisen (2004) observed that changes in OA concentration in liquid medium coincided with changes in the amount of mRNA, as affected by period of incubation and a range of NaCl concentrations. However, changes in temperature which led to upregulation of mRNA production did not always result in increased OA, thus, weakening the predictive value of mRNA quantification.

At present, molecular techniques are unlikely to generate a rapid test for OA contamination in grapes that could be used at receival in wineries. However, detection of toxigenic species such as *A. carbonarius* could be helpful in identifying loads that require further sampling and analysis to establish the presence and concentration of OA. Development of a rapid and robust PCR-based identification system for toxigenic isolates of *A. carbonarius* and *A. niger* may also have some application for the early identification of these strains in Aspergillus bunch rots.

4 Survival, growth and toxin production by *Aspergillus carbonarius* and *A. niger*

4.1 Introduction

4.1.1 Effect of temperature, water activity and sunlight on survival of *Aspergillus carbonarius* spores

The black aspergilli are saprophytic and have been isolated from soils around the world (Klich and Pitt, 1988; Varga et al., 1994). Thus it is no surprise, that, of the multitude of potential substrates in vineyards, soil is the most frequent source of *A. carbonarius* (Leong et al., 2005a). Black *Aspergillus* propagules probably overwinter in soil. Preliminary studies have demonstrated that soil temperature and moisture significantly affect the survival of *A. carbonarius.* Kazi et al. (2003b) inoculated soil with *A. carbonarius* on milled oats as a carrier, and noted an optimum survival temperature of 25 °C. Increasing the moisture content of the soil reduced survival of *A. carbonarius.* When vineyards were irrigated, counts of *A. carbonarius* plummeted but returned to pre-irrigation levels as the soil dried. Greater reductions were observed in clay loam soils, which retain more moisture, than in sandy soils.

Spores are blown from soil onto the surface of berries (Leong et al., 2005b), where particular relative humidities and temperatures may be deleterious. This, coupled with exposure to UV radiation in sunlight, may affect the frequency of isolation of black *Aspergillus* spp. from berries during maturation. In vineyards worldwide, these fungi were isolated more frequently as berries mature (Nair, 1985; Battilani et al., 2003a; Serra et al., 2003; Bellí et al., 2004c). However, geography and changes in temperature and relative humidity also contributed to variations in fungal incidence and OA contamination (Battilani et al., 2003a; Roset, 2003; Serra et al., 2003; Sage et al., 2004; Battilani et al., 2005b; Bellí et al., 2005a; Serra et al., 2005a). Overhead irrigation increased the relative humidity within the vine canopy and also the frequency of isolation (N. Bellí, pers. comm. 23/09/03). The sporocidal effect of both artificial and natural UV radiation (in sunlight) has been demonstrated for *A. niger* spores (Rotem and Aust, 1991). Clumps of spores were more resistant than single spores, and the melanised, thick-walled spores of *A. niger* were more resistant to UV radiation than the hyaline, thin-walled spores of *Botrytis cinerea.* The resistance of *A. carbonarius* to UV radiation has not been demonstrated, however, in *A. carbonarius*,

the spores are also melanised with thick walls, hence could be expected to show some resistance.

Complex interactions between temperature, moisture and microbiota may play a role in the survival of *A. carbonarius* in soil and on berry surfaces. By removing the confounding factors of the soil matrix and the canopy environment, it is possible to study in isolation the interaction between water activity and temperature on the survival of *A. carbonarius* propagules. Interactions were observed over a range of water activities (0.4-1.0) and temperatures (1-37 °C) likely to occur during the year in Australian viticultural regions. *A. carbonarius* spores were also exposed to sunlight and their survival noted over time.

4.1.2 Effect of temperature and water activity on growth and ochratoxin A production by *Aspergillus carbonarius* and *A. niger*

In the right conditions, spores on berry surfaces germinate and grow. Once *A. carbonarius* has penetrated the berry, temperature and water activity play a major role in regulating fungal growth and toxin production.

Several researchers have noted that OA is more frequently detected in wines from warmer regions (generally, southern Europe) than in wines from cooler regions (section 1.3). Battilani et al. (2003a) and Serra et al. (2003) reported increased incidence of OA in wine grapes from regions subject to warm, humid conditions or during warm, humid seasons. OA was absent from grapes grown in cool, dry climates. Such observations do not distinguish between the potential effects of temperature and water activity on incidence of toxigenic fungi in the environment, infection processes, growth and toxin production, nevertheless, examining the latter two factors in isolation is worthwhile. Temperatures differ among viticultural regions, among seasons, during stages of berry maturation and, for dried vine fruit production, during drying. Water activity within the berry decreases during maturation upon accumulation of berry sugars, and further decreases when berries are dried. Understanding the potential for OA production during drying is critical for minimising contamination of dried grapes. Bucheli et al. (2000) observed OA production during drying of coffee cherries and Joosten et al. (2001) studied OA

production in coffee cherries by *A. carbonarius*, but few data are available regarding OA production during drying of grapes.

To date, few studies have characterised the effects of temperature and/or water activity on OA directly in grapes (Battilani et al., 2004). However, effects have been studied on a synthetic grape juice medium (SGM) designed to simulate grape juice during early veraison. The growth and toxigenicity of isolates of *A. carbonarius* and *A. niger* from vineyards in Europe and Israel have been characterised on SGM within a matrix of temperatures and water activities (Battilani et al., 2003c; Mitchell et al., 2003, 2004; Bellí et al., 2004b,d, 2005b). The effect of temperature on growth of Australian isolates of *A. niger* and *A. carbonarius* has been reported (Leong et al., 2004); however, these isolates were from a single viticultural region, and significant intra-specific variation has been reported in European studies. Furthermore, isolates were grown on a standard mycological medium, CYA, which would not reflect the potential for growth and OA production in grapes. Choice of medium has a significant effect on OA production (Bragulat et al., 2001).

The aim of the experiment reported here was to characterise growth and OA production by Australian isolates of *A. carbonarius* and *A. niger* on SGM at various temperatures and water activities, in order to compare the growth and toxigenic potential of Australian isolates with those from Europe. Reports of the time taken to reach maximum toxin production on SGM are contradictory. For example, Bellí et al. (2004d) observed that OA maxima at 25 °C and water activity above 0.98 always occurred at 5 d of incubation, whereas Mitchell et al. (2004) suggested that 10 d was more appropriate for testing over a range of water activities and temperatures. Both authors noted a decrease in OA after the maximum, suggesting degradation of the toxin, a metabolic process which has previously been observed for black *Aspergillus* spp. (Varga et al., 2000c; Abrunhosa et al., 2002; Varga et al., 2002). Understanding the pattern of OA production, as well as the potential for subsequent degradation, may aid in decisions regarding crops affected by Aspergillus bunch rot.

4.2 *Effect of temperature, water activity and sunlight, on survival of* Aspergillus carbonarius *spores*

4.2.1 Methods

4.2.1.1 Effect of temperature and water activity

A. carbonarius isolates from grapes in the Sunraysia region, Victoria (FRR 5374, FRR 5573, FRR 5574, previously described by Leong et al. (2004)) were grown on CYA in the dark at 25 °C for 8 d in a preliminary experiment, and for 11 d when the trial was repeated, after which spores were harvested as described in section 2.3. The spore suspension was diluted in cold, sterile water containing 0.05% (w/v) Tween-80® to give a final concentration of 4-5 x 10^5 spores/mL. The volume of suspension prepared was 2-5 L, and this was mixed in a large beaker with a magnetic stirrer. Aliquots (10 mL) of spore suspension were filtered under vacuum onto individual sterile filter membranes (pore size 0.45 µm, 40 mm diameter, mixed cellulose esters; Millipore, Billerica, MA, USA). The membranes were placed into 9 cm plastic Petri dishes with the lids removed and dried at 37 °C for 60-120 min, then the lids were replaced until use.

Small bowls of saturated salt solutions were placed in closed plastic boxes and allowed to equilibrate at the temperatures shown in Table 4.1 to generate environments of particular water activities. The Petri dishes holding the filters were uncovered, and were placed in the boxes in such a way that no dish was covered, that is, the airspace above each filter was contiguous with the air above the saturated salt solution. Salt solutions were refilled as required. Eighteen filters were prepared at each water activity x temperature combination.

To establish the initial spore load, 40 filters were sampled prior to incubation. During incubation, filters were sampled in triplicate at intervals up to 618 d. Given that 18 filters were incubated in each condition, each water activity x temperature combination could be sampled a maximum of six times. At each sampling point, spores were dislodged from membranes into 100 mL sterile peptone solution using the stomacher and *A. carbonarius* colonies were enumerated as described in section 2.1. Samples were taken less frequently in conditions that were likely to extend survival of the spores, and were taken more frequently when a decrease in viable spore count was

observed. Where counts were expected to be small, colonies were enumerated on filters directly plated onto DRBC.

Due to irregular sampling points, these data were not amenable to statistical analysis.

Table 4.1: Saturated solutions and water activities generated at various temperatures

Desired water activity	Saturated salt solutions and resultant water activities			
	1 °C	15 °C	25 °C	37 °C
1.00 (moist)	water	water	water	water
0.90	$BaCl_2$ 0.920[a]	$BaCl_2$ 0.911[a]	$BaCl_2$ 0.900[b]	$BaCl_2$ 0.893[a]
0.80	$(NH_4)_2SO_4$ 0.825[b]	$(NH_4)_2SO_4$ 0.810[b]	$(NH_4)_2SO_4$ 0.800[b]	$(NH_4)_2SO_4$ 0.790[d]
0.60	$Na_2Cr_2O_7$ 0.595[b]	NaBr 0.610[b]	NH_4NO_3 + $AgNO_3$ 0.615[b]	$NaNO_2$ 0.615[d]
0.40	$CaCl_2$ 0.400[b]	NaI 0.409[c]	K_2CO_3 0.430[b]	K_2CO_3 0.400[d]

[a] calculated from Young (1967)
[b] Winston and Bates (1960)
[c] Greenspan (1977)
[d] values from Winston and Bates (1960) for solutions at 40 °C

4.2.1.2 Effect of sunlight

A. carbonarius isolates (FRR 5374, FRR 5573, FRR 5574, as for section 4.2.1.1) were grown on CYA in the dark at 25 °C for 7 d. Spores were harvested as described in section 2.3 and lodged on filter membranes as described in section 4.2.1.1. Each membrane was placed into a closed 9 cm diameter plastic Petri dish and dried at 37 °C overnight.

The Petri dishes were supported on metal racks, the lids removed, and the filter membranes exposed to direct sunlight outdoors, including cloudy days when UV radiation was less intense. The first experiment conducted over 5 d was curtailed when rain wet the filters. When the experiment was repeated, UV radiation was less intense hence the exposure period was extended to 9 d. Controls comprised membranes covered with aluminium foil to shield the spores from sunlight while undergoing temperature fluctuations similar to the test membranes. The estimated UV radiation (290-400 nm) for each day was obtained from the Australian Bureau of

Meteorology website (http://www.bom.gov.au/weather/nsw/forecasts.shtml, accessed 12/03-04/04). At each sampling point, two test membranes and a single control membrane were randomly collected from each of the three replicate racks. Spores were dislodged from membranes into 100 mL sterile peptone solution using the stomacher and *A. carbonarius* colonies were enumerated as described in section 2.1. Where counts were expected to be small, colonies were enumerated on filters directly plated onto DRBC. Due to irregular sampling points, these data were not amenable to statistical analysis.

4.2.2 Results

4.2.2.1 Temperature and water activity

The effects of water activity and temperature on the survival of spores of *A. carbonarius* are shown in Fig. 4.1. The limit of detection was 500 cfu/filter. When no colonies were detected by this method, an arbitrary value of half the detection limit was assigned (250 cfu/filter). If available, additional filters were directly plated onto DRBC to assess for the presence of any viable spores, hence the presentation of some data of less than 100 cfu/filter.

Over the range of water activities, 37 °C did not support the survival of *A. carbonarius* spores; as the temperature decreased, spores survived for longer periods. At 1 °C, spores survived for well over a year at 0.9 a_w and below. The relationship between water activity and survival was more complex. It was evident that the lowest water activity, 0.4, best supported the survival of spores over the temperatures examined, but the converse, that the highest water activity was most deleterious, did not hold true. Survival at 0.9 and 0.8 a_w at temperatures above 15 °C appeared to be poorer than survival at 1.0 a_w, although there was insufficient material to assess long-term survival at 1.0 a_w / 15 °C and 1.0 a_w / 25 °C. Water activity 0.6 also was not conducive for long term survival and, at 37 °C, decline in viable spores occurred most rapidly at 0.6. Of note was the effect of moisture (1.0 a_w) on long term survival at 1 °C. A gradual decrease in spore viability was observed during both experiments A and B. Similarly, towards the end of the experiment, a decrease in viability at 1 °C and 0.9 a_w was observed. There were no decreases in viability at lower water activities. Complex relationships between temperature and water activity clearly affect the survival of spores.

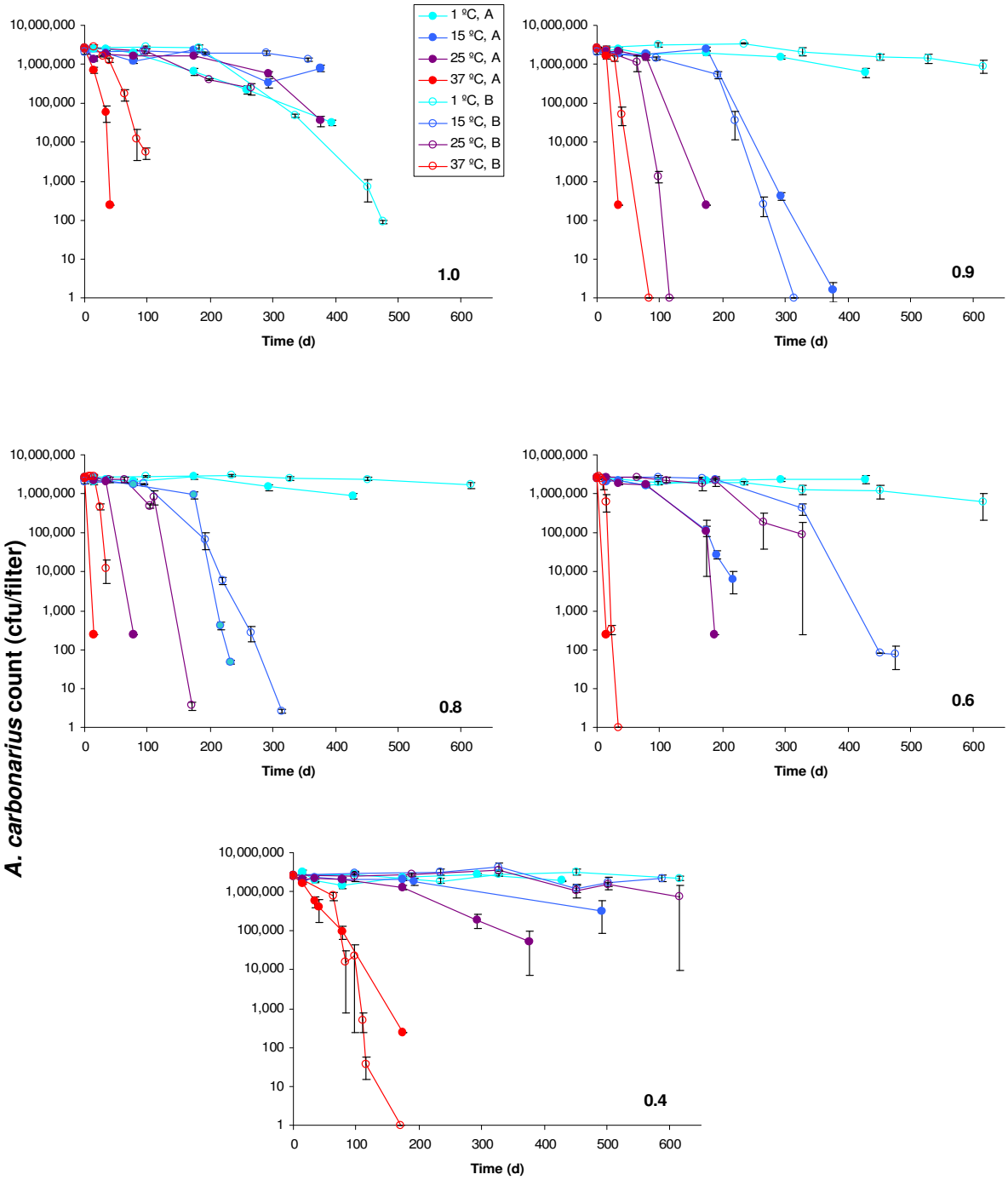


Figure 4.1: Effect of water activity and temperature on survival of *Aspergillus carbonarius* spores on filter membranes. The experiment was conducted twice (A, B). Mean of counts from three replicate 40 mm diameter filter membranes. Error bars denote the standard error of the mean

4.2.2.2 Sunlight

Spores of *A. carbonarius* supported on filter membranes were killed by exposure to sunlight (Fig. 4.2) and also became lighter in colour, compared with covered spores (Fig. 4.3). The rate of decline was greater when the experiment was repeated (B) over 9 d, when a large decrease (over 10^5) in spore viability was observed after 10 mWh cumulative exposure to UV radiation. Only a small part of this decrease (approximately 15%) was attributed to wind blowing a proportion of spores from the filter membranes (estimated from Fig. 4.3).

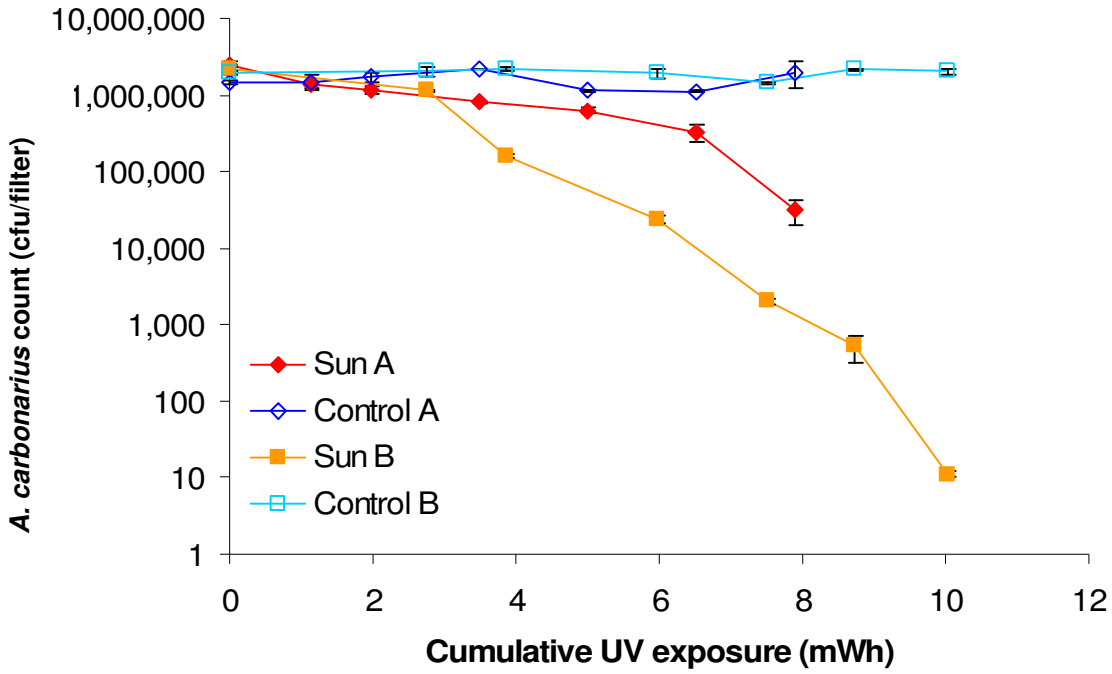


Figure 4.2: Survival of *Aspergillus carbonarius* spores on filter membranes exposed to sunlight. Cumulative UV exposure quoted for 290-400 nm. The experiment was conducted twice (A, B). Control plates were shielded with aluminium foil. Mean of counts from 40 mm diameter filter membranes on three replicate racks; error bars denote the standard error of the mean (at each time point, two filters exposed to sunlight plus one control per rack were sampled)

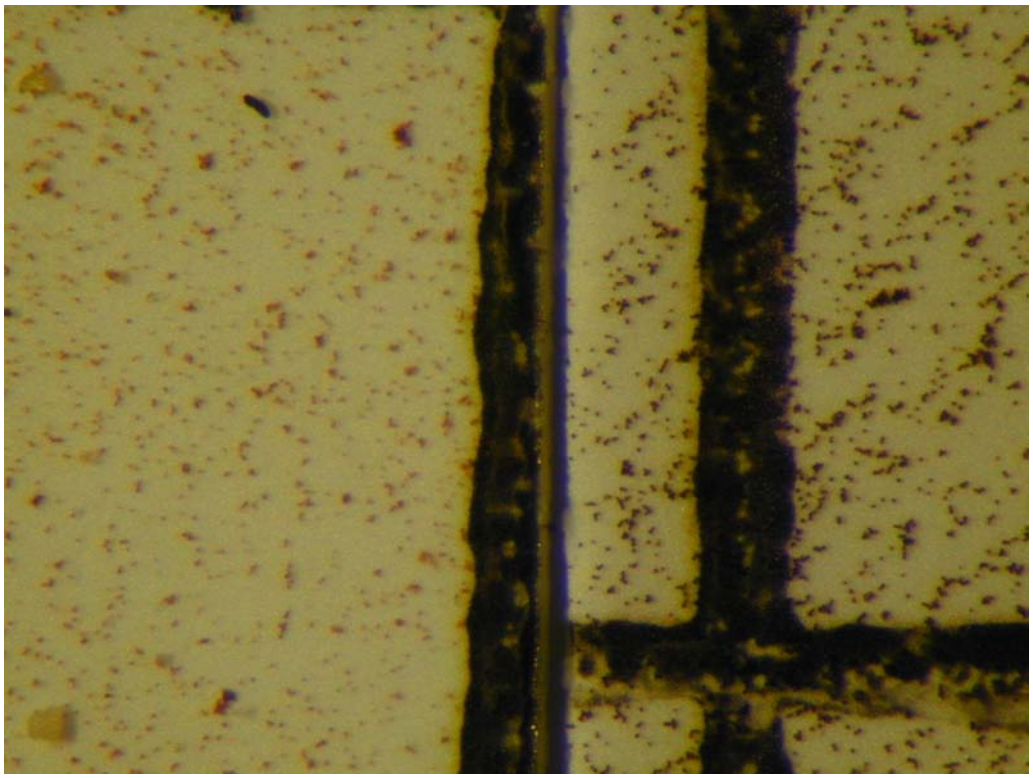


Figure 4.3: Bleaching of *Aspergillus carbonarius* spores on filter membranes exposed to sunlight for 9 d (left) compared with covered spores (right). (Photograph taken through a Leica Wild M3C stereomicroscope, magnification x 16 (Heerbrugg, Switzerland) with direct illumination (Intrulux®500, Switzerland))

4.3 Effect of temperature and water activity on growth and ochratoxin A production by Aspergillus carbonarius *and* A. niger

4.3.1 Methods

4.3.1.1 Medium preparation

Glycerol was added to SGM (modified from Mitchell et al. (2003); Bellí et al. (2004b); Appendix A) to generate water activities of 0.965, 0.95 and 0.92 (glycerol added at 54.64 g/L, 108.21 g/L and 218.00 g/L, respectively). The water activity of unadjusted SGM was 0.98, as determined using an Aqualab CX3 water activity meter (Decagen Devices, Inc., Pullman, Washington, USA). Media were prepared in 5 L volumes (S9000, AES Laboratoire, Combourg, France) and 20 mL were delivered into 9 cm diameter plastic Petri dishes by an automatic plate pourer (APS300, AES Laboratoire, Combourg, France). Plates were allowed to dry at room temperature for 4 hours, after which plates of the same water activity were sealed in plastic sleeves and stored at 4 °C until use.

4.3.1.2 Preparation of inoculum

Ochratoxigenic isolates of *A. carbonarius* (FRR 5682, FRR 5690, FRR 5691, FRR 5692, FRR 5693) and *A. niger* (FRR 5694, FRR 5695) from Australian vineyards were selected to represent genetically distinct strains (Fig. 3.6) from different viticultural regions (Table 3.3). Strong producers of OA and atypical weak producers were assessed. Isolates were grown on SGM in the dark at 25 °C for 7 d. For each isolate, a spore suspension was prepared as described in section 2.3 in sterile glycerol solution (60% w/w). The suspension was filtered through sterile glass wool to remove hyphal fragments, and was diluted in additional glycerol solution to a concentration of approximately 10^5 spores/mL. Aliquots (1 mL) of the suspension were stored in sterile Eppendorf tubes at -80 °C until use.

4.3.1.3 Inoculation and incubation

SGM plates were allowed to equilibrate at room temperature overnight and the spore suspensions thawed and mixed by vortexing. The spore suspension (5 µL) was delivered to the centre of the plate using an automatic pipette (Genex Beta, Finland) with sterile plugged pipette tips to avoid cross-contamination. Inoculations were performed on duplicate plates for replicates A and B, and on triplicate plates for replicate C. Replicates A, B and C were set up on three consecutive days, and freshly thawed aliquots of spore suspension were used for each replicate. The edge of each plate was sealed with low density polyethylene film (Cling Wrap, Homebrand, Woolworths, Yennora, NSW, Australia) to minimise moisture loss while allowing free gaseous exchange. Plates of the same water activity were incubated in low density polyethylene bags (Cospak, Minto, NSW, Australia) in stacks of no more than seven Petri dishes. Plates were incubated at 15 °C, 25 °C, 30 °C and 35 °C, thus yielding a full factorial design of four temperatures x four water activities x seven isolates. Uninoculated plates were incubated in identical conditions and no change in water activity was observed after 15 d.

4.3.1.4 Growth and estimation of ochratoxin A

In order to calculate the linear growth rate of each isolate under each condition, colony diameter was recorded for every plate at intervals appropriate to the growth rate of that isolate. Isolates incubated at 30 °C and 35 °C were measured up to three times daily, whereas isolates incubated at 15 °C were measured every 2-3 d. Linear

growth rates were calculated by plotting radial extension (mm) against time (d). Growth rates were analysed as described in section 2.6.

OA production on SGM was assessed by the agar plug method described in section 2.4. Samples were taken in order to estimate the maximum OA yield under each condition; hence, sampling ceased after a decline in OA production was observed. Additional samples were taken at time points of interest for *A. carbonarius* isolates FRR 5690 and FRR 5692 and *A. niger* isolate FRR 5694, as these isolates produced more OA than the other isolates tested. Sampling ceased after 36 d. Agar plugs were weighed in order to calculate OA yield per gram of medium.

Contour plots for mean growth rate and mean maximum OA yield over the range of water activities and temperatures examined were prepared in Sigmaplot (v 9.01, Systat Software, Inc., Point Richmond, CA, USA).

4.3.2 Results

4.3.2.1 Growth

The interaction between temperature, water activity and isolate was highly significant ($P < 0.001$); hence, the main effects were not tested independently. Growth rates for all the isolates are listed in Table 4.2. The maximum growth rate achieved was 11.35 mm/d by *A. niger* isolate FRR 5695 at 0.98 a_w and 35 °C. The minimum growth rate observed in this study was 0.27 mm/d, calculated for *A. carbonarius* FRR 5682 at 0.92 a_w and 15 °C. Growth by *A. niger* isolates was significantly faster ($P < 0.05$) than growth by *A. carbonarius* isolates at 30 °C and 35 °C over the range of water activities examined, whereas at 20 °C and 15 °C, growth achieved by some isolates of both species was similar at most water activities. Some significant differences in growth rate were observed between the five isolates of *A. carbonarius* at 25 °C and above, whereas the growth rates of the two isolates of *A. niger* were generally similar, except at 35 °C at all water activities and, at 30 °C and 25 °C at 0.98 a_w only. No single isolate grew significantly faster or slower than other members of the same species in every condition, although *A. carbonarius* FRR 5692 and *A. niger* FRR 5694 grew fastest in most conditions.

Between 15 °C and 30 °C, growth rate increased significantly with temperature for all isolates. Growth for most isolates of *A. carbonarius* was significantly greater at 30 °C than 25 °C, whereas for *A. niger* at most water activities, growth at 35 °C was approximately equal to or greater than that displayed at 30 °C. Growth for all isolates was significantly greater at water activity 0.95 than 0.92, and generally increased with increasing water activity, though, above 0.95 a_w, these increases were not significant for every isolate over the 0.15 a_w increment. At 35 °C, three isolates of *A. carbonarius* grew more slowly at 0.98 a_w than at 0.965.

These trends are summarised in Fig. 4.4, where data for all isolates of each species are combined. Most rapid growth for *A. carbonarius* isolates occurred around 0.965 a_w and 30 °C, whereas for *A. niger* isolates, most rapid growth observed within the parameters of this trial occurred at 0.98 a_w and 35 °C.

4.3.2.2 Ochratoxin A production

Whereas growth maxima were observed at 30 °C and above and at 0.965 a_w and above for both species, maxima for OA production were observed at the lowest temperature tested, 15 °C, and around 0.96 a_w for *A. carbonarius* and 0.95 a_w for *A. niger* (Fig. 4.4). For *A. carbonarius*, temperatures between 15 °C and 25 °C favoured OA production at higher water activities (0.965-0.98), whereas for *A. niger*, the most OA was produced at 0.95 a_w regardless of temperature. OA was produced by all isolates at 0.92 a_w at 15 °C; however, little OA was produced at this water activity as the temperature increased. Relatively little OA was produced above 30 °C at any water activity.

The isolates examined showed varying abilities to produce OA (Fig. 4.5). The maximum OA produced was 21 µg/g, by *A. carbonarius* FRR 5692 at 0.95 a_w and 15 °C after 15 d, whereas the maximum OA produced by an isolate of *A. niger* was 15 µg/g (FRR 5694), in the same conditions. Maximum OA production occurred at 0.95 or 0.965 a_w, 15 °C and after 15 d for all isolates, except for *A. niger* FRR 5695, when the maximum was observed after 22 d.

Production of OA over time is shown in Fig. 4.6, indicating a strong trend for all strains in nearly all conditions tested to produce OA up to a maximum, followed by a

decrease in OA concentration over time. As noted previously, maximum toxin production generally occurred at 15 °C, however, *A. carbonarius* isolates FRR 5682, FRR 5690 and FRR 5692 produced comparable levels of OA at 25 °C and higher water activities. These three isolates were relatively strong OA producers (Fig. 4.5). It was demonstrated that, at the higher water activities and lower temperatures which favoured maximum toxin production, OA could also be degraded rapidly, with well over half the toxin degraded within 5-7 d of the observed maximum. The trends for OA production and degradation at 30 °C and 35 °C are not visible in Fig. 4.6, as relatively little OA was produced. An exception to this general trend of OA production and degradation was *A. niger* isolate FRR 5695 at 0.98 or 0.965 a_w and 15 °C, where OA increased until the final sampling point at 22 d. Isolates incubated at 15 °C and 0.92 a_w accumulated OA until 36 d when the trial ended. Germination and growth in these conditions were slow; hence, it was several days before visible colonies could be sampled for analysis.

Trends in maximum OA production could not be linked to a specific time, due to the strong effect of temperature on germination and growth rate; namely, isolates at 15 °C produced maximum OA after a longer period than isolates at 25 °C (10-15 d *cf* 5 d) (Fig. 4.6). To remove the confounding effect of growth rate, the relationship between OA concentration and colony size is shown in Fig. 4.7 for the three most toxigenic isolates in this trial. Data for 0.92 a_w and 35 °C are not shown as little OA was produced in these conditions. For the two isolates of *A. carbonarius*, OA at 10-100% of the maximum was primarily observed at 15 °C and 25 °C. Maximum OA for every condition occurred when the colony radius was roughly 13-20 mm, after which OA reached a plateau and/or decreased. A radius of 20 mm represents colonisation of approximately 25% of the agar surface. This growth was achieved in less than 5 d at 30 °C, but took 10-15 d at 15 °C. For the same extent of growth, OA yield at 30 °C was far less than at 15 °C. *A. niger* FRR 5694 showed trends slightly different from the two isolates of *A. carbonarius.* OA at 10-100% of the maximum was only observed at 15 °C and, as for *A. carbonarius*, maximum OA occurred when the colony radius was roughly 13-20 mm. Less OA was produced at 25 °C and 30 °C; however, OA continued to accumulate until the colony radius was greater than 30 mm at 30 °C and until the medium was fully colonised at 25 °C.

Table 4.2: Effect of water activity and temperature on linear growth rates (mm/d) of *Aspergillus carbonarius* and *A. niger*

Water activity	Temperature	Isolate						
		A. carbonarius					*A. niger*	
		FRR 5682[a]	FRR 5690	FRR 5691	FRR 5692	FRR 5693	FRR 5694	FRR 5695
0.92	15 °C	0.27[b] ± 0.00[1c]	0.50 ± 0.01[1]	0.40 ± 0.02[1]	0.65 ± 0.01[1,2]	0.31 ± 0.01[1]	1.09 ± 0.01[2]	1.05 ± 0.01[2]
	25 °C	2.21 ± 0.04[5,6,7,8,9]	2.59 ± 0.03[9,10,11]	2.96 ± 0.05[10,11,12]	3.67 ± 0.01[14,15]	3.03 ± 0.06[12,13]	4.48 ± 0.03[17,18,19,20]	4.87 ± 0.02[19,20,21,22]
	30 °C	3.24 ± 0.06[12,13,14]	3.25 ± 0.01[12,13,14]	3.95 ± 0.03[15,16]	4.25 ± 0.06[16,17,18]	3.12 ± 0.03[12,13]	6.62 ± 0.02[39,40,41]	6.38 ± 0.02[37,38,39,40]
	35 °C	2.57 ± 0.09[8,9,10]	2.41 ± 0.03[7,8,9]	3.43 ± 0.05[13,14]	3.02 ± 0.04[11,12,13]	3.02 ± 0.02[11,12,13]	7.23 ± 0.04[42,43,44]	5.84 ± 0.10[28,29,30,31,32,33,34,35]
0.95	15 °C	1.68 ± 0.01[3]	1.68 ± 0.00[3]	1.78 ± 0.02[3,4]	1.96 ± 0.02[3,4,5,6]	1.85 ± 0.01[3,4,5]	1.85 ± 0.01[3,4,5]	1.87 ± 0.00[3,4,5]
	25 °C	4.37 ± 0.01[16,17,18]	4.64 ± 0.01[18,19,20,21]	5.13 ± 0.02[22,23,24]	5.99 ± 0.08[31,32,33,34,35,36,37,38]	5.34 ± 0.07[23,24,25,26,27]	5.57 ± 0.08[25,26,27,28,29,30,31]	5.69 ± 0.10[26,27,28,29,30,31,32,33]
	30 °C	5.99 ± 0.03[31,32,33,34,35,36,37,38]	5.55 ± 0.02[24,25,26,27,28,29,30]	6.36 ± 0.03[37,38,39,40]	7.00 ± 0.07[41,42,43,44]	7.34 ± 0.16[44,45]	8.03 ± 0.02[46]	7.92 ± 0.33[46]
	35 °C	5.19 ± 0.02[22,23,24,25]	4.98 ± 0.05[21,22]	5.76 ± 0.02[27,28,29,30,31,32,33,34]	5.54 ± 0.09[23,24,25,26,27,28,29,30]	5.69 ± 0.02[26,27,28,29,30,31,32,33]	9.27 ± 0.02[48]	7.90 ± 0.07[46]

0.965	15 °C	1.98 ± 0.02[3,4,5,6,7]	2.01 ± 0.01[3,4,5,6,7]	2.06 ± 0.01[3,4,5,6,7]	2.32 ± 0.03[6,7,8,9]	2.02 ± 0.02[3,4,5,6,7]	2.01 ± 0.01[3,4,5,6,7]	2.18 ± 0.04[4,5,6,7,8,9]
	25 °C	4.47 ± 0.07[17,18,19]	5.13 ± 0.02[22,23]	5.48 ± 0.06[23,24,25,26,27,28,29]	6.30 ± 0.01[36,37,38,39]	5.85 ± 0.05[28,29,30,31,32,33,34,35]	5.63 ± 0.09[26,27,28,29,30,31]	5.62 ± 0.04[25,26,27,28,29,30,31]
	30 °C	6.79 ± 0.07[40,42,42]	5.87 ± 0.04[29,30,31,32,33,34,35,36]	6.88 ± 0.01[41,42,43]	7.69 ± 0.02[45,46]	7.92 ± 0.22[46]	8.51 ± 0.05[47]	8.81 ± 0.02[47]
	35 °C	5.58 ± 0.02[25,26,27,28,29,30,31]	5.66 ± 0.07[26,27,28,29,30,31,32]	5.96 ± 0.08[30,31,32,33,34,35,36,37,38]	6.20 ± 0.02[35,36,37,38,39]	6.09 ± 0.02[32,33,34,35,36,37,38]	9.60 ± 0.02[48,49]	8.78 ± 0.07[47]
0.98	15 °C	1.95 ± 0.01[3,4,5,6]	2.17 ± 0.04[4,5,6,7,8,9]	2.16 ± 0.01[4,5,6,7,8,9]	2.31 ± 0.01[6,7,8,9]	2.31 ± 0.01[6,7,8,9]	2.21 ± 0.00[4,5,6,7,8,9]	2.15 ± 0.00[4.5,6,7,8]
	25 °C	4.18 ± 0.04[16,17]	5.42 ± 0.04[23,24,25,26,27,28]	5.20 ± 0.07[22,23,24,25]	6.15 ± 0.02[34,35,36,37,38]	5.95 ± 0.07[30,31,32,33,34,35,36,37]	5.49 ± 0.04[23,24,25,26,27,28,29]	6.12 ± 0.05[33,34,35,36,37,38]
	30 °C	5.82 ± 0.12[28,29,30,31,32,33,34,35]	5.95 ± 0.02[30,31,32,33,34,35,36,37]	6.39 ± 0.05[38,39,40]	7.27 ± 0.04[43,44,45]	7.42 ± 0.09[44,45]	8.51 ± 0.06[47]	9.29 ± 0.04[48]
	35 °C	5.70 ± 0.21[26,27,28,29,30,31,32,33]	4.87 ± 0.08[20,21,22]	5.29 ± 0.19[22,23,24,25,26]	6.10 ± 0.05[33,34,35,36,37,38]	5.34 ± 0.03[23,24,25,26,27]	11.35 ± 0.06[50]	9.76 ± 0.02[49]

[a] FRR culture collection, Food Science Australia, North Ryde, NSW, Australia

[b] standard error of mean from three replicate growth rates

[c] growth rates with different superscript numerals differ significantly, calculated according to Tukey's "honestly significant difference" ($P < 0.01$) (http://faculty.vassar.edu/lowry/ch14pt2.html, accessed 01/05; critical values for the Studentized range were estimated using http://calculators.stat.ucla.edu/studrange.php, accessed 01/05)

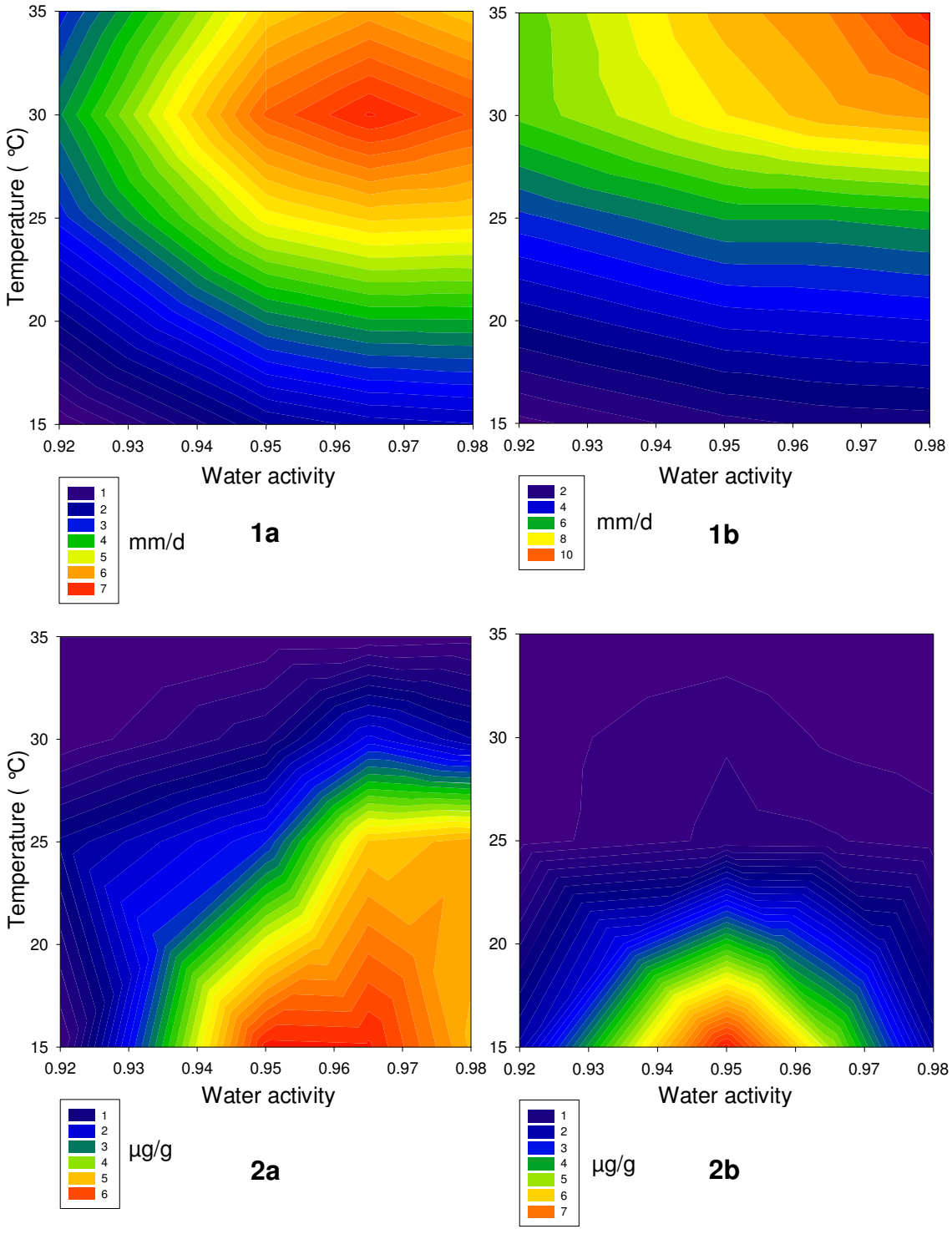


Figure 4.4: Mean growth rate (mm/d) (1) and (2) mean maximum ochratoxin A yield (µg/g) produced on synthetic grape juice medium within 36 d for (a) *Aspergillus carbonarius* FRR 5682, FRR 5690, FRR 5691, FRR 5692, FRR 5693 (data pooled) and (b) *A. niger* FRR 5694, FRR 5695 (data pooled). For OA analysis, samples below the limit of quantification in which a small peak corresponding to OA was observed were assigned a value of 0.0009 µg/g, half the limit of quantification. Samples in which OA was not detected were assigned a value of 0.0002 µg/g, half the limit of detection

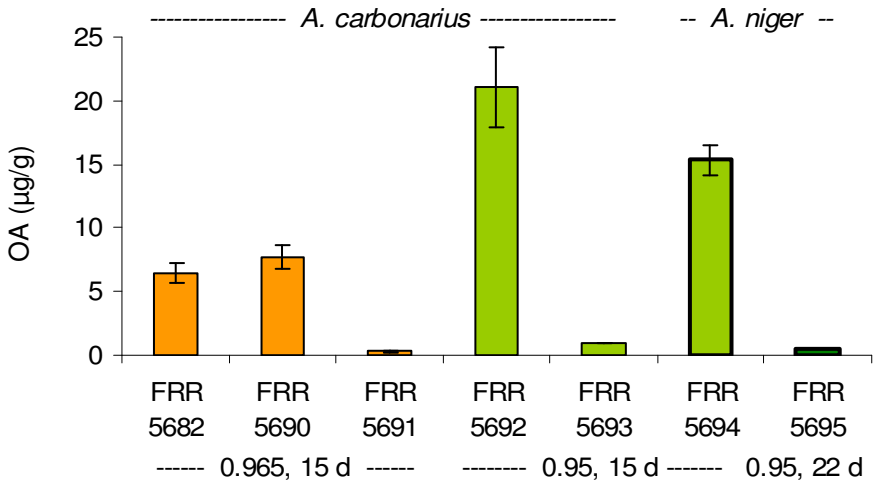
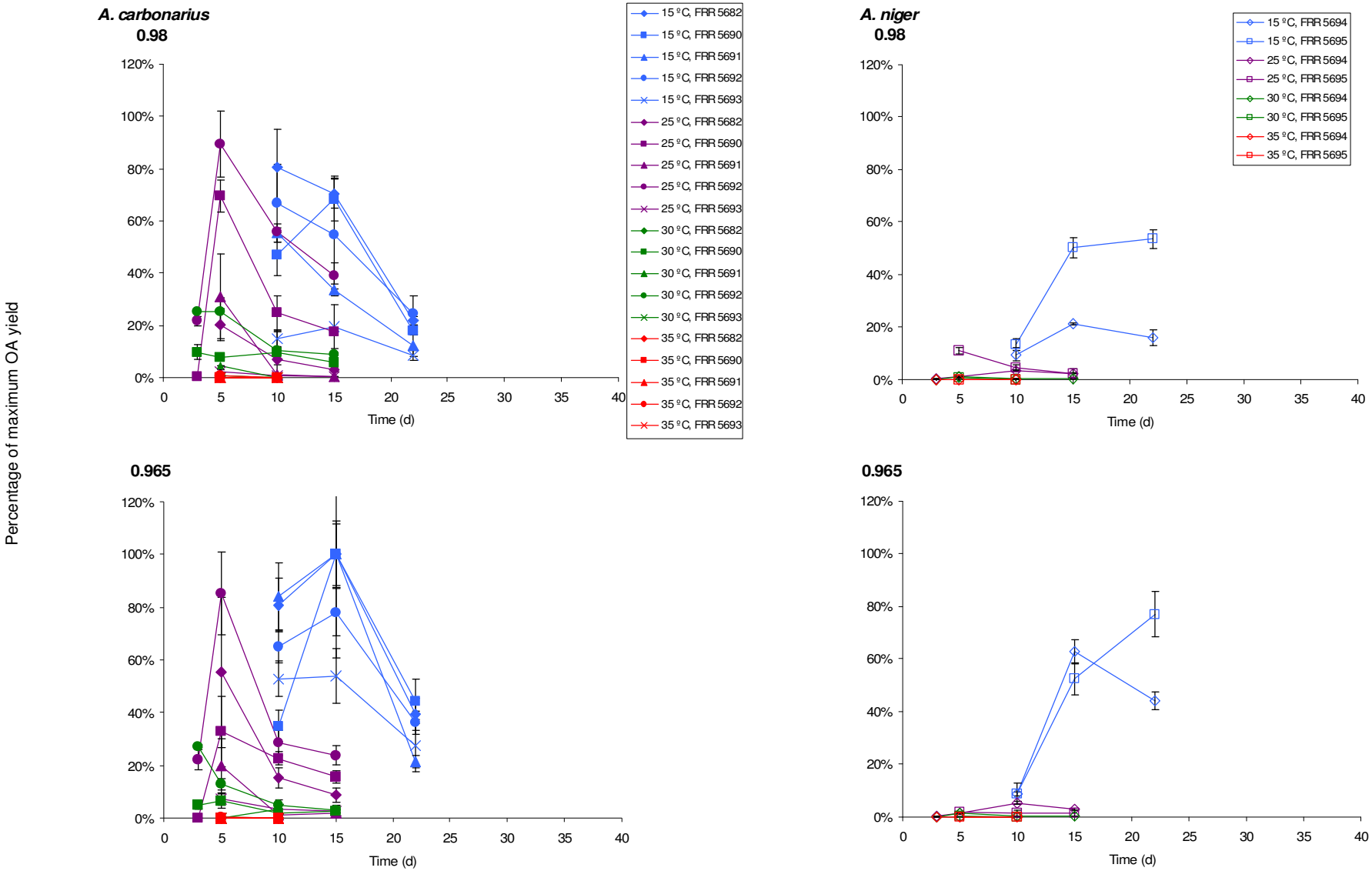


Figure 4.5: Maximum ochratoxin A produced by isolates of *Aspergillus carbonarius* and *A. niger* on synthetic grape juice medium. Maximum OA was produced at 15 °C, in conditions as noted below the *x*-axis. Isolates with OA maxima at water activity 0.965 are shown in orange; those maxima at 0.95 are shown in green. Error bars denote the standard error of the mean of three replicates


A. carbonarius
0.98
0.965
A. niger
0.98
0.965
Percentage of maximum OA yield
Time (d)
15 ºC, FRR 5682
15 ºC, FRR 5690
15 ºC, FRR 5691
15 ºC, FRR 5692
15 ºC, FRR 5693
25 ºC, FRR 5682
25 ºC, FRR 5690
25 ºC, FRR 5691
25 ºC, FRR 5692
25 ºC, FRR 5693
30 ºC, FRR 5682
30 ºC, FRR 5690
30 ºC, FRR 5691
30 ºC, FRR 5692
30 ºC, FRR 5693
35 ºC, FRR 5682
35 ºC, FRR 5690
35 ºC, FRR 5691
35 ºC, FRR 5692
35 ºC, FRR 5693
15 ºC, FRR 5694
15 ºC, FRR 5695
25 ºC, FRR 5694
25 ºC, FRR 5695
30 ºC, FRR 5694
30 ºC, FRR 5695
35 ºC, FRR 5694
35 ºC, FRR 5695

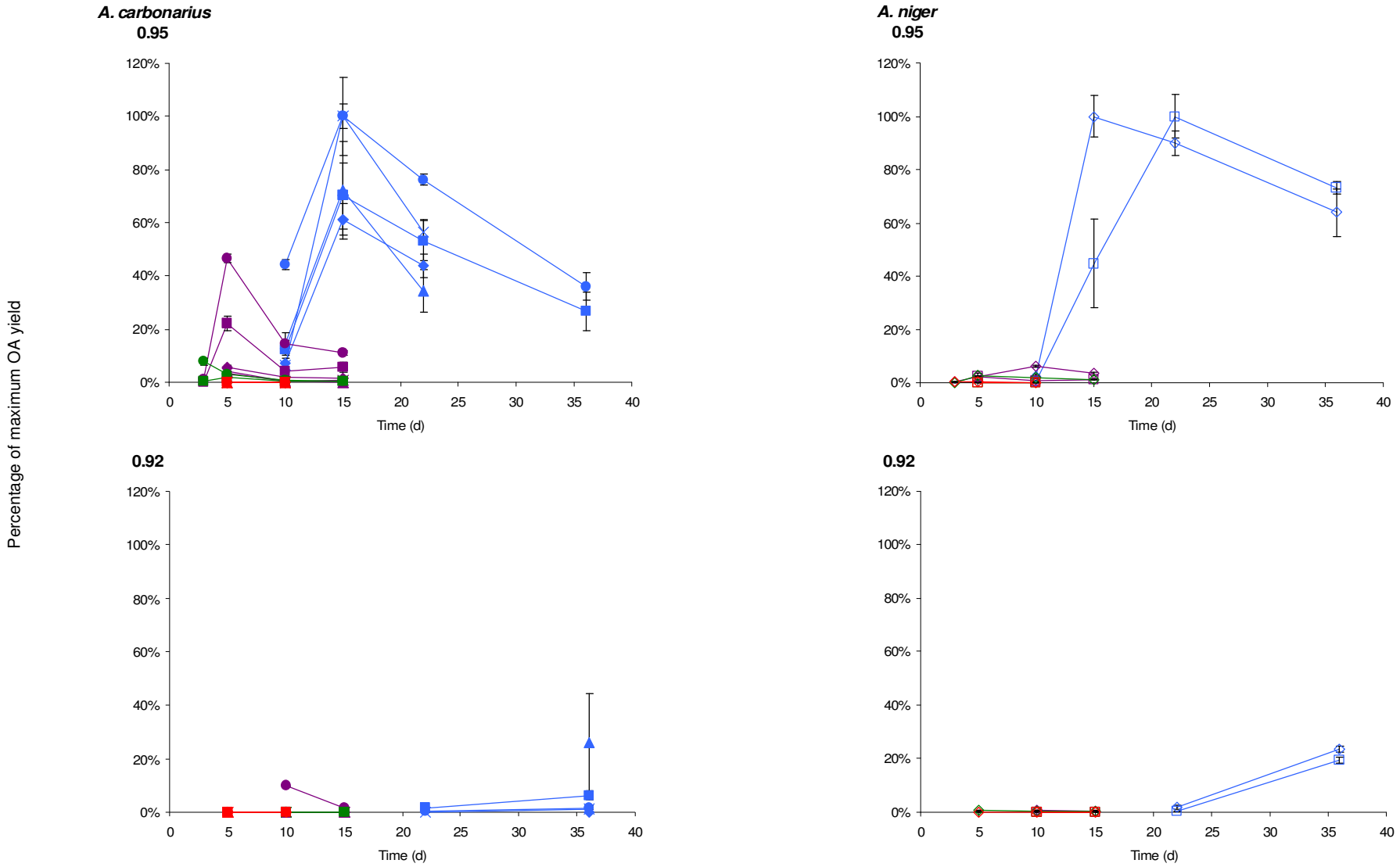


Figure 4.6 (including facing page): Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* on synthetic grape juice medium over time at various temperatures and water activities, expressed as a proportion of the maximum OA yield for each isolate (Fig. 4.5). Error bars denote the standard error of the mean of three replicates

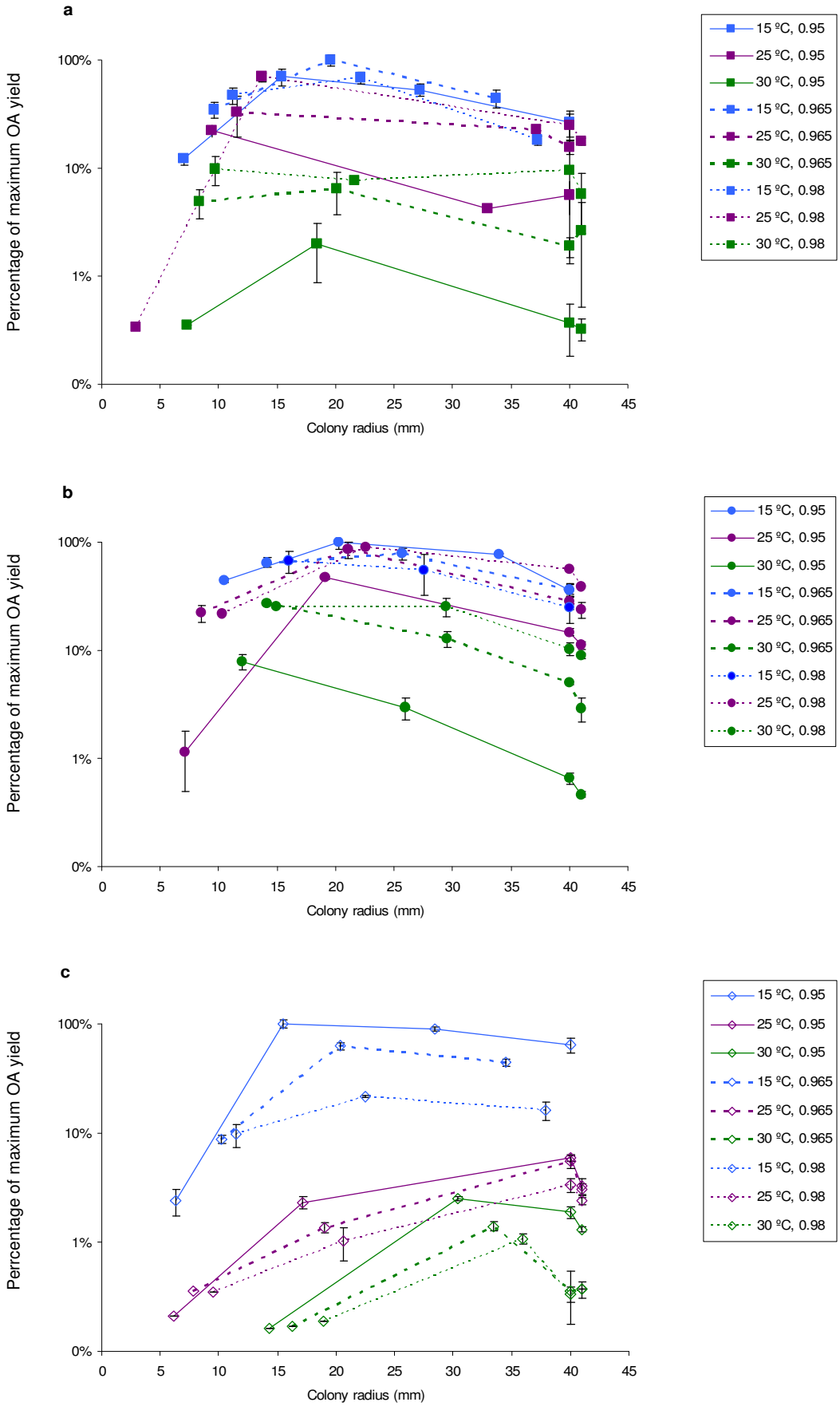


Figure 4.7: Ochratoxin A production by (a) *Aspergillus carbonarius* FRR 5690, (b) *A. carbonarius* FRR 5692 and (c) *A. niger* FRR 5694 on synthetic grape juice medium at various colony sizes for three temperatures and three water activities, expressed as a proportion of the maximum OA yield for each species (Fig. 4.5) and plotted on a logarithmic scale. Error bars denote the standard error of the mean of three replicates. Where two successive samples were taken on fully colonised plates, the first sample was plotted at radius 40 mm and the second at radius 41 mm

4.4 Discussion

4.4.1 Survival of *Aspergillus carbonarius* spores

The effects of temperature and water activity on survival of *A. carbonarius* spores can be broadly summarised by the observation that survival was prolonged at low temperatures and at water activity below 0.6. Little data are available regarding the effect of temperature and water activity on survival and subsequent germination of *A. carbonarius*; however, germination data for the related species, *A. niger,* may provide some indication of the limits for black *Aspergillus* spp. in general. Snow (1949) and Reiss (1986) reported germination limits of 0.84 a_w and 10 °C for *A. niger*, whereas Ayerst (1969) reported germination down to 0.78 a_w. Given that *A. carbonarius* seems less tolerant of low water activity than *A. niger*, as demonstrated by the slower growth of *A. carbonarius* at 0.92 a_w, it is reasonable to assume that *A. carbonarius* would not germinate at water activity below that of the limit for *A. niger* (0.78 a_w), and would be dormant below this limit. Mitchell et al. (2004) observed that 0.88 a_w was the limit of growth for many strains of *A. carbonarius*. The longevity of *A. carbonarius* spores at low water activities and temperatures could be attributed to exogenous dormancy.

Temperatures above the optimum for growth generally represent a stress for fungi. *A. carbonarius* grew at 35 °C in these trials; however, Leong et al. (2004) reported that 37 °C severely retarded growth and 42 °C was sporocidal. Hence, heat stress at 37 °C was likely to contribute to the relatively rapid death of *A. carbonarius* spores, as even *A. niger* spores died after prolonged incubation at 45 °C (Rotem and Aust, 1991). This effect of temperature is common to many genera (Hong et al., 1997).

The effect of intermediate water activities is more puzzling. Survival at low water activities can be associated with spore dormancy, but survival at 1.0 a_w was also typically greater than at 0.6-0.9 a_w. This phenomenon has occasionally been noted in other genera (Hong et al., 1997). Germination is typically most rapid at 1.0 a_w and decreases with decreasing water activity (Snow, 1949; Ayerst, 1969). Given that the limit for growth of *A. carbonarius* is approximately 0.88 a_w, it is noted that 0.6-0.9 a_w represent water activities just below or around the limits for growth and germination. It is possible that spores in this intermediate or borderline range are subject to the stress of cycling between true dormancy and low levels of metabolic activity. A

similar rationale could explain the relatively poor survival of spores at 1.0 a_w and 1 °C, compared with lower water activities and 1 °C at which spores are truly dormant, or, 1.0 a_w at 15 °C and above, at which spores are metabolically active.

Reports of survival of *A. carbonarius* inoculated into soils (Kazi et al., 2003b) show some similarities to and some points of divergence from our data. In soils, survival decreased at temperatures above 25 °C, but increased in dry soil (low water activity), in keeping with this *in vitro* study. The poor survival observed in soil at low temperatures or high water activities (moist soil) was not supported by our data and may be related to stimulation of soil microflora (Greaves and Jones, 1944) which may, in turn, compete with or be antagonistic to *A. carbonarius.*

An observation of minor interest from this trial was that spores in the repeated experiment (B) tended to survive longer than those in the first experiment (A). Spores in B were harvested after an additional incubation of 3 d and appeared to be slightly more resistant. This observation, if experimentation proves it to be consistent, may have relevance in the vineyard, where spore dispersal by strong winds may occur quite some time after sporulation, resulting in fairly resistant spores being deposited on the surface of berries.

Spores on the surface of berries may survive for weeks over a range of water activities (relative humidities), even at 37 °C, but UV radiation in sunlight is deleterious for *A. carbonarius* spores, despite this species possessing thick, melanised spore walls. A cumulative exposure of 10 mWh of UV irradiation, which resulted in an approximate 10^5 fold decrease in viability, could be achieved in one week of high UV intensity with cloudless skies, such as typically occurs during summer in Australia. However, the exposure to UV light of spores on bunches would be less than on flat filter membranes, and would be influenced by bunch and canopy architecture. The same cumulative UV exposure resulted in a greater decrease in spore viability during the repeated experiment (B) than during the first (A). Exposure during B comprised lower daily UV intensity over an extended period (9 d compared with 5 d), suggesting that duration of exposure may play a role in spore death.

4.4.2 Growth

Factors that affect spore survival have a different effect on fungal growth. UV light is likely to have little influence on mould growth within the berry. The influence of temperature and water activity on growth rate of Australian isolates of *A. carbonarius* and *A. niger* was significant, and *A. niger* grew more rapidly than *A. carbonarius*, as reported for European isolates (Battilani et al., 2003c; Mitchell et al., 2003, 2004; Bellí et al., 2004b). Mitchell et al. (2003, 2004) reported slight differences in growth rate among isolates of *A. carbonarius* from Greece, Israel, Italy and Portugal, and this was also observed for Australian isolates. Bellí et al. (2005b) likewise reported slight differences in growth rate and optimal conditions for growth among isolates of *A. carbonarius* from France, Italy, Portugal and Spain; however, these were not statistically significant. Growth rate data for Australian isolates of *A. carbonarius* did not exactly fit the model based on data from European isolates (Bellí et al., 2005b).

The optimum conditions for growth of Australian isolates of *A. carbonarius* on SGM (0.965 a_w, 30 °C) were similar to those reported and/or predictively modelled by Bellí et al. (2004b) (0.97 a_w, *ca* 32 °C), Mitchell et al. (2004) (0.97-0.985 a_w, 25-35 °C) and Bellí et al. (2005b) (0.98 a_w, *ca* 28 °C) for European isolates grown on the same medium. Battilani et al. (2003c) reported an optimum temperature of 30 °C for Italian isolates of *A. carbonarius*. Australian *A. niger* isolates examined here demonstrated an optimum for growth at the highest water activity and temperature combination within the limits of this trial, namely, 0.98 and 35 °C. Likewise, Bellí et al. (2004b) observed maximum growth for European *A. niger* isolates at 0.97 a_w and 35 °C, and Battilani et al. (2003c) reported the same optimal growth temperature. Optima for growth of *A. niger* on other media have been reported by Ayerst (1969) (1.0 a_w, 35 °C) and Reiss (1986) (0.96-0.98 a_w, 35-37 °C). The reproducibility of these growth optima indicates that *A. niger* has a higher optimum growth temperature than *A. carbonarius* and possibly also takes greater advantage of high water activities for rapid growth. This study has also confirmed the greater tolerance of *A. niger* for lower water activities reported by Bellí et al. (2004b). Reducing the water activity from 0.98 to 0.92 resulted in a 32% decrease in growth rate for *A. niger*, compared with a 50% decrease for *A. carbonarius*, as also observed by Mitchell et al. (2004). Whereas *A. niger* was more tolerant of low water activities, *A. carbonarius* was slightly more tolerant of low temperatures, most probably due to its lower optimum growth

temperature. *A. carbonarius* isolates at 15 °C grew at 27% of the rate at the optimum (30 °C) whereas *A. niger* isolates at 15 °C grew at 21% of the rate at the optimum (35 °C).

In general, Australian isolates of *A. carbonarius* and *A. niger* displayed growth rates similar to European isolates. When examining the extremes of growth for comparison, combined data from Bellí et al. (2004b) and Bellí et al. (2005b) for a total of 12 isolates from France, Italy, Portugal and Spain are given in square brackets: rates for five Australian isolates of *A. carbonarius* at 0.92 a_w and 15 °C were 0.27-0.65 mm/d [0.25-1.12 mm/d at 0.93 a_w] and rates at 0.98 a_w and 30 °C were 5.82-7.42 mm/d [3.45 at 0.98 a_w, to a maximum value at 0.99 a_w of 10.1 mm/d]. Rates for two Australian isolates of *A. niger* at 0.92 a_w and 15 °C were both 1.05-1.09 mm/d [0.91-1.13 mm/d at 0.93 a_w for three European isolates], and rates at 0.98 a_w and 30 °C were 8.51-9.29 mm/d [6.14-9.34 mm/d]. The twelve European isolates of *A. carbonarius* displayed a wider range of growth rates than the five Australian isolates. Bellí et al. (2004b) noted that one strain displaying the rapid growth rate of 9.3 mm/d at 0.98 a_w and 30 °C was similar to strains of the new species tentatively designated *A. ibericus* (see section 3.4.1). For growth of eight *A. carbonarius* isolates at 25 °C, Mitchell et al. (2004) reported a range of *ca* 4-11 mm/d at 0.98 a_w and *ca* 3-8 mm/d at 0.93 a_w, whereas all five Australian isolates all grew within the range *ca* 4-6 mm/d at 0.98 a_w and *ca* 2-4 mm/d at 0.92 a_w. Four isolates from Portugal, Israel and Greece grew faster than 6 mm/d at 0.98 a_w and three isolates grew faster than 4 mm/d at 0.93 a_w. Characterisation of additional Australian isolates of *A. carbonarius* may yield similar strains capable of faster growth. At present, suffice to say that differences in growth rate do exist among *A. carbonarius* isolates, although the wide variation among European isolates is yet to be demonstrated among Australian strains. No relationship between growth rate and region of origin has been observed for European strains (Bellí et al., 2004b; Mitchell et al., 2004). The growth rates for Australian isolates of *A. carbonarius* from the Hunter Valley (FRR 5682) and Riverina (FRR 5690) appeared to be similar, whereas the rates for isolates from the Riverland (FRR 5691), Sunraysia (FRR 5693) and Queensland (FRR 5692) showed some differences, although not to the same extent as European isolates. It is not likely that these regional differences in growth rate would be preserved upon examination of additional isolates.

4.4.3 Ochratoxin A production

The values for mean maximum OA yield in Fig. 4.4 should not be taken to imply that *A. niger* isolates produce, on average, more OA than *A. carbonarius* isolates. Only two *A. niger* isolates were examined and atypical low OA producing isolates of *A. carbonarius* were included in this study.

The conditions for maximum OA production by *A. carbonarius* on SGM (*ca* 0.96 a_w, 15 °C) were similar to those reported by Mitchell et al. (2004) (*ca* 0.95-0.98 a_w, 15-20 °C) and Bellí et al. (2005b) (*ca* 0.95-0.99 a_w, 20 °C). The optimum temperature for OA production by *A. niger* was also 15 °C. Battilani et al. (2003c), likewise, reported 15-20 °C as the optimum for OA production by black *Aspergillus* spp. on SGM, and Esteban et al. (2004) reported maximum toxin production at 15-25 °C on YES and CYA. Our data support the optimal water activities for OA production reported by Bellí et al. (2004d) for two isolates of *A. carbonarius* grown on SGM at 25 °C (0.95-0.98 a_w). In contrast, the optimal water activity observed for *A. niger* (0.98-0.995) by Bellí et al. (2004d) was higher than demonstrated by our data, *viz.* a strong optimum at 0.95 a_w, regardless of temperature. Optima for OA production by other fungi differ from the black aspergilli: *A. ochraceus* has optima around 0.95-0.99 a_w and 25-37 °C (Bacon et al., 1973; Northolt et al., 1979; Aziz and Moussa, 1997; Lee and Magan, 2000) and *P. verrucosum*, 0.95-0.99 a_w or 0.92 a_w, depending on substrate, and 24 °C (Northolt et al., 1979; Patterson and Damaglou, 1986). This indicates that ochratoxigenic species differ in their response to changes in water activity and temperature.

The most toxigenic Australian isolates of *A. carbonarius* and *A. niger* produced OA on SGM in the same order of magnitude as the most toxigenic isolates reported from Europe to date. At 0.95 a_w, *A. carbonarius* FRR 5692 produced 21 µg/g at 15 °C and 10 µg/g at 25 °C, and Bellí et al. (2005b) similarly reported OA production of 20.5 µg/g at 0.95 a_w and 20 °C after 7 d by an isolate from Spain. Eight European isolates examined by Mitchell et al. (2004) produced far less OA, with maximum OA at 20 °C of only *ca* 0.75 µg/g. At 25 °C, the maximum OA yield by *A. carbonarius* FRR 5692 was 10 µg/g, as mentioned above, and the maximum yield by *A. niger* FRR 5694 was 5.6 µg/g. Similarly, Bellí et al. (2004b) reported maximum yield by Spanish isolates of *A. carbonarius* and *A. niger* incubated at 25 °C to be 5.06 µg/g and 2.26 µg/g,

respectively. To highlight the importance of medium for mycotoxin production, the maximum yield reported by Esteban et al. (2004) was 485 µg/g on CYA for *A. carbonarius* CBS 127.49 and 98 µg/g on YES for *A. foetidus* CBS 618.78 (belonging to the *A. niger* aggregate). Bellí et al. (2004d), Esteban et al. (2004), Mitchell et al. (2004) and this study have all utilised the same method for OA extraction from agar plugs followed by detection and quantification by HPLC (Bragulat et al., 2001), with only minor modifications; hence, it is unlikely that the major variation reported between strains results from methodological considerations.

OA production on natural substrates may not reach the same concentrations as on rich mycological media. Joosten et al. (2001) reported a maximum yield of 4.8 µg/g OA on coffee cherries inoculated with *A. carbonarius* and incubated at 0.99 and 25 °C, and Serra et al. (2005b), likewise, reported a maximum yield of 5.8 µg/g on homogenised grapes collected at early veraison and inoculated with *A. carbonarius*. This is a similar concentration to that observed on SGM. However, maximum concentrations of OA reported on whole, damaged or intact grapes inoculated with *A. carbonarius* were *ca* 0.2 µg/g (Battilani et al., 2004) to 2.4 µg/g (section 5.5.2). Maximum OA concentrations observed naturally occurring in grapes at early veraison and harvest were lower still: 0.00065-0.013 µg/g (Battilani et al., 2003a, 2005b; Serra et al., 2005b).

At temperatures of 25 °C and above, maximum OA production on SGM occurred 3-5 d after inoculation for all five Australian isolates of *A. carbonarius.* At 15 °C, maxima occurred from 10 d onwards; the time till maximum OA yield (accumulation time) increased with lower water activities due to longer lag times (Mitchell et al., 2004) and slower growth. Poor toxin production at 15 °C reported by Mitchell et al. (2004) and Bellí et al. (2005b), particularly at lower water activities, may have resulted from shorter incubation times, 10 d and 7 d, respectively, than those in this study (10-22 d). As found in this study, Bellí et al. (2004d) reported maxima after incubation at 25 °C for 5 d for water activity 0.95 and above, whereas at 0.90 a_w, maxima occurred after 10 d. Maxima for *A. niger* isolates in the same trial also occurred after 5-10 d, depending on strain; however, there was a tendency for longer accumulation times at lower water activities. The two Australian *A. niger* isolates displayed maxima at 3-5 d above 30 °C, but it is of note that at 25 °C, maxima sometimes occurred after 10 d

(after the maxima of *A. carbonarius*) and again, at 15 °C, maxima were sometimes delayed compared with *A. carbonarius*. *A. niger* FRR 5695 displayed the longest accumulation times for OA on SGM. Other authors have noted accumulation times of 10-14 d to reach maximum OA on SGM (Battilani et al., 2003c; D. Mitchell, pers. comm. 27/10/04). On CYA, Esteban et al. (2004) observed that *A. niger* yielded maximum OA after 5 d of incubation at 25 °C as above, with increasing accumulation times at lower temperatures. No trends for *A. carbonarius* isolates grown on YES were observed due to significant differences among strains. Téren et al. (1996) also reported strain differences in the time till maximum OA yield in YES broth - some strains of both *A. carbonarius* and *A. niger* produced maximum levels at 4 d, others, after 10 d. Varga et al. (2002) detected OA as early as 4 d, with maxima for *A. niger* in YES broth after 7 d, and for *A. carbonarius* after 10 d. Time for maximum OA production by other species varies and is dependent on medium and strain. Varga et al. (2002) reported data for several species. For one example, OA production was detected after 4 d for *A. ochraceus* in YES broth with maxima at 7-10 d depending on strain, whereas Harris and Mantle (2001) reported OA detection after 8 h with the maximum yield after 3 d in potato dextrose broth, suggesting that OA production during the early stages of growth is a phenomenon common to toxigenic species.

The isolates of *A. carbonarius* and *A. niger* in this study all demonstrated a decrease in OA following the peak of production, except where samples were not taken after the maxima. This decrease in OA was presumed to be due to degradation of the toxin (Varga et al., 2000c; Abrunhosa et al., 2002), and has also been observed by Varga et al. (2002), Battilani et al. (2003c), Bellí et al. (2004d) and Esteban et al. (2004). This phenomenon is not restricted to the black aspergilli and has been observed for other *Aspergillus* and *Penicillium* spp. (Harris and Mantle, 2001; Saxena et al., 2001; Varga et al., 2002; Geisen, 2004); however, *A. albertensis* was capable of maintaining a constant concentration of OA in broth and, furthermore, this concentration was the same regardless of incubation temperature (Varga et al., 2002). Control of OA production and degradation is possibly governed at the genetic level by different mechanisms in *A. albertensis* compared with other *Aspergillus* spp.

This study, as well as the studies discussed above, has demonstrated that conditions for maximum growth and for maximum OA production are different. Within species,

isolates with the most rapid growth rate do not necessarily produce the most OA; *A. carbonarius* FRR 5692 grew fastest among the isolates examined and also produced the most OA, but the second highest producer, FRR 5690, generally grew more slowly than some of the other isolates. Similarly, for *A. ochraceus*, a correlation between growth rate and OA yield was dependent on strain (Pardo et al., 2004). OA production by *A. carbonarius* and *A. niger* was not related to extent of growth, as the yield in relation to colony size was strongly governed by temperature. This could also be interpreted as an effect of growth rate, given the relationship between growth and temperature. However, OA yield varied from 5 µg/g at 0.98 a_w and 15 °C to 0.004 µg/g at 0.92 a_w and 25 °C for *A. carbonarius* FRR 5690, even though growth rates were similar in both sets of conditions (2.2 mm/d and 2.6 mm/d, respectively). Hence, it cannot be conclusively stated that rapid growth suppresses OA production, as postulated by Häggblom (1982) for *A. ochraceus*; rather, the suppression may be due to the interaction between temperature and growth rate or some other factor. Removing consideration of the actual OA yield, the maximum yield in each set of conditions for *A. carbonarius* growing on SGM plates appeared to be somewhat related to colony size, or extent of growth, as reported for fumonisin production by *Fusarium* spp. (Simbarashe et al., 2005). The relationship between OA production by *A. ochraceus* and extent of growth as measured by glucosamine or ergosterol concentration has been studied; however, no consistent trends have been observed (Häggblom, 1982; Harris and Mantle, 2001; Saxena et al., 2001). Differences among species exist in the relationship between extent of growth and OA production. Our preliminary data suggested that *A. niger* accumulated OA until the medium was 60-100% colonised at temperatures above 25 °C, whereas *A. carbonarius* accumulated OA until 25% colonisation.

4.4.4 Implications for vineyard ecosystems

Several stages exist in the cycle of OA production by black *Aspergillus* spp. in grapes: 1) the propagules survive in their reservoir, most likely to be soil (Kazi et al., 2004) for extended periods, 2) they survive on the berry surface once they have been blown there by wind, 3) they infect the berry by some means, typically when the cuticle is damaged, 4) they grow and colonise the berry if conditions are conducive, 5) they produce OA while growing, again, if conditions are conducive, and 6) they sporulate and the spores are transferred to other berries or back to the soil. Strategies for the

minimisation of OA in grapes may be targeted at reduction of toxigenic fungi in soil and on berry surfaces (stages 1 and 2), thus lowering the probability of infection; reducing infection of berries by preventing berry damage (stage 3, discussed in chapter 5); and understanding parameters for fungal growth and OA production in grapes (stages 4 and 5), so that appropriate measures can be taken regarding application of sprays to control fungal growth and/or removal of the damaged bunches. If the toxin levels are likely to be low, wine made from the crop could be blended with that from uncontaminated grapes.

During vine dormancy when soil temperatures are cold and/or soil is dry, ochratoxigenic black *Aspergillus* spp. have the potential to survive for extended periods, as spores are dormant. At warmer temperatures, interactions with other members of the soil ecosystem are likely to play a major role, as suggested by the soil studies of Kazi et al. (2003b). Research into the incidence of *A. carbonarius* in various soil types and reduction of this species in soil through management of tillage, mulching and irrigation [reviewed by Leong et al. (2005a)] is continuing at the Department of Primary Industries in Mildura, Victoria.

On berry surfaces, spores may be shielded by a vigorous canopy from the lethal effects of UV radiation in sunlight, and cooler conditions within the canopy may also prolong spore survival. Increased humidity within the canopy may favour the development of bunch rots. Exposing bunches to the sun may decrease the spore load, but sun-damage to berries may increase the susceptibility to infection.

The water activity of berries from early veraison until harvest changes from approximately 0.98 to 0.95 as sugars accumulate. This estimate is based on the water activity of SGM that contained 10 g of glucose/fructose (*ca* 10 °Brix) and yielded a water activity of 0.98. The range of water activities from veraison until harvest supports optimal growth of both *A. carbonarius* and *A. niger*, with rate primarily governed by temperature. In hot weather, *A. niger* is likely to be dominant, as was reported by Leong et al. (2004) on grapes grown for drying. At temperatures below 20 °C, growth is retarded for both species.

As the optimum water activity for OA production was around 0.95-0.96, the potential for OA production increases as berries mature. However, *A. carbonarius* is capable of producing large amounts of OA as early as veraison (0.98 a_w) at temperatures below 25 °C. More OA is produced at lower temperatures, as has been noted by Battilani et al. (2004) for Italian grapes inoculated with *A. carbonarius* and incubated at 20 °C and 25 °C (0.12 µg/g and 0.03 µg/g, respectively). However, those researchers also reported an increased incidence of mould development at 20 °C compared with 25 °C, which is contrary to the trends for growth rate observed here. In the production of dried vine fruit, once desiccation begins, the water activity decreases below 0.95 and OA production is retarded. Rapid drying of potentially infected berries to below 0.88 a_w removes the threat of continued OA production, although rewetting of berries in inclement weather may reactivate toxin production. This phenomenon has been noted by Bucheli et al. (2000) during drying of coffee cherries.

OA production by black *Aspergillus* spp. was detected during the early stages of growth, and often reached maximum yield after relatively little growth had occurred. Hence the earliest signs of visible berry rot by a toxigenic isolate may be indicative of OA contamination. Fortunately, it seems that the same mould has the potential to degrade the toxin fairly rapidly after it has been produced, demonstrated in this study and by other researchers (Varga et al., 2000c; Abrunhosa et al., 2002; Varga et al., 2002; Battilani et al., 2003c; Bellí et al., 2004d; Esteban et al., 2004). In vineyards, this has been supported by anecdotal evidence for grapes inoculated with *A. carbonarius* for a winemaking trial (described in chapter 6), in which grapes harvested 8-9 d after inoculation contained *ca* 0.11 µg/g OA, whereas grapes harvested 14-21 d after inoculation contained *ca* 0.006 µg/g. Aspergillus rot that develops just prior to harvest may represent a greater risk for OA contamination than rot that develops earlier in the season.

4.4.5 Future research

A change in pH accompanies the change in water activity during berry maturation. Esteban et al. (2005a) reported that pH optima for OA production by *A. carbonarius* on standard culture media, CYA and YES, were dependent on isolate and temperature of incubation, and occurred within the approximate range 2-7. Currently, all trials using SGM were conducted around pH 3.8-4.2, whereas the pH in grapes may be

closer to 2 during early veraison. Exploring the effects of pH, temperature and water activity simultaneously could aid in the simulation of growth and toxin production in grapes.

Given that growth is favoured by warmer temperatures whereas OA production is favoured by cooler temperatures, study of the effect of diurnal fluctuations on toxin production would be of interest. Hypothetically, during the day, black *Aspergillus* spp. would rapidly colonise the available substrate, whereas the cooler night would be optimum for toxin production; toxin produced at night may, in turn, be rapidly degraded the following day. Simulated diurnal fluctuations had no direct effect on production of OA by *A. ochraceus* in raw coffee; rather, OA was increased due to the condensation on the coffee surface that resulted from the temperature changes (Palacios-Cabrera et al., 2004). For *A. ochraceus*, optima for growth and OA production coincide with daytime temperatures, whereas these optima are separated between day and night for black *Aspergillus* spp., hence diurnal fluctuations may have a marked effect.

A. niger has always been isolated more frequently than *A. carbonarius* from fresh grapes in the vineyard (section 3.1.1). Both these species have also been isolated from dried vine fruit (Heenan et al., 1998; Abarca et al., 2003; Clarke et al., 2004; Leong et al., 2004; Magnoli et al., 2004; Tjamos et al., 2004), and there is a sense among some researchers that the relative incidence of *A. carbonarius* in both Australia and Europe is increased in drying and dried grapes (Valero et al., 2005; S.L. Leong, unpublished data; R.W. Emmett, pers. comm. 10/06/04; D. Mitchell, pers. comm. 27/10/94). This is a puzzling phenomenon, as *A. carbonarius* grows more slowly than *A. niger* under all the conditions tested to date. Some other physiological factor must confer on *A. carbonarius* a competitive advantage in particular situations. One hypothesis is that *A. carbonarius* is more resistant to the lethal effects of UV radiation than *A. niger*. *A. carbonarius* spores contain more melanin, may have thicker walls, are larger and thus contain more cytoplasm to buffer any deleterious effects, and possess multiple nuclei (Kevei et al., 1996) which could compensate for UV damage to the genome (J.I. Pitt, pers. comm. 02/04/04). It remains to be tested if this, or some other mechanism, confers a competitive advantage on *A. carbonarius* in dried grape vineyards.

Regulation of OA production is not well understood at the molecular level (section 3.4.3). It is likely that the genes responsible for OA synthesis in black *Aspergillus* spp. are different from those in *A. ochraceus*, *P. nordicum* and *P. verrucosum*, and under different regulatory mechanisms [reviewed by Niessen et al. (2005)]. Future investigation of these genes would contribute much to the fundamental understanding of OA production.

All the research to date, including this study, has focussed on OA production by mould in commodities which are ingested by humans and animals. An alternative route of intoxication, inhalation, was proposed by Di Paolo et al. (1994), Richard et al. (1999) and Iavicoli et al. (2002), and spores of *P. verrucosum* were shown to contain OA (Skaug et al., 2000). Quantification of OA present in conidia of toxigenic black *Aspergillus* spp. would be of interest for the safety of viticulturalists, who may inhale these conidia from sporulating *Aspergillus* spp. in rotten bunches.

5 Factors affecting the incidence and growth of *Aspergillus carbonarius* on grapes in vineyards

5.1 Introduction

The incidence of *A. niger* increases as berries mature, and Aspergillus rot may develop post-veraison; these are well-established facts (Nair, 1985; Hewitt, 1988; Snowdon, 1990; Emmett et al., 1992). The discovery that *A. carbonarius* was the likely source of OA in grapes (see sections 1.4 and 3.4.1), however, led to re-examination of the incidence of black *Aspergillus* spp. on grapes, with particular attention to species differentiation. Several factors affect the potential for growth and toxin production of black *Aspergillus* spp. on grapes, including climate, vineyard management, berry maturity, cultivar and cuticle integrity.

The occurrence of black *Aspergillus* spp. on grapes correlated with increased temperature and, to a lesser extent, with increased humidity and rainfall, based on data from Spanish viticultural regions over three seasons (Bellí et al., 2005a). Italian data likewise showed a positive correlation between black *Aspergillus* spp. and temperature, but a negative correlation with rainfall (Battilani et al., 2005b). Black aspergilli were isolated more frequently from warmer regions with a Mediterranean climate than from temperate regions in France (Sage et al., 2004), Italy (Battilani et al., 2003a), Portugal (Serra et al., 2003, 2005a) and Spain (Bellí et al., 2005a). Bellí et al. (2005a) also reported increased isolation of black aspergilli during the warmest of three seasons. Serra et al. (2003) observed that black aspergilli were more frequently isolated from a hot, dry region than from a temperate, humid region, suggesting that the effect of temperature is stronger than that of humidity. Roset (2003) noted that OA in grape juice correlated with increased pre-harvest temperature, rainfall, proximity to the coast and later date of harvest; similar trends regarding temperature and rainfall were reported by Battilani et al. (2003a).

Several authors have reported an increase in black aspergilli on grapes from berry set until harvest (Nair, 1985; Battilani et al., 2003a; Serra et al., 2003; Bellí et al., 2004c, 2005a; Bau et al., 2005a; Serra et al., 2005a), although this trend was not consistent for all black *Aspergillus* spp. over three years in Italian vineyards (Battilani et al., 2005b). Incidence of black *Aspergillus* spp. in vineyard soil increased with regular

tillage (Kazi et al., 2004). Battilani et al. (2004) reported differences in susceptibility to infection and OA production *in vitro* among grape cultivars commonly grown in Italy; furthermore, OA production correlated with severity of infection for certain cultivars but not others. However, these trends were not necessarily reflected by infection and OA contamination in vineyards; differences among cultivars were often more strongly associated with seasonal variations in climate and time of ripening (Battilani et al., 2005b; Leong et al., 2005a).

Much of these data were collected from vineyards in Europe (Table 3.1), from which black aspergilli were isolated by direct plating of individual berries. Sporadic isolation of toxigenic *Aspergillus* spp. in those studies was exacerbated by variation among the few individual berries plated per bunch, among bunches, and among seasons (Battilani et al., 2003a). Given these constraints, it has been difficult to study the survival and growth of naturally occurring OA-producers within the bunch ecosystem. Some of these factors were addressed in the trials described in this chapter by inoculating *A. carbonarius* directly onto bunches on the vine. Incidence and distribution of black *Aspergillus* spp., and their growth and toxin production on intact and damaged berries, were examined for cultivars grown in Australian viticultural conditions.

5.2 Natural occurrence of black Aspergillus *spp. on grapes in Australia*

5.2.1 Methods

5.2.1.1 Location of vineyard trials

In 2001 and 2002, trials were conducted in vineyards in the Sunraysia region centred around Mildura, Victoria. In 2003 and 2004, trials were conducted in the Hunter Valley, New South Wales (Table 5.1).

Table 5.1: Sites of vineyard trials

Cultivar	Trellis	Irrigation	Pruning; Cultivation, where known
2001, 2002[a] Vineyard A, Commercial, Sunraysia, Victoria			
Riesling	2-wire vertical	overhead	mechanically hedged; stubble direct-drilled inter-row, herbicide
Chardonnay[a] (Schwarzmann; 8 yr)[b] Semillon[a] (Ramsay; 8 yr) Cabernet Sauvignon[a] (Schwarzmann; 7 yr) Merlot Shiraz[a] (Schwarzmann; 7 yr)	2-wire V	drip; 4-5 h every 1 - 3 d to maintain berry turgor	
2001 Vineyard B, Research / Cultivar collection, Sunraysia, Victoria			
Chardonnay Riesling Cabernet Sauvignon Merlot Pinot Noir Shiraz	2-wire vertical	under-vine sprinklers	spur
2002 Vineyard C, Commercial, Sunraysia, Victoria			
Chardonnay (Ramsay; 8 yr) Cabernet Sauvignon (Ramsay; 8 yr) Shiraz (own roots; 8 yr)	2-wire vertical	overhead; 10 h when soil had dried out, as indicated by soil moisture probe, usually every 7-9 d	mechanically hedged; regular cultivation with rotary-hoe
2003, 2004 Vineyard D, Commercial, Hunter Valley, New South Wales			
Chardonnay (> 25 yr) Semillon (> 25 yr) Shiraz (> 25 yr)	2-wire vertical	drip; 2-3 h twice weekly	cane

[a] cultivars included in trials in 2002
[b] where known, rootstock and age of vines in parenthesis

5.2.1.2 Assessment of incidence of black *Aspergillus* spp. on grapes

5.2.1.2.1 2001, 2002 - Cultivar and vineyard management

In 2001, the effect of cultivar on incidence of black *Aspergillus* spp. was examined. From Vineyards A and B (Table 5.1), five bunches were collected from each cultivar before harvest. Each bunch was treated as a replicate and the following measurements were taken to specify bunch size, architecture and maturity: bunch weight, bunch length and width, weight of 10 berries, total soluble solids (measured in °Brix using an Atago PR-32 refractometer, Tokyo, Japan), pH and titratable acidity (as assessed by titration to pH 8.2; Iland et al. (2000)). The latter three measurements were taken on juice from homogenised berries. Before analysis, the juice was clarified by filtration through muslin or by centrifugation.

Each bunch was shaken vigorously in a zip lock bag with 500 mL distilled water containing Citowett (BASF Australia Ltd, Vic, Australia) as a wetting agent for 1 min. To assess the surface mycoflora of bunches, serial dilutions of the rinsings were performed and plated onto DRBC in duplicate as described in section 2.1. The excess water was decanted, after which the berries were clipped from the bunch and surface disinfected in 10% domestic bleach solution (final concentration 0.2% active chlorine) for 2 min. The berries were rinsed three times in tap water and homogenised in a standard domestic blender (Philips HR2835/AB). The blender bowl was rinsed with 95% ethanol between samples to minimise cross-contamination by fungal spores. Serial dilutions of this homogenate were plated onto DRBC in triplicate to assess the internal mycoflora of the berries. Black *Aspergillus* spp. were enumerated as described in section 2.1.

Logarithmically transformed total counts (sum of external and internal counts; cfu/g) of black *Aspergillus* spp. were analysed by pairwise comparison (Genstat, 6th Edition, Lawes Agricultural Trust, Rothamsted, UK) with bunch parameters from Table 5.2.

In 2002, the effect of cultivar and aspects of vineyard management on incidence of black *Aspergillus* spp. was assessed. Vineyards A and C (Table 5.1), situated in the same area, were selected, as the same three cultivars (Chardonnay, Cabernet

Sauvignon, Shiraz) were grown in close proximity in each. Semillon from Vineyard A was also examined; however, it was not grown in Vineyard C. The age of vines was similar in both vineyards and, within each vineyard, the cultivars were managed identically. Three primary differences existed between the vineyards, namely, type of irrigation (drip *vs* overhead), floor management (cover crop *vs* regular tillage) and type of trellis (V *vs* vertical).

At harvest, 10 replicates, each comprising five randomly selected bunches, were collected for every cultivar. Samples were placed in zip lock bags and weighed. Surface and internal mycoflora were examined as described above, with the following amendments: the volume for rinsing bunches was 1 L for the five bunches pooled into a single sample, the bunches were not surface sterilised before homogenisation, and homogenates were plated in duplicate.

Data were analysed as described in section 2.6.

5.2.1.2.2 2003, 2004 - Cultivar and berry maturity

In 2003 and 2004, the effect of cultivar and berry maturity on incidence of black *Aspergillus* spp. was examined in Vineyard D (Table 5.1). All three cultivars were assessed in 2003, whereas in 2004, the trial was conducted with Chardonnay and Shiraz only. Samples were collected at pre-bunch closure (berries green and pea size), veraison, 11-24 d pre-harvest and at harvest. The rationale for assessing the natural incidence of black *Aspergillus* spp. in bunches was to establish a baseline against which the population of *A. carbonarius*, artificially inoculated onto bunches, could be compared (section 5.4). The trial comprised three replicate rows. Six samples, each comprising two bunches, were collected per replicate in 2003, and two samples (single bunches) were collected per replicate in 2004. Bunches were weighed, then homogenised in a stomacher (BagMixer, Interscience, France), with the addition of sterile distilled water roughly equivalent to the sample weight, for 3 min. Black *Aspergillus* spp. in the homogenate were enumerated as described in section 2.1. The mean berry weight at each growth stage was calculated by counting the total number of berries in representative bunches of known weight. The number of *A. carbonarius* colonies was expressed as cfu per berry (cfu/berry), to facilitate comparison of samples of different berry maturity and weight. The presence of *Alternaria* spp.,

Cladosporium spp. and yeasts was also noted, as these fungi are frequently isolated from grapes.

Several samples did not yield detectable black *Aspergillus* counts, hence data were not amenable to statistical analysis. Logarithmically transformed data (cfu/berry) for *Alternaria* spp., *Cladosporium* spp. and yeasts enumerated in 2004 were analysed as described in section 2.6.

5.2.2 Results

5.2.2.1 Effect of cultivar and vineyard management on incidence of black *Aspergillus* spp. at harvest

Among the black aspergilli, the predominant species isolated was *A. niger*. *A. aculeatus* was isolated from two of 30, and three of 30 bunches from Vineyards A and B, respectively, and *A. carbonarius* was isolated from five of 30 and 12 of 30 bunches from those vineyards. However, the populations of these latter two species were typically less than 100 cfu/g in 61% of bunches from which they were isolated. Hence, the results reported in Table 5.2 primarily represent contamination with *A. niger*.

Surface contamination was generally greater than internal contamination. White and red cultivars did not display consistent differences. Whereas Chardonnay and Shiraz from Vineyard B, and Riesling from Vineyard A were contaminated to a greater extent than other cultivars, no single cultivar showed increased contamination in both vineyards simultaneously. In comparing the cultivars and sites, the greatest contamination was observed in Shiraz from Vineyard B, in which bunches were also heavier than the other cultivars. During pairwise comparison, logarithmically transformed total counts of black *Aspergillus* spp. yielded correlations of 0.32 and 0.31 with bunch weight and titratable acidity, respectively.

Table 5.2: Frequency of contamination with black *Aspergillus* spp. in 2001 - number of bunches within certain ranges of contamination

Cv[a]	Vineyard	Mean[b] ± standard error of the mean							Surface contamination (cfu/g)					Internal contamination (cfu/g)				
		Total soluble solids (°Brix)	Bunch weight (g)	Berry weight (g)	Bunch length (cm)	Bunch width (cm)	pH	Titra-table acidity (g/L)	< 10	10-10^2	10^2-10^3	10^3-10^4	> 10^4	< 10	10-10^2	10^2-10^3	10^3-10^4	> 10^4
Ch	A	22.6 ± 0.3	105.7 ± 13.4	1.30 ± 0.06	15.3 ± 1.3	7.5 ± 1.1	3.9 ± 0.1	5.1 ± 0.1	3	2	-[c]	-	-	4	1	-	-	-
	B	22.1 ± 0.5	88.0 ± 24.3	1.06 ± 0.05	9.4 ± 0.7	5.9 ± 0.7	4.0 ± 0.1	5.2 ± 0.2	-	-	-	3	2	-	3	1	-	1
Rs	A	17.0 ± 0.6	96.0 ± 12.6	0.76 ± 0.06	10.5 ±0.7	6.1 ±0.3	3.7 ± 0.0	4.6 ± 0.0	3	-	1	-	1	3	1	-	-	1
	B	17.9 ± 0.4	73.7 ± 10.9	0.94 ± 0.04	10.9 ± 0.5	7.0 ± 0.2	3.8 ± 0.0	4.1 ± 0.1	1	3	-	1	-	4	1	-	-	-
Sm	A	23.9 ± 1.2	122.7 ± 22.4	1.49 ± 0.10	16.5 ± 1.0	8.4 ± 0.2	4.0 ± 0.0	3.6 ± 0.1	3	2	-	-	-	3	2	-	-	-
Sh	A	20.9 ± 0.9	128.9 ± 16.8	1.50 ± 0.11	20.5 ± 1.3	8.1 ± 0.8	3.9 ± 0.1	4.3 ± 0.2	3	2	-	-	-	5	-	-	-	-
	B	23.8 ± 0.3	241.0 ± 24.9	1.69 ± 0.08	19.2 ± 1.0	11.0 ± 0.6	4.2 ± 0.0	4.6 ± 0.2	-	-	1	-	4	-	1	-	-	4
Mr	A	23.4 ± 0.6	166.0 ± 29.2	1.19 ± 0.08	19.7 ± 0.5	10.3 ± 0.7	4.1 ± 0.0	3.7 ± 0.1	3	2	-	-	-	5	-	-	-	-
	B	21.6 ± 0.2	90.5 ± 12.2	1.16 ± 0.08	13.6 ± 1.0	5.4 ± 0.6	4.2 ± 0.0	3.4 ± 0.1	3	2	-	-	-	5	-	-	-	-
CbS	A	21.5 ± 0.7	124.1 ± 24.4	1.14 ± 0.04	17.5 ± 0.6	7.6 ± 0.5	4.0 ± 0.0	4.5 ± 0.1	2	3	-	-	-	5	-	-	-	-
	B	20.7 ± 0.6	107.2 ± 9.1	1.03 ± 0.03	14.3 ± 1.0	6.1 ± 0.9	4.0 ± 0.0	5.6 ± 0.2	1	4	-	-	-	1	4	-	-	-
Pn	B	25.6 ± 1.6	66.9 ± 12.9	0.95 ± 0.07	9.2 ± 0.6	5.1 ± 0.3	4.4 ± 0.0	3.7 ± 0.2	2	2	1	-	-	5	-	-	-	-

[a] Cv: cultivar; Ch: Chardonnay; Rs: Riesling; Sm: Semillon; Sh: Shiraz; Mr: Merlot; CbS: Cabernet Sauvignon; Pn: Pinot noir
[b] mean of five bunches
[c] - = nil bunches within that contamination range

Total soluble solids (°Brix), mean berry and bunch weight for samples collected in 2002 are reported in Table 5.5 as part of the trial with inoculated bunches (described in section 5.3.1.2). *A. niger* was the most abundant species on bunches although, overall, black *Aspergillus* spp. were not commonly isolated (Fig. 5.1).

Significant differences were not observed among vineyards and cultivars in 2002; however, the following trends were evident. The natural incidence of the black aspergilli was greater on white cultivars than on red cultivars. Cool weather delayed the ripening of the white cultivars, hence bunches nearing maturity remained on the vine for an extended period. The greatest black *Aspergillus* counts were observed on Chardonnay from Vineyard C, in which damage due to vinegar fly (*Drosophila melanogaster*) was noted. Among red cultivars, incidence was greatest in Cabernet Sauvignon from Vineyard A and Shiraz from Vineyard C.

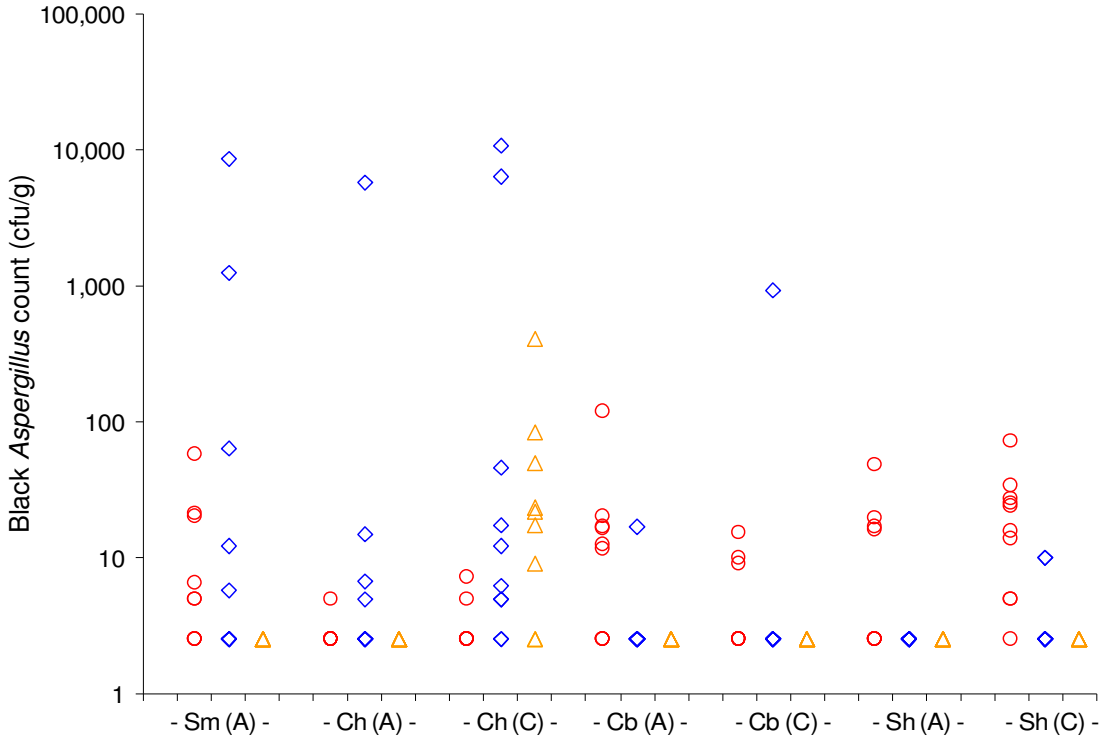


Figure 5.1: Natural incidence of black *Aspergillus* spp. on grapes at harvest, 2002. Each symbol represents the count (cfu/g) from five bunches (pooled); red circles = *Aspergillus carbonarius*, blue diamonds = *A. niger*, orange triangles = *A. aculeatus.* Samples in which black *Aspergillus* spp. were not detected are plotted at 2.5 cfu/g, half the limit of detection. Sm: Semillon, Ch: Chardonnay, Cb: Cabernet Sauvignon, Sh: Shiraz; vineyard in parenthesis

5.2.2.2 Effect of cultivar and berry maturity on fungal populations on grapes

Black *Aspergillus* spp. were seldom isolated at pre-bunch closure or veraison, and, if present, occurred at low levels (Table 5.3). Incidence of infection increased nearing harvest, and increased *A. niger* counts were observed in certain bunches. Logarithmically transformed counts (cfu/berry) for other commonly isolated fungi showed a significant three-way interaction (cultivar x stage x species; $P < 0.001$), as demonstrated by the absence of trends observed for isolation of *Alternaria* or *Cladosporium* spp. in 2004 (Fig. 5.2). When examined following a separate ANOVA, the number of yeasts isolated per berry appeared to increase nearing harvest; however, the interaction between cultivar and stage was again significant ($P < 0.001$).

Table 5.3: Natural incidence of black *Aspergillus* spp. on wine grapes from pre-bunch closure until harvest in 2003 and 2004

Cultivar	Stage	Season	Presence of black *Aspergillus* species				
			Black *Aspergillus* spp. present[a] / No. tested	No. of samples within concentration range for black *Aspergillus* spp. (cfu/berry)			
			Samples	5-100	100-1000	10^3-10^4	> 10^4
Chardonnay	Pre-bunch closure	2003	0/18				
		2004	0/6				
	Veraison	2003	0/18				
		2004	0/6				
	Pre-harvest	2003	3/18	2 *n*[b]		1 *n*	
		2004	2/6			1 *c*[b]	1 *n*
	Harvest	2003	5/18	3 *c* 2 *n*	1 *c*		
		2004	0/6				
Semillon	Pre-bunch closure	2003	2/27	1 *a* 1 *n*			
	Pre-harvest	2003	2/27	1 *c* 1 *n*			
	Harvest	2003	9/27	6 *c*			1 *a*[b] 3 *n*
Shiraz	Pre-bunch closure	2003	2/18	1 *c* 1 *n*			
		2004	0/6				
	Veraison	2003	0/18				
		2004	0/6				
	Pre-harvest	2003	6/18	4 *c*	1 *c*	1 *c*	
		2004	0/6				
	Harvest	2003	3/18	1 *n*	1 *c*	1 *c*	
		2004	2/6	1 *n*			1 *n*

[a] limit of detection 5 cfu/berry
[b] *n*: no. of samples from which *A. niger* was isolated; *c*: *A. carbonarius*; *a*: *A. aculeatus*

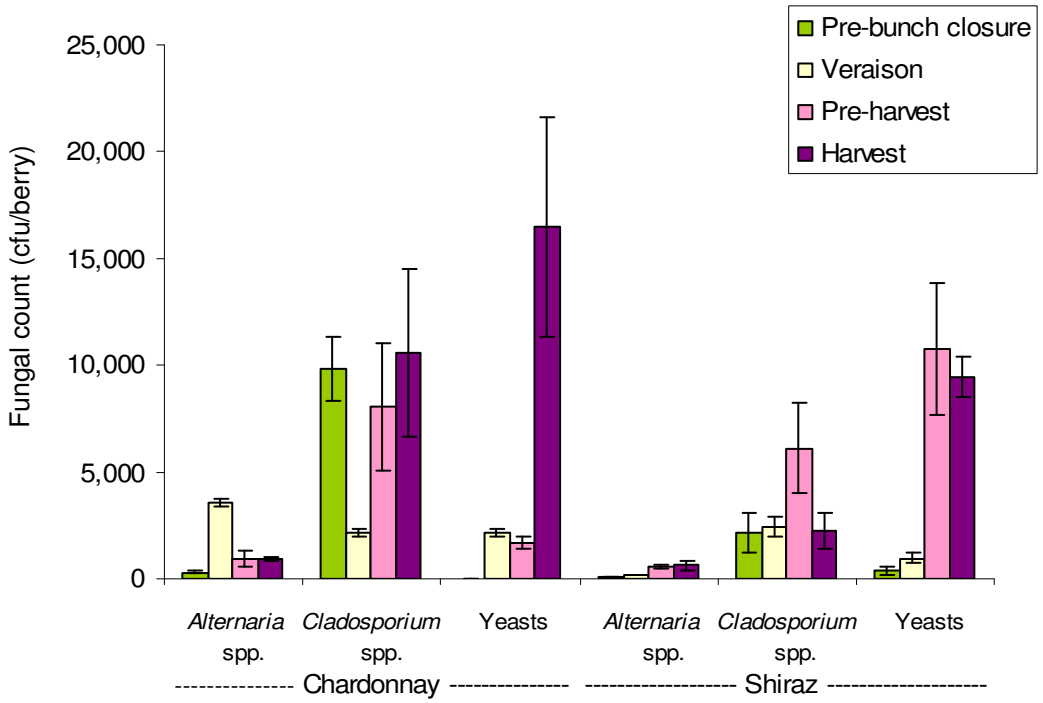


Figure 5.2: Fungi other than black *Aspergillus* spp. commonly isolated from wine grapes, from pre-bunch closure until harvest, 2004. Error bars denote the standard error of the mean of six replicates

5.3 Significance of berry damage and inoculum coverage in the development of Aspergillus rot

5.3.1 Methods

5.3.1.1 Inoculation of bunches in vineyards

This trial was conducted in 2002 in Vineyards A and C (Table 5.1). A spore suspension of *A. carbonarius* strains previously isolated from Sunraysia (FRR 5374, FRR 5573, FRR 5574) was prepared as described in section 2.3. The suspension was diluted in sterile Citowett solution to a concentration of approximately 3 x 10^5 spores/mL. The inoculum was prepared in the morning and kept on ice for use the same day. After use, the viable count of *A. carbonarius* in the remaining inoculum was determined by dilution plating (section 2.1). Bunches from white cultivars were inoculated on the same day with a suspension of viable propagules estimated at 2.7 x 10^5 cfu/mL. The red cultivars were inoculated 20 d later with a fresh spore suspension (1.2 x 10^5 cfu/mL).

For each cultivar, a seven row x 26 vine plot was selected for the trial, comprising three trial rows, each with a buffer row on either side. Twenty four treatment vines and 24 control vines, the latter being the source of bunches described in section 5.2.1.1.2, were distributed among the three trial rows, with two buffer vines between treated and control vines. On each vine, 3-4 bunches from different parts of the canopy were tagged to give a total of 80 treated bunches. Bunches were selected from the side of the vine exposed to afternoon sun, to represent the harshest conditions for spore survival on berries. Each treated bunch was inoculated 1-3 weeks before harvest by spraying the surface three to four times with spore suspension from a hand-triggered spray bottle – the total volume of spray was approximately 4 mL per bunch.

5.3.1.2 Development of Aspergillus rot in vineyards

At harvest, black *Aspergillu*s spp. were enumerated in inoculated bunches (10 replicates, each comprising five bunches randomly pooled from vines within the plot) as described for uninoculated bunches in section 5.2.1.1.2, to assess if the spores had survived on the berry surface and/or if berry infection had occurred.

Logarithmically transformed data (cfu/berry) were analysed as described in section 2.6.

5.3.1.3 Development of Aspergillus rot *in vitro*

At harvest, sets comprising 10 uninoculated or inoculated bunches were collected in individual zip lock bags and were either slit with a sterile scalpel to mimic splitting of the berries, or were submerged in distilled water for 24 h. Preliminary experiments (data not shown) had suggested that submersion in distilled water was a suitable method to induce splitting similar to that caused by rain in susceptible cultivars (Clarke et al., 2003). Some bunches, not inoculated previously, were inoculated after damage by immersion in a suspension of *A. carbonarius* spores at *ca* 1-2 x 10^5 cfu/mL. The treatments are shown in Table 5.4.

Table 5.4: Bunch treatments - inoculation with *Aspergillus carbonarius* and damage to bunches

Pre-damage treatment	Type of damage	Post-damage treatment
nil	berries slit with sterile scalpel	nil
nil	bunches submerged in distilled water (24 h) to induce berry splitting	nil
nil	berries slit with sterile scalpel	bunches immersed in a suspension of *A. carbonarius* spores
nil	bunches submerged in distilled water (24 h) to induce berry splitting	bunches immersed in a suspension of *A. carbonarius* spores
inoculation with *A. carbonarius* on the vine	berries slit with sterile scalpel	nil
inoculation with *A. carbonarius* on the vine	bunches submerged in distilled water (24 h) to induce berry splitting	nil

Excess moisture was drained from the bunches, and bags were opened to dry overnight. Slightly damp paper towel was placed in the bag to increase humidity and to absorb juice from damaged berries. The bags were sealed and incubated at room temperature for 6-8 d, after which bunches were inspected for splitting and/or mould. The percentage of infected berries in each bunch was scored according to the key shown in Fig. 5.3, and the degree of sporulation classified as sparse, moderate or abundant. For bunches that were slit and inoculated either before or after damage, the percentage of infected berries was directly related to the number of slit berries, rather than being indicative of fungal incidence and severity of infection. Disease severity according to the key shown in Fig. 5.3 was not assessed for such bunches; degree of sporulation and incidence of infection (number of bunches affected) were noted. Some bunches were incubated for an additional 7-20 d to monitor further mould development.

Data on incidence and severity of infection with black *Aspergillus* spp. were expressed in various forms appropriate to each treatment, thus were not amenable to statistical analysis.

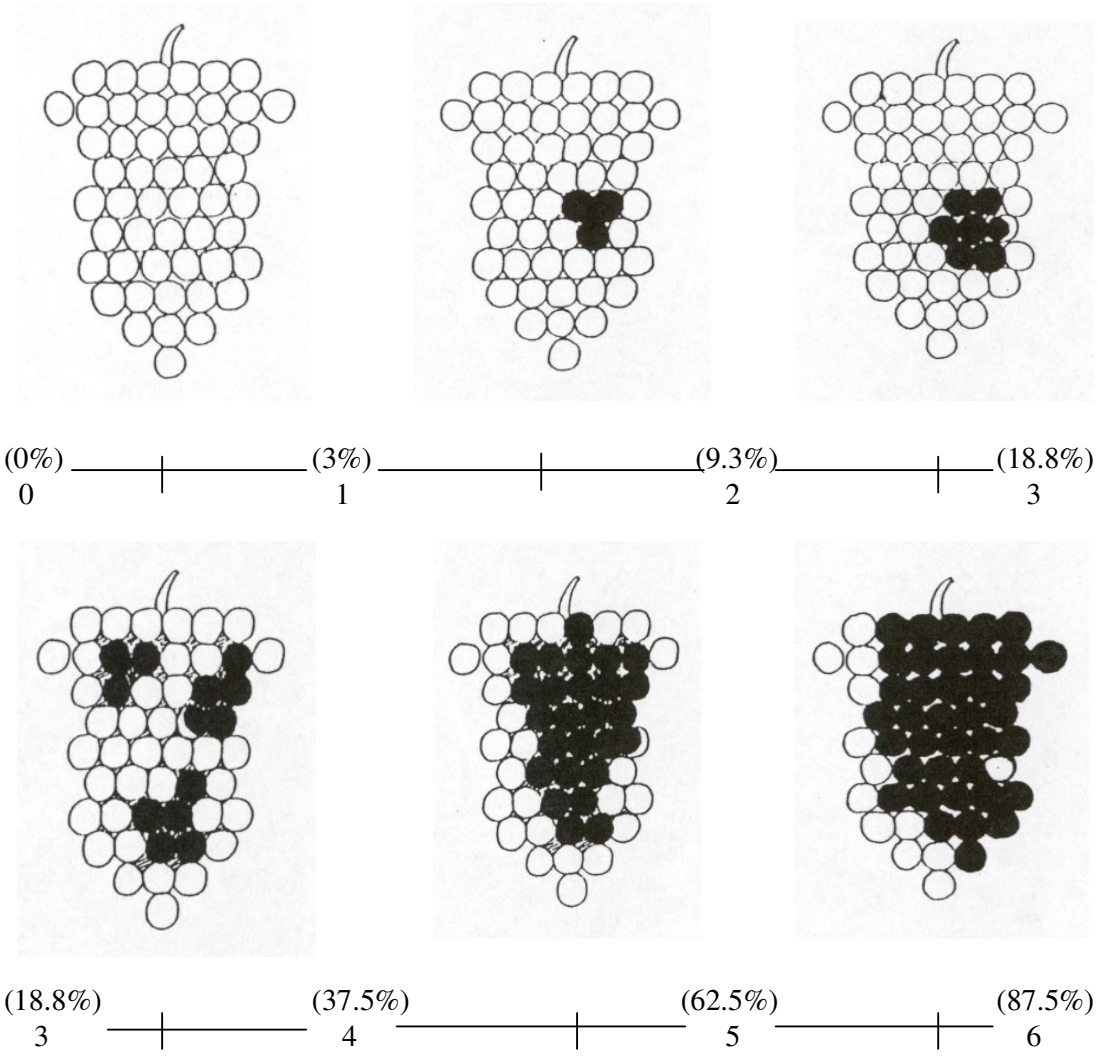


Figure 5.3: Diagrammatic key for the assessment of disease severity on grape bunches based on proportion of surface area affected (R.W. Emmett, pers. comm. 29/01/02)

5.3.2 Results

5.3.2.1 Development of Aspergillus rot in vineyards

Parameters of the bunch samples collected in 2002 are listed in Table 5.5. Counts of *A. carbonarius* on inoculated grapes were generally greater than the background level of any black *Aspergillus* spp. on uninoculated grapes (Fig. 5.4 *cf* Fig. 5.1). The concentration of *A. carbonarius* inoculum on bunches was estimated to be *ca* 10^4 cfu/g, whereas the concentration at harvest was less than 5 x 10^3 cfu/g on all the cultivars for the majority of samples. Occasionally, infection of bunches with *A. carbonarius* increased the number of propagules above the inoculated level of *ca* 10^4 cfu/g. This was observed in two samples of Chardonnay and two samples of Cabernet Sauvignon from Vineyard C, and four samples of Semillon from Vineyard A. Significant differences in *Aspergillus* count between the two vineyards and among inoculated bunches of Chardonnay, Cabernet Sauvignon and Shiraz were not observed. However, counts on Semillon bunches were significantly greater than on other cultivars in Vineyard A ($P < 0.05$). The majority of bunches spray-inoculated with *A. carbonarius* did not develop obvious bunch rot over 2 weeks or more. These symptomless bunches typically yielded few colonies, and the majority of fungal propagules were loosely associated with the skin, rather than tightly bound to the skin and in the pulp (Fig. 5.5). In contrast, samples yielding numerous black aspergilli had more propagules associated with the berry homogenate than with the berry surface.

Table 5.5: Bunch parameters at harvest, 2002

Cultivar (Vineyard)	Total soluble solids at inoculation (°Brix)	Time since inoculation (d)	Total soluble solids at harvest (°Brix)	Mean[a] berry weight (g)	Mean[a] bunch weight (g)
Chardonnay (A)	18.9	16	23.7	1.2	126.9
Semillon (A)	16.1	30	21.1	1.8	181.8
Chardonnay (C)	15.0	22	20.6	1.1	148.5
Shiraz (A)	25.0	7	23.9	1.0	88.6
Cabernet Sauvignon (A)	21.8	14	24.7	1.3	127.8
Shiraz (C)	21.4	18	24.5	9.6	127.3
Cabernet Sauvignon (C)	23.3	16	26.6	1.0	107.5

[a] means derived from weights of pooled berries (100) and bunches (50), hence standard errors could not be calculated

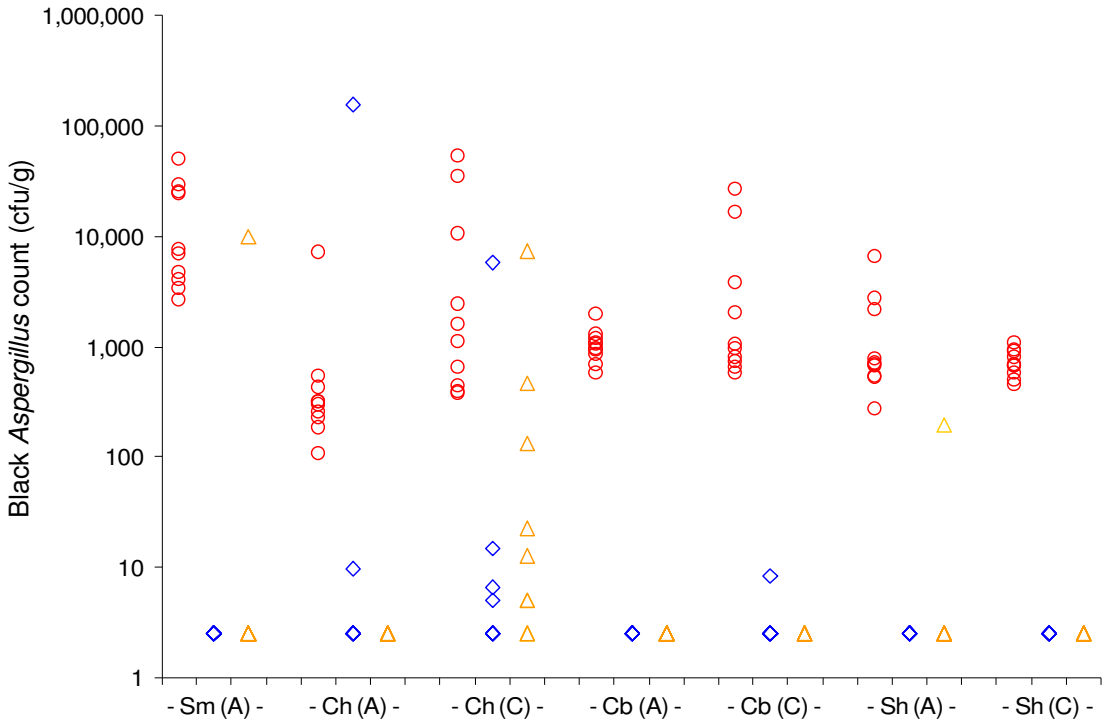


Figure 5.4: Incidence of black *Aspergillus* spp. on inoculated grapes at harvest, 2002. Each symbol represents the count (cfu/g) from five bunches (pooled); red circles = *A. carbonarius*, blue diamonds = *A. niger*, orange triangles = *A. aculeatus*. Samples in which black *Aspergillus* spp. were not detected are plotted at 2.5 cfu/g, half the limit of detection. Sm: Semillon, Ch: Chardonnay, Cb: Cabernet Sauvignon, Sh: Shiraz; vineyard in parenthesis

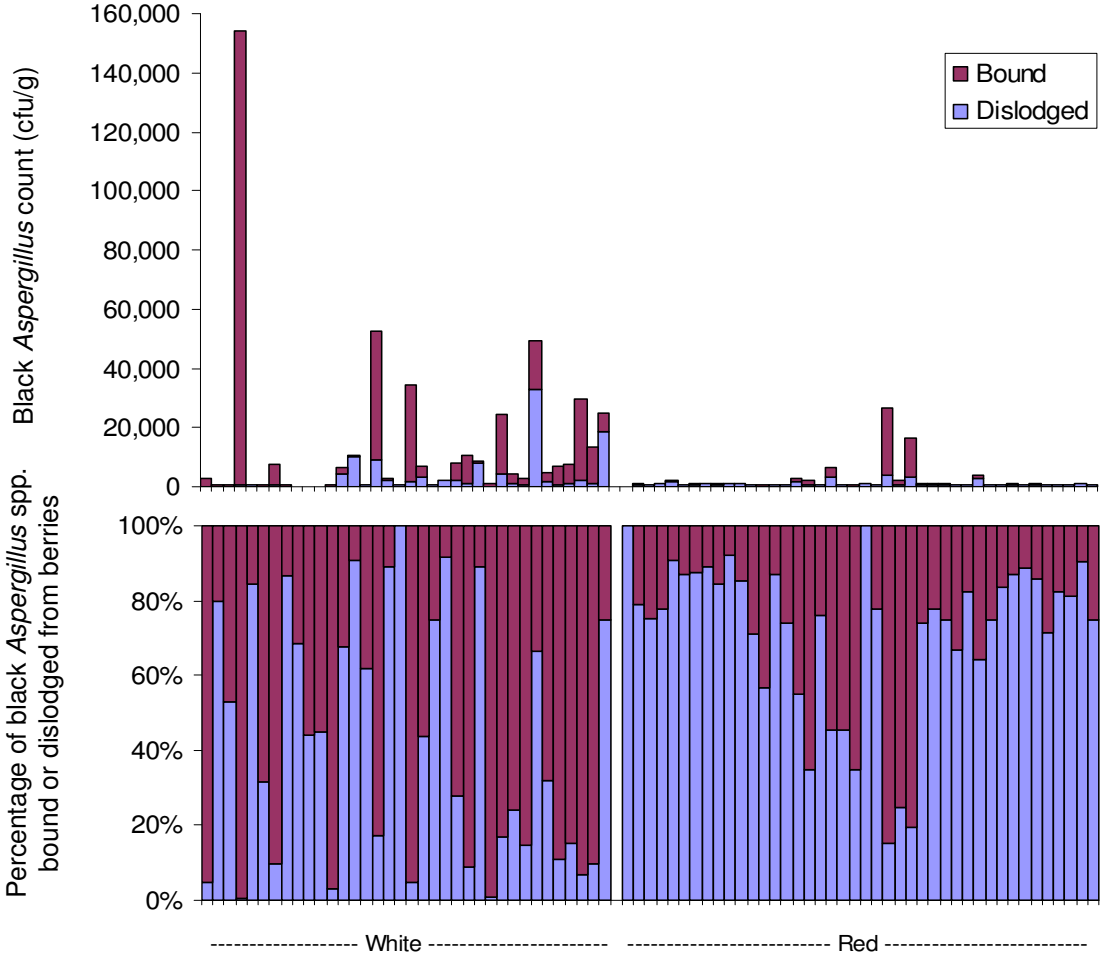


Figure 5.5: Comparison of propagules of black *Aspergillus* spp. bound to the surface or in the pulp of homogenised berries (Bound), with those dislodged from the surface of berries by vigorous shaking in water (Dislodged), for white (Chardonnay and Semillon) and red (Cabernet Sauvignon and Shiraz) cultivars. Results from uninoculated and inoculated fruit showing significant levels of black *Aspergillus* spp. are shown, significant levels being defined as more than five colonies of black *Aspergillus* spp. on the lowest dilution when plated out. Below this, differences due to a single additional colony rendered the relative proportions meaningless

5.3.2.2 Development of Aspergillus rot *in vitro*

After moist incubation of bunches for several days, visible growth of *A. carbonarius* was observed on nearly all inoculated bunches, regardless of the time and method of inoculation, method of damage and cultivar (Table 5.6a,b,d,e) . The exception to this was Chardonnay from Vineyard B, in which the incidence of infection was 40-90%. For this set of bunches, damaged berries, yeast-like odours, and the presence of vinegar flies in the vineyard suggested that yeast populations had increased. These yeasts outcompeted *A. carbonarius* in the inoculated samples and, even where *A. carbonarius* grew, sporulation was impeded (Fig. 5.6).

Figure 5.6: Yeast growth on Chardonnay berries slit with a scalpel and moist incubated at room temperature for 8 d; (a) uninoculated berries; (b) berries inoculated by immersion in a suspension of *Aspergillus carbonarius* spores after slitting, demonstrating poor sporulation by the mould

Bunches that had been spray-inoculated with *A. carbonarius* 14-30 d before harvest showed little mould, even though spores on the surface of these bunches were still viable (section 5.3.2.1). However, once these bunches were slit, profuse growth and sporulation were observed (Table 5.6b). Little variation in degree of sporulation was observed between bunches that had been spray-inoculated before slitting, and bunches that had been immersion-inoculated after slitting. The latter can be assumed to represent the maximum growth and sporulation potential of *A. carbonarius* spores in the conditions tested, as indicated by the rapid sporulation observed within 4 d.

The primary difference between bunches that had been inoculated pre- and post-slitting was in the proportion of the bunch infected, termed the “severity” of infection. Inoculation by immersion ensured greater coverage of the bunch by the spores, and

hence all the slits were infected (Fig. 5.7). In comparison, where bunches had been spray-inoculated, some slits showed no fungal growth at all, or growth of fungi other than *A. carbonarius*. This general difference between inoculation pre- and post-damage was also observed where the damage was simulated rain damage rather than slitting.

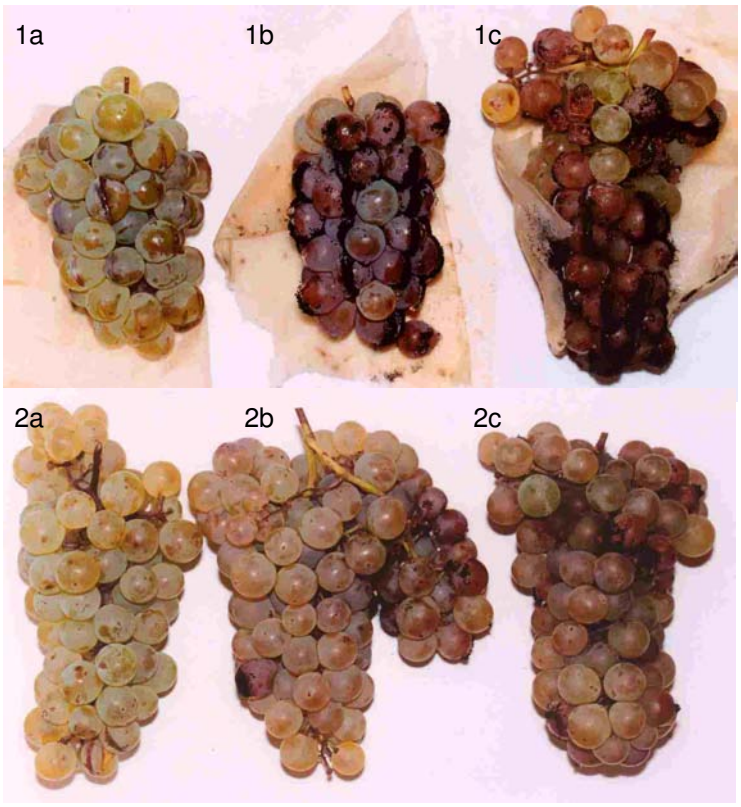


Figure 5.7: Effect of damage and inoculation with *Aspergillus carbonarius* on Chardonnay bunches moist incubated at room temperature for 8 d. Bunches were damaged (1) by slitting with a scalpel or (2) by simulated rain damage; (a) uninoculated control; (b) spray-inoculation on the vine pre-harvest; (c) immersion-inoculation post-damage

The incidence of infection with the black aspergilli was always less than 100% in sets of uninoculated bunches, even when bunches were incubated for extended periods (Table 5.6c,f). Similarly, the severity of infection in uninoculated bunches was typically less than 50% of the bunch. All three species of the black aspergilli were isolated. Other fungi commonly isolated included *B. cinerea*, *Cladosporium* spp., *Penicillium* spp. and *Rhizopus* spp.

Table 5.6a: Incidence of black *Aspergillus* infection on slit berries, inoculated with a suspension of *Aspergillus carbonarius* spores *in vitro*

Cultivar (Vineyard)	Infection with black *Aspergillus* spp.			Comment
	Growth (no. of bunches)	Sporulation (no. of bunches, degree)	Site on berry (most common site shown in **bold**)	
Chardonnay (A)	10/10	9/10, abundant 1/10, sparse	**slit**; through undamaged skin	
Semillon (A)	10/10	10/10, abundant	**slit; through undamaged skin;** pedicel	consistently vigorous sporulation
Chardonnay (C)	9/10	6/9, sparse 3/9, moderate	**slit**	berries browning due to yeast infection
Shiraz (A)	10/10	10/10, abundant	**slit; through undamaged skin**	consistently vigorous sporulation
Cabernet Sauvignon (A)	10/10	10/10, abundant	**slit**; through undamaged skin; pedicel	
Shiraz (C)	10/10	10/10, abundant	**slit**; through undamaged skin	
Cabernet Sauvignon (C)	10/10	10/10, abundant	**slit**; through undamaged skin; pedicel	

Table 5.6b: Incidence of black *Aspergillus* infection on berries spray-inoculated on the vine with a suspension of *A. carbonarius*, harvested and slit

Cultivar (Vineyard)	Infection with black *Aspergillus* spp.			Comment
	Growth (no. of bunches)	Sporulation (no. of bunches, degree)	Site on berry (most common site shown in **bold**)	
Chardonnay (A)	10/10	10/10, abundant	**slit**; through undamaged skin	
Semillon (A)	only 9 tested 9/9	9/9, abundant	**slit; through undamaged skin;** pedicel; wizened berries	consistently vigorous sporulation
Chardonnay (C)	7/10	7/7, abundant	**slit**; ranging from three berries to all slits in bunch	severe yeast infection caused berry browning
Shiraz (A)	10/10	10/10, abundant	**slit**; through undamaged skin; pedicel	
Cabernet Sauvignon (A)	10/10	10/10, abundant	**slit**; through undamaged skin; pedicel	
Shiraz (C)	10/10	8/10, abundant 2/10, moderate	**slit**; through undamaged skin	in some bunches, fewer than 10% berries infected with *A. carbonarius*
Cabernet Sauvignon (C)	10/10	10/10, abundant	**slit**; pedicel	

Table 5.6c: Incidence and severity of black *Aspergillus* infection on uninoculated berries, harvested and slit

Cultivar (Vineyard)	Incubation (d)	Infection with black *Aspergillus* spp.					Comment[b]
		No. of bunches infected	Mean severity (% area diseased ± standard error of the mean)[a]		Sporulation (no. of bunches, degree)	Site on berry (most common site shown in **bold**)	
			of infected bunches	of all bunches			
Chardonnay (A)	8	2/10	0.7 ± 0.1	0.1 ± 0.1	2/2, sparse	**slit**	*A. carbonarius* present
	14	7/10	2.7 ± 0.1	1.9 ± 0.6			
Semillon (A)	7	10/10	3.9 ± 1.4	3.9 ± 1.4	9/10, abundant 1/10, sparse	**slit**; wizened berries	
Chardonnay (C)	8	1/10	0.5	0.1 ± 0.1	1/1, moderate	**slit**	yeasts
	11	2/10	0.7 ± 0.1	0.1 ± 0.1			
Cabernet Sauvignon (A)	8	5/10	1.3 ± 0.3	0.7 ± 0.3	3/5, moderate 2/5, sparse	**slit**; wizened berries	*A. carbonarius* and *A. niger* present
	14	6/10	9.1 ± 3.9	5.4 ± 2.7			
Shiraz (A)	7	9/10	2.9 ± 0.5	2.6 ± 0.5	9/9, abundant	**slit**; through undamaged skin	*A. carbonarius* present
Cabernet Sauvignon (C)	6	3/10	1.1 ± 0.6	0.7 ± 0.4	4/4, abundant	**slit**	*A. carbonarius* and *A. niger* present
	25	9/10	9.7 ± 3.9	8.7 ± 3.6			
Shiraz (C)	6	2/10	1.2 ± 0.5	0.2 ± 0.2	2/2, moderate	**slit**	*A. aculeatus* and *A. carbonarius* present
	27	8/10	16.4 ± 5.4	13.1 ± 4.8			

[a] assessed according to Fig. 5.3

[b] conidia of black *Aspergillus* spp. from certain samples were mounted on slides and identified by microscopy. This is not indicative of their incidence on the bunches, as the number of slides examined was not proportional to the infection observed

Table 5.6d: Incidence and severity of black *Aspergillus* infection on bunches subjected to simulated rain damage, followed by inoculation with a suspension of *A. carbonarius* spores *in vitro*

Cultivar (Vineyard)	Infection with black *Aspergillus* spp.						Comment
	No. of bunches within category range[a]			Mean severity (% area diseased ± standard error of the mean)[a]	Sporulation (no. of bunches, degree)	Site on berry (most common site shown in **bold**)	
	0.5-1.5	2-3	3.5-4.5				
Chardonnay (A)	2	6	2	16.2 ± 0.1	10/10, abundant	**pedicel; through undamaged skin**	
Semillon (A)	0	5	5	26.9 ± 4.5	9/10, abundant 1/10, moderate	through undamaged skin; split; pedicel	
Chardonnay (C)	1	2	3	11.3 ± 4.5 (mean of six infected bunches, 20.3 ± 5.3)	3/6, abundant 2/6, moderate 1/6, sparse	**through undamaged skin**	severe yeast infection observed
Cabernet Sauvignon (A)	0	0	10	32.5 ± 2.8	10/10, abundant	**split; through undamaged skin**	significant splitting of berries observed
Shiraz (A)	0	3	7	29.4 ± 3.8	10/10, abundant	**through undamaged skin**; pedicel	
Cabernet Sauvignon (C)	1	7	2	16.2 ± 2.1	8/10, abundant 2/10, sparse	through undamaged skin	
Shiraz (C)	0	2	8	27.5 ± 2.3	10/10, abundant	through undamaged skin; pedicel	

[a] assessed according to Fig. 5.3

Table 5.6e: Incidence and severity of black *Aspergillus* infection on berries spray-inoculated on the vine with a suspension of *A. carbonarius* spores, harvested and subjected to simulated rain damage

Cultivar (Vineyard)	Infection with black *Aspergillus* spp.						Comment
	No. of bunches within category range[a]			Mean severity (% area diseased ± standard error of the mean)[a]	Sporulation (no. of bunches, degree)	Site on berry (most common site shown in **bold**)	
	0.5-1.5	2-3	3.5-4.5				
Chardonnay (A)	2	8	0	12.8 ± 2.0	8/10, abundant 2/10, moderate	**pedicel**; through undamaged skin	
Semillon (A)	3	7	0	9.9 ± 1.4	10/10, abundant	especially on wizened and shrivelled berries	
Chardonnay (C)	4	0	0	1.7 ± 0.7 (mean of four infected bunches, 3.8 ± 0.8)	3/4, abundant 1/4, moderate	wizened berries	
Cabernet Sauvignon (A)	0	5	5	25.7 ± 2.7	10/10 abundant	**split**	berry splitting observed
Shiraz (A)	2	3	5	20.0 ± 3.1	10/10, abundant	through undamaged skin; wizened berries; cap scar	
Cabernet Sauvignon (C)	2	8	0	9.6 ± 1.0	6/10, abundant 1/10, moderate 3/10, sparse	wizened berries; through undamaged skin	some splitting observed
Shiraz (C)	2	8	0	12.5 ± 1.6	3/10, abundant 7/10, moderate	cap scar; stem; wizened berries	some splitting observed

[a] assessed according to Fig. 5.3

Table 5.6f: Incidence and severity of black *Aspergillus* infection on uninoculated bunches, harvested and subjected to simulated rain damage

Cultivar (Vineyard)	Incubation (d)	Infection with black *Aspergillus* spp.					Comment[a]
		No. of bunches infected	Mean severity (% area diseased ± standard error of the mean)[a]		Sporulation (no. of bunches, degree)	Site on berry (most common site shown in **bold**)	
			of infected bunches	of all bunches			
Chardonnay (A)	8	0/10	0	0		not noted	
	14	4/10	2.3 ± 0.4	0.9 ± 0.4	3/3, sparse		
Semillon (A)	7	5/10	1.5 ± 0.6	0.75 ± 0.4	2/5, abundant 3/5, moderate		*A. niger* present
	14	8/10	1.7 ± 0.4	1.3 ± 0.4			
Chardonnay (C)	11	0/10	0	0			no black *Aspergillus* spp.; severe yeast infection observed
Cabernet Sauvignon (A)	8	1/10	3.9	0.4	1/1	including wizened berry	*A. aculeatus* and *A. carbonarius* present
	14	4/10	1.8 ± 0.7	0.7 ± 0.4			
Shiraz (A)	8	2/10	0.9 ± 0.1	0.2 ± 0.1	2/2	not noted	*A. niger* present
	29	6/10	5.9 ± 1.4	3.5 ± 1.2			
Cabernet Sauvignon (C)	6	1/10	0.8	0.1	1/1, sparse	aborted wizened berries	
	25	5/10	1.7 ± 0.4	0.8 ± 0.3			
Shiraz (C)	6	2/10	2.0 ± 0.3	0.4 ± 0.3	1/2, not noted 1/1, sparse	aborted wizened berries	*A. niger* present
	27	5/10	2.3 ± 0.8	1.2 ± 0.6			

[a] assessed according to Fig. 5.3

For harvested bunches subjected to simulated rain damage by submersion in distilled water for 24 h, splitting was observed in more than 22 of 30 Cabernet Sauvignon bunches from Vineyard A. Splitting was not observed in Chardonnay and Shiraz from Vineyard A, and was observed in less than 3 of 30 bunches for other cultivar sets, including Cabernet Sauvignon from Vineyard C, where bunches contained the greatest total soluble solids (Table 5.5). Splitting in Shiraz bunches was infrequent, because several of the berries had shrivelled slightly, hence, were not at maximum turgor.

A direct comparison between white cultivars in terms of *in vitro* infection with *A. carbonarius* was not possible since Semillon was not grown in Vineyard C. However, it was observed that the incidence and severity of infection in Semillon from Vineyard A was worse than Chardonnay from both vineyards for nearly all the bunch treatments. During experimentation, it was also noted that Semillon berries were loosely attached to the pedicel, the pedicel being a common site of prolific sporulation (Table 5.6a,b,d). Wizened berries, common in Semillon, were another site of sporulation (Fig. 5.8).

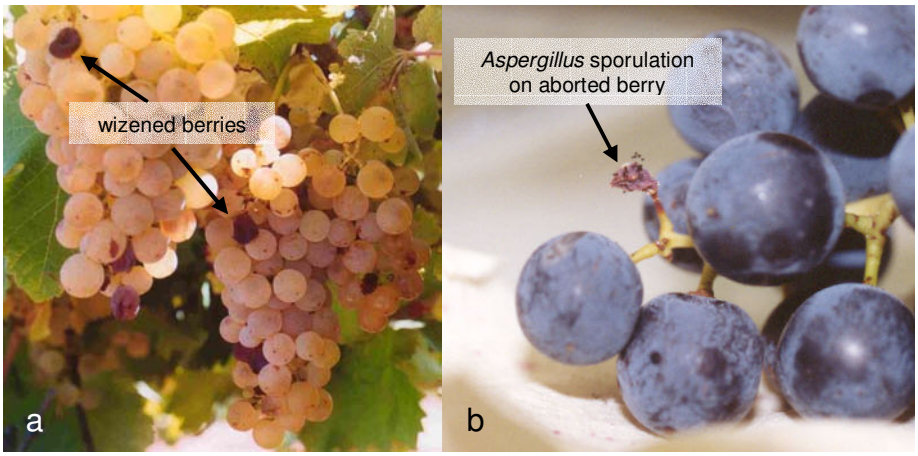


Figure 5.8: Wizened berries (a) in Semillon bunches on the vine and (b) growth of black *Aspergillus* spp. on aborted and wizened Cabernet Sauvignon berry in moist incubation conditions

Among the red cultivars examined, the most vigorous growth of *A. carbonarius* was observed in inoculated bunches of Cabernet Sauvignon from Vineyard A (Fig. 5.9). The splitting induced in these bunches by submersion was also the worst among the cultivars examined. However, no consistent differences were observed between Cabernet Sauvignon and Shiraz from both Vineyards A and C.

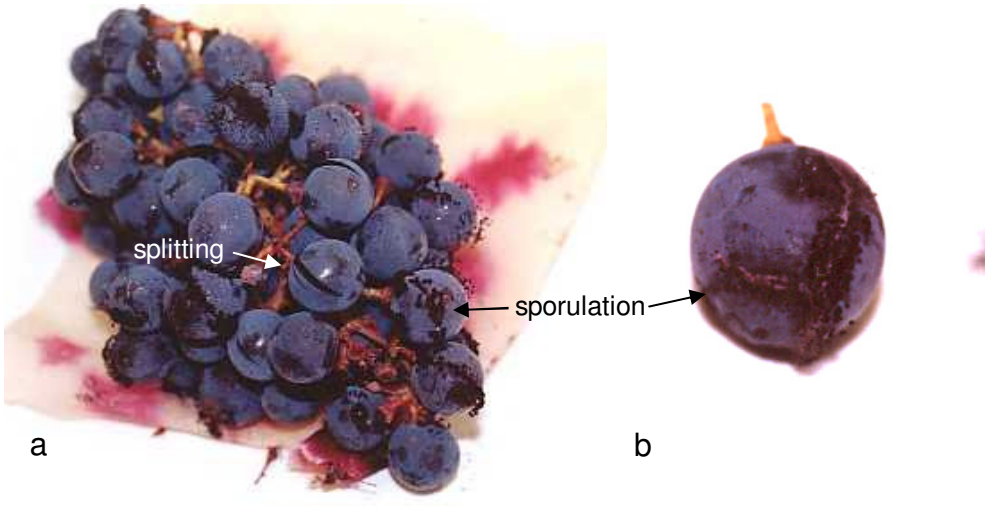


Figure 5.9: Berry splitting and fungal growth (a) on Cabernet Sauvignon grapes spray-inoculated with *Aspergillus carbonarius* on the vine pre-harvest and subjected to simulated rain damage, followed by moist incubation at room temperature for 8 d; (b) splitting of, and sporulation on, a single berry

5.4 Survival and growth of Aspergillus carbonarius on wine grapes before harvest

5.4.1 Methods

This trial was conducted in 2003 and 2004 in Vineyard D (Table 5.1). Three rows were used per cultivar (Chardonnay and Shiraz). A spore suspension at 2-4 x 10^5 spores/mL of *A. carbonarius* strains FRR 5682 and FRR 5683, previously isolated from Vineyard D, was prepared as described in section 2.3.

Bunches on both sides of the vine were inoculated by immersion in 1 L of spore suspension contained within a polyethylene bag. The same inoculum was used for up to 40 bunches without a detectable decrease in the spore concentration (assessed by plating on DRBC as described in section 2.1, data not shown). Bunches in each row were inoculated at pre-bunch closure (berries green and pea size), veraison and 11-24 d pre-harvest. After the inoculum had dried, bunches were sampled to quantify the initial *A. carbonarius* spore load. To assess the spore load over time, inoculated bunches were sampled at each of the subsequent stages and at harvest. A sample comprised two bunches combined into a single sample in 2003, and single bunches in 2004. Six samples were collected per replicate row, resulting in 18 samples for analysis at each stage. A set of uninoculated samples was also collected at every stage, described in section 5.2.1.2.2.

Samples were homogenised and black *Aspergillus* spp. enumerated as described in section 5.2.1.2.2. The number of viable propagules of *A. carbonarius* was expressed as cfu/berry to facilitate comparison of samples of different berry maturity and weight.

A similar trial was conducted in 2003 with Semillon vines, to examine the effect of two fungicide spray programs on *A. carbonarius* spore survival and development of Aspergillus rot. The trial was replicated over three blocks. In one section of each block, rows were sprayed with the grower's standard program and, in another section, sprays were substituted with those provided by Syngenta Crop Protection Pty Ltd (Switzerland) (Appendix E). As a control, sprays for two rows were omitted after flowering. Inoculation and sampling were performed as described for the trials with cultivars Chardonnay and Shiraz, with the amendment that the veraison sample was omitted, that is, samples were taken at pre-bunch closure, 21 d pre-harvest and at harvest.

Logarithmically transformed data (cfu/berry) for the Chardonnay and Shiraz trial, and percentage of berries infected per bunch for the Semillon trial, were analysed as described in section 2.6.

5.4.2 Results

All colonies of *A. carbonarius* recovered from inoculated bunches by this technique were assumed to be solely the result of inoculation, as the natural incidence of *A. carbonarius* (Table 5.3) was deemed unlikely to inflate the colony count. Fungi other than *A. carbonarius* were seldom isolated from inoculated bunches, as they were present at levels below that of the inoculum. Bunch and berry weights are listed in Table 5.7, and were greater in 2004 than in 2003.

Four-way ANOVA (factors: year, time of inoculation, time of harvest, cultivar) of data demonstrated a significant three-way interaction between year x time of inoculation x time of harvest ($P < 0.001$). Although cultivar was not present in this higher order interaction, significant two-way interactions were demonstrated for cultivar x time of inoculation, and cultivar x time of harvest. The presence of so many significant interactions in this trial precludes comment on the statistical significance of main effects, such as cultivar, when tested independently. However, the following

trends were noted. A decrease in counts of *A. carbonarius* was consistently observed between pre-bunch closure and veraison (Fig. 5.10a), but from veraison to pre-harvest, mean *A. carbonarius* counts increased (Fig. 5.10a,b). Sporulation on berries was visible in a proportion of these bunches (data not shown). Abundant sporulation within bunches was often associated with foci of insect damage (Fig. 5.11).

Mean counts of *A. carbonarius* between pre-harvest and harvest stages did not show consistent trends, with increases observed in seven cases, and decreases or no change in five cases (Fig. 5.10a,b,c). Strong year and cultivar effects were observed during this stage. The strongest effect of year was evident in the results for Chardonnay in 2003 and 2004. In 2004, *A. carbonarius* counts increased between veraison and harvest, regardless of time of inoculation, and these increases were greater than those observed in 2003. Chardonnay bunches comprised 26% more berries in 2004 than in 2003, and were 44% heavier at harvest (Table 5.7). Drought conditions preceding the growing season for the 2003 vintage led to sparse canopies and smaller bunches in both Chardonnay and Shiraz. Differences between these two cultivars were observed from pre-harvest till harvest in 2004: counts on Chardonnay bunches always increased from pre-harvest till harvest, whereas counts on Shiraz increased to a lesser degree or even decreased (Fig. 5.10b). It was noted that during this period, Chardonnay vines were sprayed with potassium metabisulphite, whereas Shiraz vines were sprayed with procymidone (Appendix E, Table E.2), a compound which severely restricted growth of *A. carbonarius* when incorporated into CYA at 10 ppm (data not shown). No sprays were applied between 11-16 d pre-harvest and harvest in 2003 (Appendix E, Table E.1).

Whereas the mean counts of *A. carbonarius* at pre-harvest and harvest were generally greater than at veraison, it is important to note the presence of some samples with counts lower than 100 cfu/berry at both pre-harvest and harvest (Fig. 5.10). These represent bunches in which the spore load decreased from the initial inoculum of 1,000-10,000 cfu/berry.

The overall trend for increased counts at pre-harvest and harvest was also observed in the Semillon trial for bunches with and without fungicides applied after flowering (Fig. 5.12). Sprays applied after flowering appeared to inhibit the increase in fungal

infection slightly compared with the unsprayed control; however, the differences in severity of rot measured as percentage of berries infected (Fig. 5.13) were not significant. From inoculation at pre-bunch closure until harvest, the sprays applied in the program designed by Syngenta Pty Ltd (Switzerland) and in the grower's standard program were virtually identical. The residual effect of fungicides in the Syngenta program applied until flowering (Appendix E, Table E.1) appeared to suppress fungal growth from pre-bunch closure until pre-harvest, whereas this suppression was not observed for bunches that received the grower's standard program (Fig. 5.12a). This suppression did not continue until harvest, and counts from bunches that received the Syngenta program were slightly greater than those from bunches that received the grower's standard program. Delfin® WG (*Bacillus thuringiensis* var. *kurstacki*; Thermo Trilogy, Columbia, USA) was the only treatment applied between pre-harvest and harvest, and this was applied to vines under both the Syngenta and standard programs.

Table 5.7: Mean bunch and berry weights of grapes at designated growth stages, 2003 and 2004

Stage	Weight (g)	Chardonnay		Shiraz		Semillon
		2003	2004	2003	2004	2003
Pre-bunch closure	Bunch	29.2 ± 1.7[a] n = 36	49.5 ± 4.0 n = 24	43.5 ± 2.7 n = 36	56.1 ± 4.6 n = 24	60.8 ± 2.7 n = 54
	Berry	0.33[b]	0.44 ± 0.05 n = 3[b]	0.28	0.34 ± 0.01 n = 3	0.40
Veraison	Bunch	43.3 ± 2.0 n = 54	80.7 ± 3.9 n = 42	69.7 ± 2.8 n = 54	138.7 ± 9.3 n = 42	not assessed
	Berry	0.52	0.66 ± 0.07 n = 3	0.51	0.88 ± 0.06 n = 3	not assessed
Pre-harvest	Bunch	81.3 ± 3.2 n = 72	117.4 ± 5.4 n = 60	131.3 ± 4.7 n = 72	148.4 ± 7.4 n = 60	159.3 ± 7.9 n = 81
	Berry	0.84	1.03 ± 0.06 n = 3	1.02	1.20 ± 0.04 n = 3	0.80
Harvest	Bunch	81.8 ± 3.6 n = 72	118.2 ± 6.5 n = 60	127.7 ± 4.1 n = 72	143.2 ± 8.6 n = 60	181.0 ± 7.6 n = 81
	Berry	0.88	1.06 ± 0.17 n = 3	0.90	0.94 ± 0.07 n = 3	1.39
No. of berries per bunch (mean calculated over no. of growth stages)		91 ± 3 n = 4	115 ± 3 n = 4	141 ± 6 n = 4	149 ± 9 n = 4	161 ± 20 n = 3

[a] standard error of the mean derived from the no. of samples denoted by "n"

[b] In 2003, mean berry weight calculated by counting the number of berries in two bunches weighed as a single sample. In 2004, mean berry weight calculated by counting the number of berries in three individual bunches weighed separately

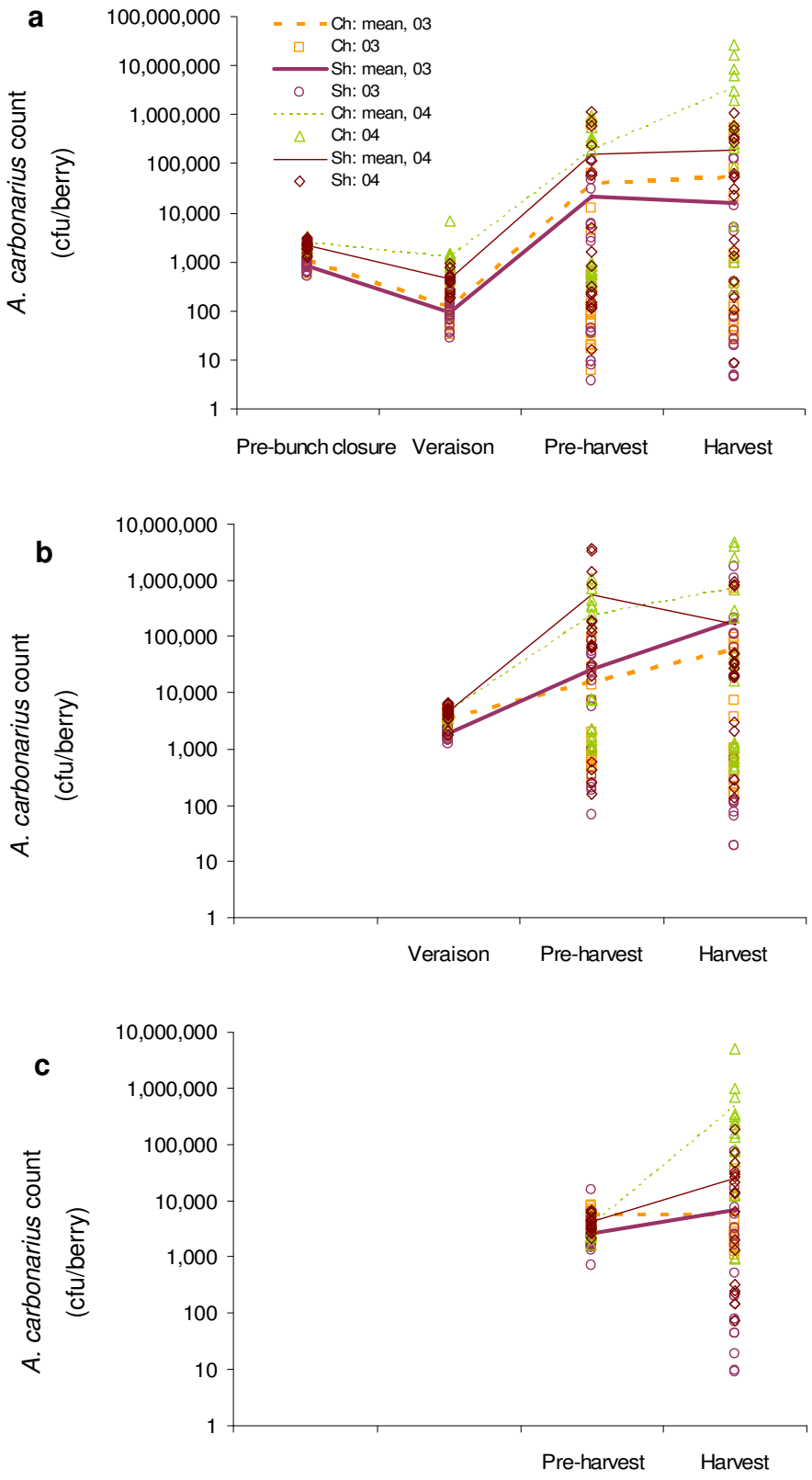


Figure 5.10: Counts of *Aspergillus carbonarius* in 2003 and 2004 following immersion-inoculation of grapes at (a) pre-bunch closure, (b) veraison and (c) pre-harvest. Symbols show individual sample results, means of six samples denoted by lines

Figure 5.11: Insect casing indicative of insect damage, a focus for berry rot developing over 24 d in a Chardonnay bunch inoculated by immersion in *Aspergillus carbonarius* spore suspension at veraison

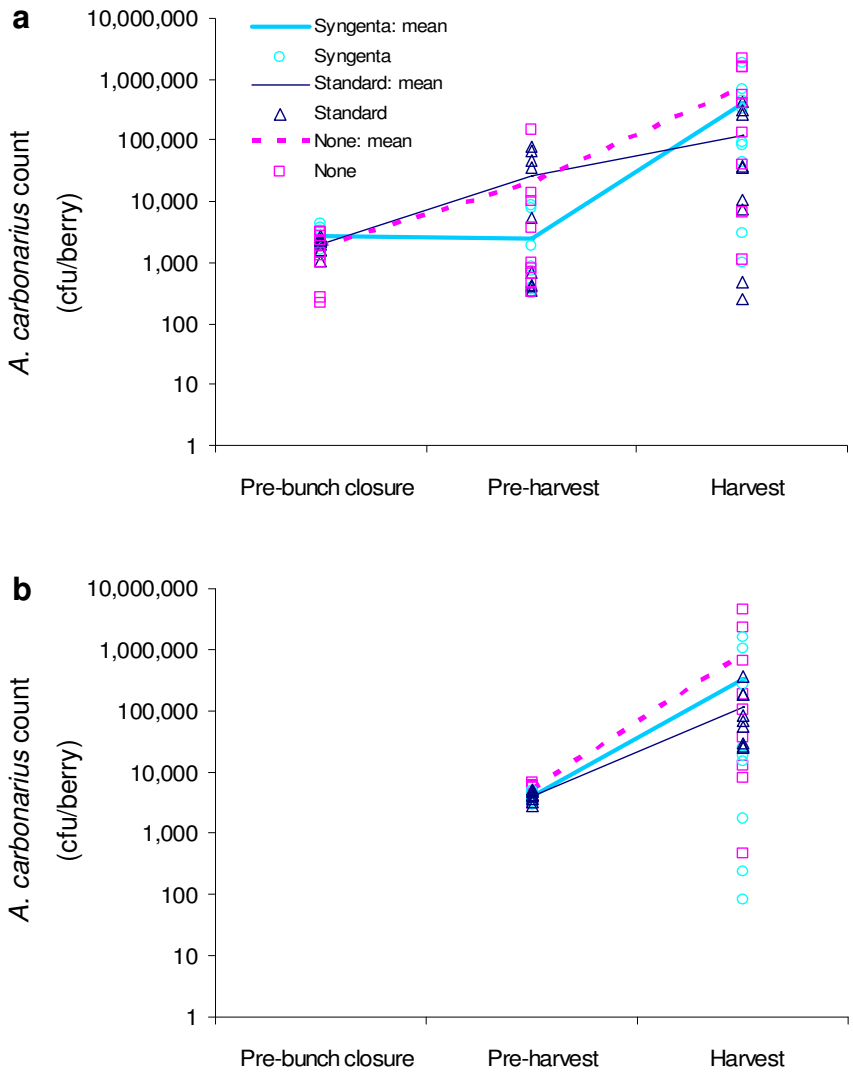


Figure 5.12: Effect of Syngenta Crop Protection Pty Ltd (Switzerland) and grower's standard spray programs on survival of *Aspergillus carbonarius* spores immersion-inoculated onto Semillon bunches at (a) pre-bunch closure and (b) pre-harvest, and subsequent growth. Sprays were withheld after flowering on control vines (None). Symbols show individual sample results, means of nine samples denoted by lines

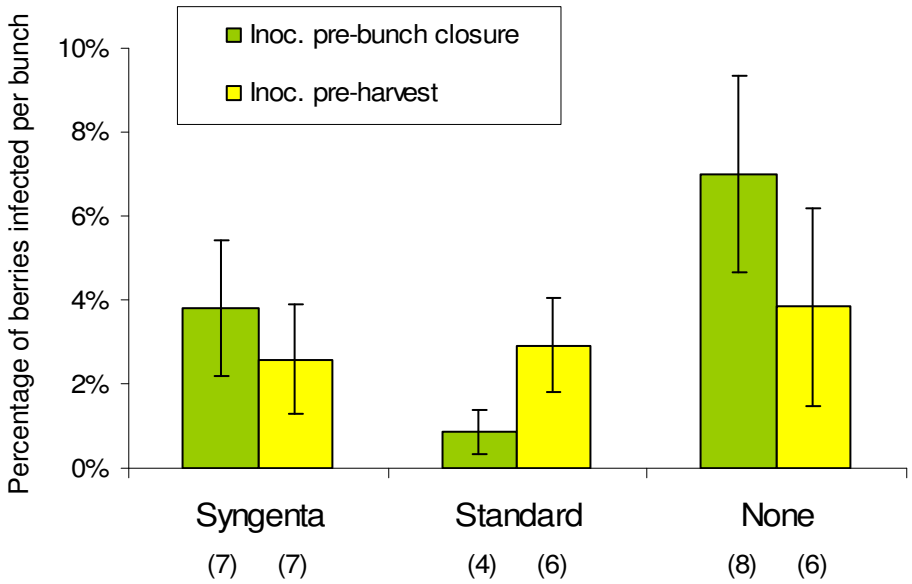


Figure 5.13: Effect of Syngenta Crop Protection Pty Ltd (Switzerland) and grower's standard spray programs on development of bunch rot caused by *Aspergillus carbonarius* spores immersion-inoculated onto Semillon bunches at pre-bunch closure and pre-harvest. Sprays were withheld after flowering on control vines (None). Severity of rot at harvest based on the number of visibly mouldy berries expressed as a percentage of the estimated total number of berries per bunch. Error bars denote the standard error of the mean of 18 bunches. Numbers in parenthesis beneath the *x*-axis denote the number of bunches displaying visible mould

5.5 Effect of damage and berry maturity on Aspergillus rot and ochratoxin A formation in Semillon bunches

5.5.1 Methods

This trial was conducted in 2004 with three rows (replicates) of Semillon vines in Vineyard D (Table 5.1). Approximately one month before harvest, bunches were inoculated by immersion in a suspension of *A. carbonarius* spores as described in section 5.4.1. Inoculation of additional samples was performed at 20 and 10 d pre-harvest. Each set of samples comprised six bunches, namely, two inoculated bunches; two inoculated bunches in which the majority of berries were damaged by puncturing with a pin; one control bunch immersed in distilled water; and one control bunch immersed in distilled water and in which berries were punctured with a pin. Sufficient sets of samples were inoculated such that one set could be collected at each 10 d interval until harvest.

At each sampling point, bunches were weighed and examined for berry discoloration typical of *Aspergillus* infection. The number of mouldy berries was noted and the

bunch given a score for mould severity (Fig. 5.3). Berries were carefully removed from the bunch with scissors to examine the extent of internal rot. The number and weight of infected berries was noted. Each bunch was stored at -20 °C until analysis for OA as described in section 2.5.

Ten days before harvest, a total of four additional bunches were inoculated by immersion in a suspension of *A. carbonarius* spores as described in section 5.4.1. Harvested bunches were stored at 1 °C until examination as described above, with the following modification. Berries were visually sorted into four categories; namely, healthy berries, berries showing slight discolouration of unknown origin, plump berries partially discoloured in the manner typically associated with infection with *A. carbonarius*, and berries heavily infected with *A. carbonarius* as indicated by complete discolouration of the berry, with or without visible sporulation of *A. carbonarius* (Fig. 5.14). For every bunch, each category of berries was analysed for OA as described in section 2.5. The rachides from these bunches were also pooled and analysed for OA by the same method.

Figure 5.14: Four categories of berries from a single Semillon bunch immersion-inoculated with a suspension of *Aspergillus carbonarius* spores 10 d before harvest; (a) healthy berries, (b) partially discoloured berries, (c) partially discoloured berries showing some evidence of infection with *A. carbonarius*, and (d) discoloured berries heavily infected with *A. carbonarius*

The following variables were examined by pairwise comparison: bunch weight, number of days between inoculation and sample collection, weight of berries displaying visible infection with black *Aspergillus* spp., number of berries displaying visible infection and total OA per bunch. Multiple linear regression was also performed with the response variable total OA per bunch. The backward elimination method was used, with elimination of insignificant variables at $P < 0.05$ and omitting five points of high leverage.

5.5.2 Results

Bunches that were not inoculated with *A. carbonarius* spore suspension did not show visible signs of Aspergillus rot, even when berries were damaged (Fig. 5.15a). Aspergillus bunch rot (Fig. 5.15c) was observed in 55 of 72 inoculated bunches. All bunches inoculated at 30, 20 and 10 d before harvest that were damaged by puncturing with a pin developed Aspergillus rot, whereas some bunches that were inoculated with *A. carbonarius* spores but undamaged did not display symptoms of infection (Fig. 5.15b). The severity of rot was greater when estimated by visual inspection than by weight of mouldy berries - this overestimation occurred in 46 of 55 infected bunches.

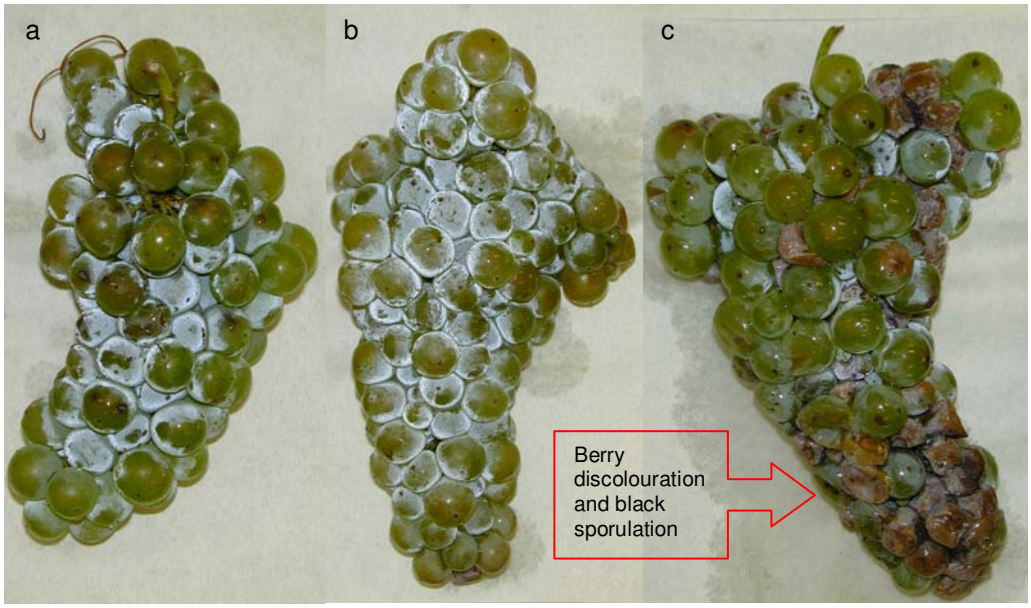


Figure 5.15: Development of Aspergillus rot in Semillon bunches inoculated by immersion in *Aspergillus carbonarius* spore suspension 20 d pre-harvest. Control (a) immersion in distilled water and damaged by puncturing with a pin; (b) immersion in spore suspension; (c) immersion in spore suspension and damaged by puncturing with a pin

Severity of Aspergillus rot (Fig. 5.16a), as well as incidence, was increased by damage to inoculated bunches. Susceptibility to rot increased towards harvest, as rot was more severe in bunches inoculated at 20 and 10 d pre-harvest than at 30 d preharvest and, in undamaged bunches, rot developed when inoculated 10 d pre-harvest. Rot appeared to be self-limiting within the bunch, as, after the initial infection (*ca* 5% and 23% of bunches inoculated at 30 d and 20 d pre-harvest, respectively), the proportion of infected berries did not continue to increase at the same rate over the course of the trial.

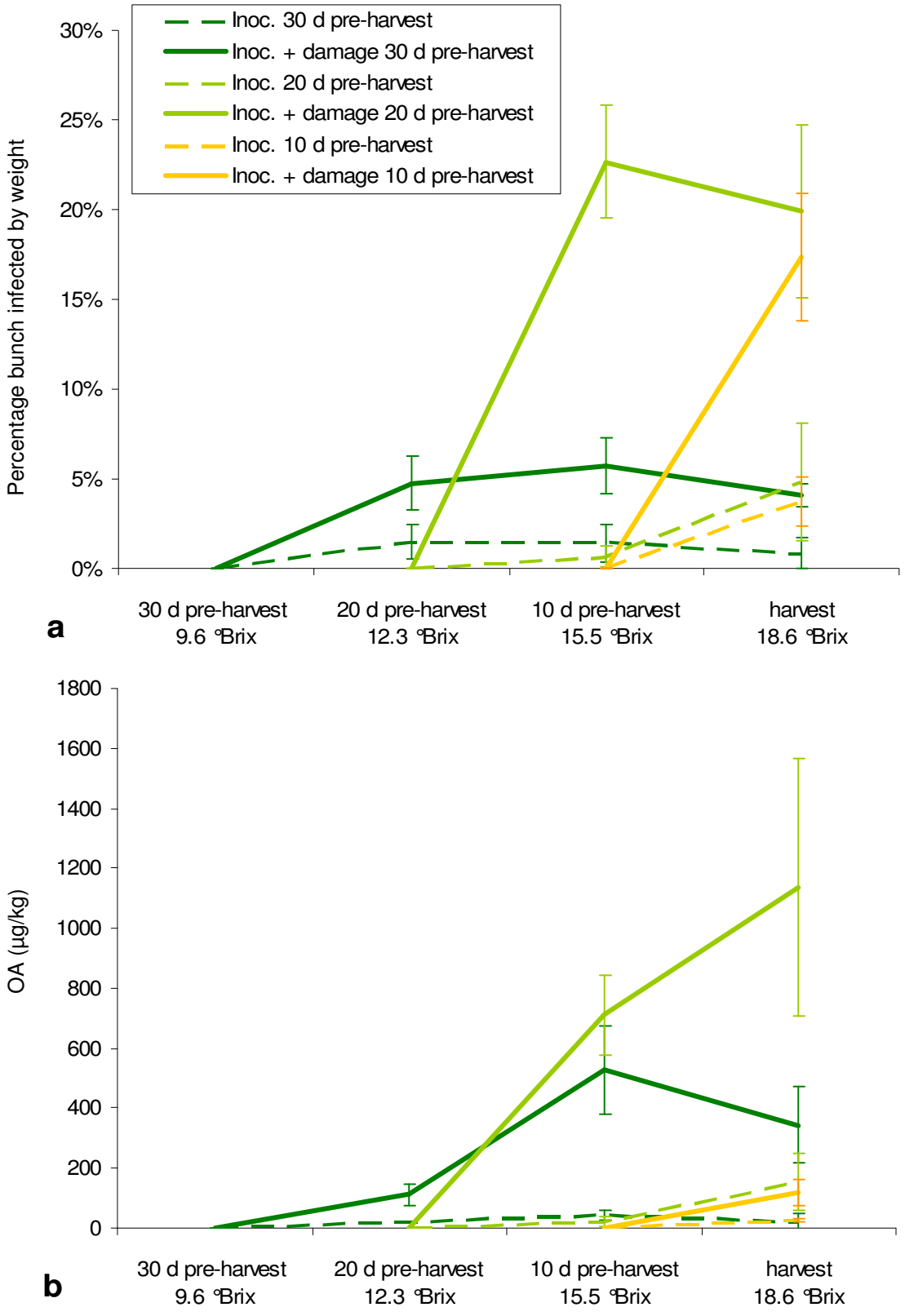


Figure 5.16: Severity of infection (a) and (b) ochratoxin A in Semillon bunches inoculated by immersion in *Aspergillus carbonarius* spore suspension before harvest, with and without berry damage. Infection and OA were assigned zero values at inoculation. Error bars denote the standard error of the mean of six bunches

At 20 d and 10 d pre-harvest and at harvest, OA was assayed for a representative selection of control bunches that had not been inoculated with *A. carbonarius* spores, including both undamaged and damaged bunches. OA was not detected in five of twelve bunches, and was present only at low concentrations in the remaining seven (mean ± standard error of the mean 0.63 ± 0.34 µg/kg, maximum 2.26 µg/kg).

Nineteen inoculated, undamaged bunches did not display clear indications of berry infection with *A. carbonarius*, that is, no berries with visible infection were segregated and weighed. Of these, OA was not detected in two bunches. An additional 10 bunches were contaminated at concentrations below 0.5 µg/kg (mean 0.2 µg/kg) and a single bunch contained 12 µg/kg OA. The remaining six bunches were contaminated within the range 0.3-22 µg/kg, and it was noted that discoloured berries, initially not attributed to infection with *A. carbonarius*, had been observed in these bunches.

These relatively low OA concentrations in inoculated, undamaged bunches resulted in lower overall means than those of inoculated, damaged bunches (Fig. 5.16b). As observed for severity of Aspergillus rot (Fig. 5.16a), the period between 20 and 10 d before harvest appeared to be optimum for the formation of OA in bunches inoculated and damaged at 30 and 20 d pre-harvest. For bunches inoculated and damaged at 20 d pre-harvest, OA increased until harvest. Bunches inoculated and damaged at 10 d pre-harvest did not reach high OA concentrations despite the rapid development of rot (Fig. 5.16b *cf* 5.16a).

Pairwise comparison with total OA per bunch yielded correlations of 0.86 and 0.71 with number and weight of *Aspergillus*-infected berries, respectively. The relationship between total OA per bunch and the number of infected berries is shown in Fig. 5.17. The model generated by multiple linear regression, with variables, number and weight of *Aspergillus*-infected berries (Table 5.8), accounted for 82% of the variance.

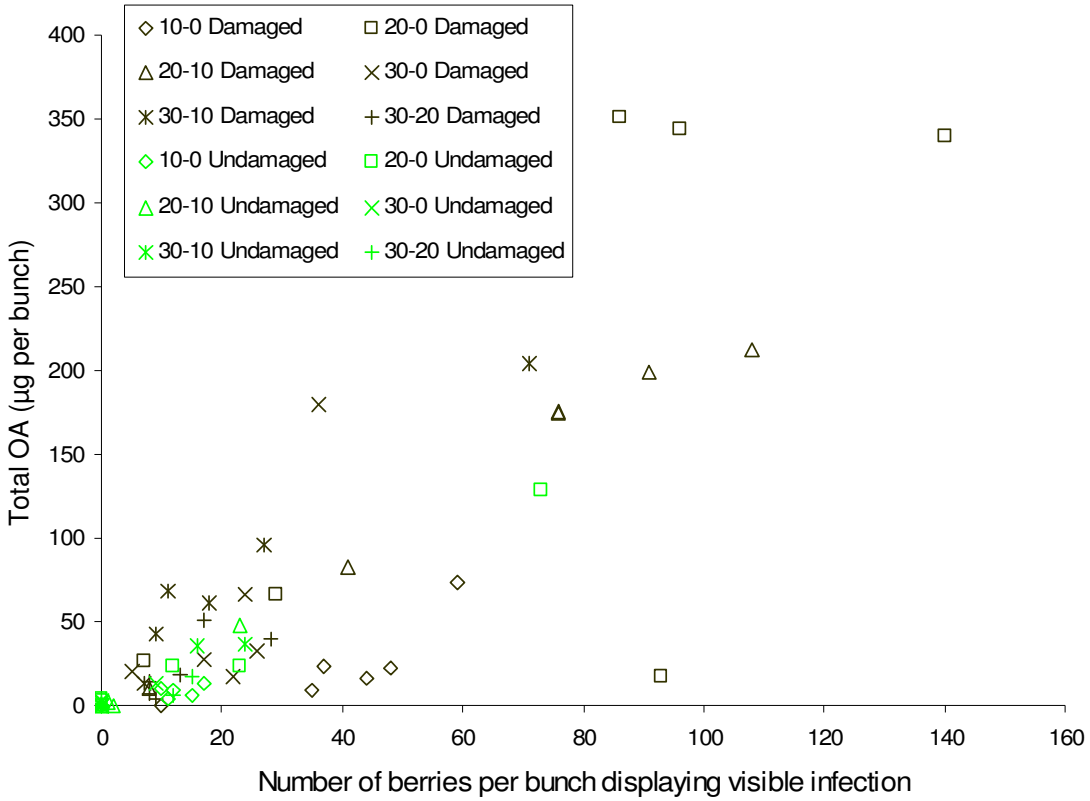


Figure 5.17: Relationship between ochratoxin A in Semillon bunches inoculated by immersion in *Aspergillus carbonarius* spore suspension before harvest and number of berries displaying visible infection with black *Aspergillus* spp. In the figure legend, the first and second numerals refer to the inoculation and sample collection times, respectively, in days before harvest. "Damaged" refers to bunches in which berries were punctured with a pin after inoculation

Table 5.8: Multiple linear regression model describing the total ochratoxin A per bunch (µg) for Semillon bunches inoculated by immersion in *Aspergillus carbonarius* spore suspension during the 30 d before harvest, 2004

Source of variation	Degrees of freedom	Mean of squares	F probability
Regression	2	182099	< 0.001
Residual	69	1674	
Total	71	6757	
Estimate of parameters		Standard error	t probability
Constant	-1.83	3.4	0.592
No. of visibly infected berries	3.768	0.317	< 0.001
Weight of visibly infected berries (g)	-2.974	0.495	< 0.001

When berries from inoculated bunches were segregated and analysed separately, OA contamination was observed to increase with severity of mould, from healthy and discoloured berries to those displaying evidence of slight and severe mould; the mean OA concentrations (± standard error of the mean of four bunches) were 1.4 ± 0.1 µg/kg, 3.6 ± 0.6 µg/kg, 27.4 ± 9.5 µg/kg and 1890 ± 194 µg/kg for the four categories of berries. Thus the berries displaying severe mould were the source of nearly all the OA in the bunch, even though these berries comprised less than 20% of the bunch weight (Fig. 5.16). OA in the single sample of pooled rachides was estimated to be 93 µg/kg.

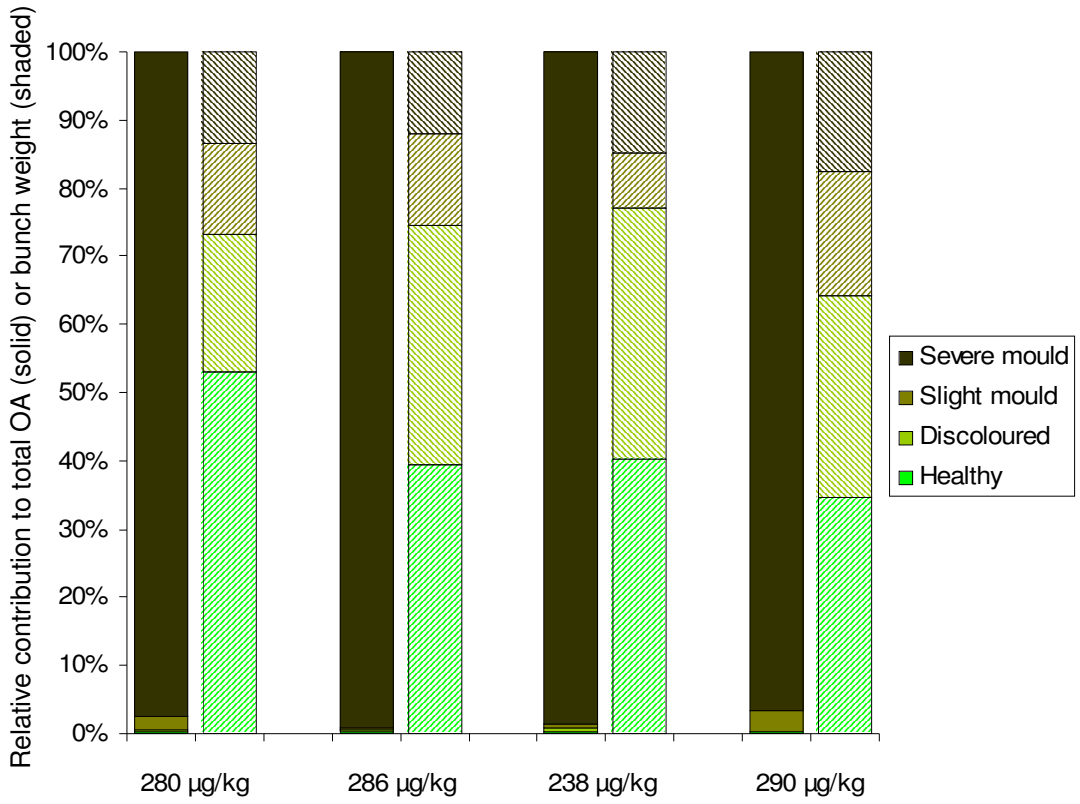


Figure 5.18: Ochratoxin A in berries visually sorted from four Semillon bunches inoculated by immersion in a suspension of *Aspergillus carbonarius* spore suspension 10 d before harvest. The relative OA contribution weight of each berry category is given in the left column of each pair, and the relative weight contribution of each category, in the right column (diagonal stripes). The estimated OA concentration for each bunch was calculated based on the OA concentration and weight of each berry category, and is given on the *x*-axis

5.6 Discussion

5.6.1 Occurrence of black *Aspergillus* spp. on bunches and development of Aspergillus rot

The incidence of *A. niger* occurring naturally on grape bunches was typically greater than that of both *A. carbonarius* and *A. aculeatus* (section 5.1.1), as noted for grapes grown in Europe and South America, and for soils from Australian viticultural regions (discussed in sections 3.1.1 and 3.4.1). Black *Aspergillus* spp. were not universally associated with all berries of all bunches, as indicated by incidence and severity of infection below 100% and 50%, respectively, observed for a range of cultivars with induced berry damage (Table 5.6c,f). These fungi were generally present on the berry surface without invading the pulp, hence were isolated less frequently after surface sterilisation of the berries (Table 5.2). Magnoli et al. (2003), Bau et al. (2005a) and Serra et al. (2005b), likewise, reported infrequent isolation of black *Aspergillus* spp. from surface sterilised berries or from seeds plated aseptically.

No obvious association between cultivar and incidence of black *Aspergillus* spp. was observed; however, counts of these fungi were greater on white cultivars examined in 2002 than on red cultivars (Fig. 5.1). This may have been an effect of climate rather than cultivar, as cool weather extended the ripening time for white, but not red, cultivars during that season. Delayed ripening may, in turn, have increased the probability of fungal contamination from vineyard soil and the potential for berry damage. The increased counts of black *Aspergillus* spp. observed on Chardonnay grapes from Vineyard C were likely to have been a result of vinegar flies (*D. melanogaster*) spreading fungal spores throughout damaged grapes left on the vine for extended periods during ripening (Buchanan and Amos, 1992); other insect vectors have also been implicated in the spread of *A. carbonarius* (Lataste et al., 2004). Battilani et al. (2005b), likewise, noted that seasonal variations affecting ripening periods overrode associations observed *in vitro* between cultivar and infection with *A. carbonarius.*

Although significant differences in incidence of *Aspergillus* were not observed between Vineyards A and C, differences were observed for certain cultivars. Counts of black *Aspergillus* spp. appeared to be greater on Chardonnay and Shiraz grapes

grown in Vineyard C under overhead irrigation and with frequent soil tillage, than on those cultivars grown in Vineyard A under drip irrigation and with minimal tillage (Fig. 5.1). This may be explained by the more frequent isolation of *A. carbonarius* from vineyard soil that was tilled regularly (Kazi et al., 2004) and from grapes grown under overhead irrigation (N. Bellí, pers. comm. 23/09/03). However, no clear differences were observed between incidence and severity of infection by black *Aspergillus* spp. for Cabernet Sauvignon grown in either vineyard.

The frequency of isolation of black *Aspergillus* spp. increased with berry maturity in 2003 and 2004 (Table 5.3), as reported by other authors (section 5.1). Other fungi commonly isolated from grapes included *Alternaria* spp., *Cladosporium* spp. and yeasts, also reported by Da Rocha Rosa et al. (2002), Sage et al. (2002), Magnoli et al. (2003), Bellí et al. (2004c), Sage et al. (2004) and Bau et al. (2005a). Data from the Hunter Valley trial in 2004 suggested that yeasts increased with berry maturity (Fig. 5.2), supporting the findings of Bellí et al. (2005a) and Duncan et al. (1995) and, at harvest, yeasts were more abundant than the other two genera, a finding reported by Da Rocha Rosa et al. (2002) and Duncan et al. (1995) for grapes from certain areas but not others. In contrast, Bellí et al. (2005a) reported that *Alternaria* spp. infected the greatest proportion of berries in Spanish grapes, followed by yeasts, whereas *Cladosporium* spp. were isolated infrequently. For grapes from the Hunter Valley trial, *Cladosporium* spp. were generally more abundant than *Alternaria* spp., a finding which mirrors data of Abrunhosa et al. (2001) for Portuguese grapes and Duncan et al. (1995) for Californian grapes. However, in other studies on Portuguese and Spanish grapes, little difference in the extent of infection with *Alternaria* spp. and *Cladosporium* spp. was reported (Bau et al., 2005a; Serra et al., 2005a). Some authors have reported decreases in infection with these two species towards harvest (Bau et al., 2005a; Bellí et al., 2005a), whereas others have not observed any clear trends (Serra et al., 2005a), as was apparent for data obtained from the Hunter Valley in this study. Conflicting data for grapes from different seasons and regions in a single country suggest that local conditions may affect the incidence of these fungi.

Inoculation of bunches in 2002 by spraying with a suspension of *A. carbonarius* spores, in most cases, did not lead to development of Aspergillus rot. Rather, there appeared to be a slight decrease in counts from the estimated inoculum concentration

(10^4 cfu/g fruit; Fig. 5.4); this is likely to be due to overestimation of the initial inoculum, as a proportion of spores in the diffuse spray would not have become attached to berries. In these symptomless bunches, spores were loosely associated with the surface of berries, whereas in bunches showing elevated *Aspergillus* counts, a greater proportion of spores was bound to the berry skin and pulp. This suggested that elevated counts were associated with fungal penetration and infection within the berry.

Given the absence of infection from a large number of both uninoculated and inoculated bunches at harvest, two factors may mediate this fungal penetration and infection, namely, damage to the berry skin and the presence of spores at the wound. Indeed, these two factors affected severity of infection in the *in vitro* damage-inoculation trial: first, damage by slitting was greater than the slight splitting induced by submerging the bunches in water and, second, inoculation by immersion provided better coverage of the berry surfaces than spraying, thus ensuring the presence of spores at the wound site. The observed ranking of the treatments in increasing order of severity of infection was: uninoculated then simulated rain damage < uninoculated then slit < inoculated then simulated rain damage < simulated rain damage then inoculated < inoculated then slit ≈ slit then inoculated.

This ranking demonstrated that the primary determining factor for severity of infection was damage, with spore coverage being the secondary factor. The importance of damage to infection was also reported by Leong et al. (2004) and Battilani et al. (2004). The sites susceptible to infection were wounds where the berries had been slit or where submersion had induced splitting, the latter particularly observed in Cabernet Sauvignon bunches from Vineyard A (Fig. 5.9), in which a relatively large proportion of berries split. It is also of note that degree of sporulation on bunches that had been spray-inoculated on the vine 1-3 weeks before slitting was equivalent to that of bunches inoculated with a fresh suspension of spores after slitting. This abundant sporulation observed in both treatments suggested that the environment on the surface of bunches had not injured the spores nor diminished their potential for vigorous growth when the berry skin was breached.

Of the two white cultivars examined in 2002, consistently high infection levels were noted for Semillon in both uninoculated (Fig. 5.1, Table 5.6c,f) and inoculated samples (Fig. 5.4, Table 5.6a,b,d,e). Shrivelled and wizened Semillon berries (Fig. 5.8a), a common site of infection in uninoculated bunches, may represent a specific niche for saprophytic black aspergilli in the bunch (Fig. 5.8b). The base of the pedicel was another common site of infection, and cultivars, such as Semillon, in which the pedicel is loosely attached may be more susceptible to fungal infection at this site (Sarig et al., 1998). No consistent differences between infection of Cabernet Sauvignon and Shiraz were observed, apart from those associated with the greater splitting induced in Cabernet Sauvignon from Vineyard A. Cabernet Sauvignon from Vineyard C was harvested when more mature; however, it did not show the same extent of splitting. Hence, berry maturity is possibly only one factor in susceptibility to splitting. Cabernet Sauvignon was one cultivar reported to be particularly susceptible to *A. carbonarius* infection in the *in vitro* trials of Battilani et al. (2004). Those authors observed differences in susceptibility to *A. carbonarius* infection and OA production among cultivars grown in Italy; however, no associations between susceptibility to infection and grape colour, bunch structure or thickness of berry skin were observed.

During inoculation trials in 2003 and 2004, it was assumed that, once present on the berry surface, *A. carbonarius* spores would not be dislodged easily by wind or rain, due to the strong hydrophobic interactions between the tuberculate spores and the waxy cuticle of the berry. Thus the decreases in *A. carbonarius* counts observed between pre-bunch closure and veraison (Fig. 5.10a) suggested that berries during early stages of development provided a hostile environment for the survival of *Aspergillus* spores. This interpretation is supported by findings of Abdelal et al. (1980), who demonstrated that juice from immature berries inhibited germination of *A. niger* spores, and that infection did not develop even when such berries were wounded; however, Battilani et al. (2001) presented data contrary to those findings, demonstrating that *A. carbonarius* could grow on wounded pea size berries *in vitro*. The decrease in spore load which continued on selected bunches until harvest could have been due, in part, to the sporocidal effect of UV radiation in sunlight upon exposed, intact berries (section 4.2.2.2). Such changes could be detected by this

method, which was more sensitive than simple measurements of incidence of infection and degree of sporulation reported in Table 5.6a,b.

Susceptibility to Aspergillus rot appeared to commence at or after veraison (Fig. 5.10), an observation mirrored by the increased frequency of isolation of black *Aspergillus* spp. from maturing berries (section 5.1). Increased susceptibility to infection may be mediated by a number of factors, including promotion of spore germination and fungal growth by juice of ripe berries (Abdelal et al., 1980) and, as berries mature, decreased production of compounds, such as phytoalexins (Jeandet et al., 2002) and phenolics (Goetz et al., 1999; Kortekamp and Zyprian, 2003), involved in resistance to fungal pathogens. Barbetti (1980) noted an association between increased bunch weight and severity of bunch rot. The large, compact Chardonnay bunches in 2004 would be susceptible to berry splitting within the humid environment of a dense canopy (Duncan et al., 1995) and, thus, susceptible to development of Aspergillus rot.

The fungicidal sprays applied to Semillon bunches in 2002 in the program designed by Syngenta Pty Ltd (Switzerland) and in the grower's standard program did not significantly reduce the severity of Aspergillus rot at harvest in inoculated bunches, compared with bunches where sprays were withheld after flowering (Fig. 5.13). As the differences between the Syngenta and grower's standard programs concerned sprays applied before bunch closure (Appendix E, Table E.1), the relatively low counts at pre-harvest from the bunches that received the Syngenta program (Fig. 5.12a) could, perhaps, be attributed to the residual effect of the Syngenta sprays applied at 80% flowering, some 38 d before inoculation. Switch® (cyprodinil + fludioxonil; Syngenta Pty Ltd, Switzerland) was applied at that stage, and has been shown to be active against Aspergillus rot (Tjamos et al., 2004; Battilani et al., 2005b); however, application of Switch® to Chardonnay and Shiraz in 2004 between pre-bunch closure and veraison did not appear to reduce counts to a greater extent than in 2003, when Switch® was not applied (Fig. 5.10a). From pre-harvest until harvest, the mean count from bunches sprayed with *B. thuringiensis* var. *kurstaki* was slightly less than that from unsprayed bunches. Bae et al. (2004) demonstrated that, although applied to vines as a biological insecticide, *B. thuringiensis* was also antagonistic to *A. carbonarius*. Whereas this antagonism may have decreased counts slightly in this study, it was not strong enough to reduce the development of berry rot (Fig. 5.13). *B.*

thuringiensis applied 2 d after fungal inoculation did not appear to have any effect on Shiraz in 2004 between veraison and pre-harvest (Fig. 5.10b). Application of procymidone (Spiral Aquaflow, Farmoz Pty Ltd, St Leonards, NSW, Australia) to Shiraz but not to Chardonnay between pre-harvest and harvest in 2004 may explain the apparent differences between the cultivars during this period, namely, that counts on Chardonnay increased, whereas, counts on Shiraz decreased or increased to a lesser extent (Fig. 5.10). It should be noted that the increases in counts observed during this stage in 2003 were still less than in 2004, despite the absence of spray application in 2003. Thus the sparse canopy in 2003, which facilitated air movement and decreased relative humidity around bunches, was possibly a stronger determining factor for reduced fungal infection than application of fungicides. A correlation between an open canopy and reduction in *Aspergillus* count was reported by Duncan et al. (1995).

The importance of berry damage (Battilani et al., 2004; Leong et al., 2004) for the development of Aspergillus rot in Semillon bunches during the month before harvest was again demonstrated in Fig. 5.16a. Berry maturity also appeared strongly to increase the potential for development of rot, with rot beginning to develop even in undamaged bunches in the 10 d before harvest. The slow development of rot on bunches inoculated 30 d pre-harvest could be attributed either to decreased susceptibility to infection of immature berries, or application of *B. thuringiensis* 2 d after inoculation. Given that *A. carbonarius* counts on Shiraz berries still increased after the same spray application (discussed above), it is likely that berry maturity played a greater role in limiting berry rot. However, the antagonism between *B. thuringiensis* and *A. carbonarius* may have reduced the number of infective propagules somewhat, and thus slightly reduced the incidence of berry infection (measured for the Semillon trial), if not the final degree of sporulation (measured for the Shiraz trial); this is an area for further investigation. Procymidone applied 9 d pre-harvest, i.e. 1 d after the final inoculation, did not hinder development of rot in damaged bunches. Apparent decreases in mean severity of rot observed for certain treatments during the 10 d before harvest may be due to shrivelling of infected berries, thus reducing their proportional representation by weight. Shrivelling of infected berries probably also contributed to the overestimation of rot by visual assessment compared with the proportion of infected berries by weight.

Shrivelling concentrated OA in berries displaying severe mould (Fig. 5.14d, 5.18a), such berries being the primary source of OA in the bunch. It is unclear whether the increase in OA displayed during the 10 d before harvest in bunches inoculated and damaged 20 d pre-harvest (Fig. 5.16b) was due to continued formation of OA or concentration of OA as berries shrivelled, or both effects simultaneously; a role for berry shrivelling is supported by the decrease in proportional weight of infected berries (Fig. 5.16a). A bunch with slightly mouldy, non-shrivelled (heavy) berries would hypothetically contain less OA than a bunch with severely mouldy, shrivelled (light) berries. This effect could explain apparent discrepancies between rot and OA in bunches inoculated with and without damage 10 d before harvest, whereby OA content of damaged and undamaged bunches was similar (Fig. 5.16b), although infected berries comprised 14% more of the bunch weight in damaged than in undamaged bunches (Fig. 5.16a). Perhaps the number of severely infected berries was similar in damaged and undamaged bunches; however, damaged bunches also contained several discoloured (heavy) berries which inflated the severity of rot. The multiple linear regression model (Table 5.8) was able to account for the effect of severely infected (shrivelled) berries by offsetting the positive parameter, number of infected berries, with the smaller negative parameter, weight of infected berries. For example, for a bunch with 20 infected berries (positive parameter 3.768 x 20 berries), the weight parameter might be -2.974 x 10 g for slightly infected (heavy) berries, but -2.974 x 5 g for severely infected (shrivelled, light) berries, resulting in OA contamination of 44 µg or 58 µg in the slightly or severely infected bunches, respectively.

The data presented in Fig. 5.16b suggested that the potential for OA formation increased in berries at greater than 12.3 °Brix. This effect was particularly noticeable in berries inoculated and damaged 30 d pre-harvest, as infection was present at 12.3 °Brix, yet the largest increase in OA occurred after this point. Roset (2003) and Lataste et al. (2004) warned of the increased risk of OA formation with increasing berry maturity, as occurs with the late harvest of grapes; however, Serra et al. (2005b) presented data contrary to this finding, demonstrating that OA in berries was typically greater at early veraison than at harvest. In that study, OA was also detected in some berries at the green pea size stage, whereas the data here suggested that this stage was

hostile for the survival of *A. carbonarius* spores on berry surfaces. Serra et al. (2005b) further demonstrated that grapes collected at early veraison, homogenised and inoculated with *A. carbonarius*, supported greater OA production than similar grapes collected at ripeness; grapes homogenised at 4 °Brix and 11 °Brix supported the greatest OA formation (2892 µg/kg and 5781 µg/kg, respectively). Even more surprising was the greatest formation of OA on homogenised grapes at the pea size stage, in the presence of low pH and little available sugar for growth. These conflicting data highlight the complexity of studying OA production at various stages of berry maturity, with changes in pH (Esteban et al., 2005a), sugar content and thus water activity (section 4.4.3), and phytoalexins (Bavaresco et al., 2003), all known to affect both fungal growth and OA production. The wide range of conditions encountered in vineyards, such as temperature fluctuations, combined with the possibility of fungal remetabolism of OA (section 4.4.3) add further layers of complexity.

Another set of puzzling results arose from symptomless bunches. As expected, many symptomless bunches, both uninoculated and inoculated but undamaged, contained no or little OA, confirming that *A. carbonarius* is not strongly pathogenic; rather, it typically requires berry damage for rot to develop and for subsequent formation of OA. Yet OA was detected in some symptomless bunches, and Serra et al. (2005b) even detected OA in samples from which no ochratoxigenic fungi were detected once berries were surface sterilised. It is not clear if the source of OA in these bunches was the same berry infection, albeit at a much earlier stage before symptoms occurred, that eventually would have led to rot. Perhaps symptoms were delayed due to a phytoalexin response to fungal invasion of berries, and these phytoalexins, in turn, stimulated OA production (Bavaresco et al., 2003), even though fungal growth was limited. Alternatively, the preliminary findings of Serra et al. (2005b) may point to a yet to be defined mode of hyphal growth and OA production on berry surfaces, whereby fungi could still be removed by surface sterilisation after OA production; fungal isolation and OA assays were not performed on identical berry samples, thus, this association requires further investigation. The detection of OA in the pooled samples of rachides of infected bunches also raised the prospect of direct infection of and OA formation in rachides, or, of the translocation of OA between berries,

although this would require verification with additional samples. Translocation of radiolabelled OA within coffee plants was reported by Mantle (2000).

5.6.2 Significance

The relatively infrequent occurrence of black *Aspergillus* spp., in particular, toxigenic *A. carbonarius* strains, on grapes in Sunraysia and the Hunter Valley suggested that the development of Aspergillus bunch rot and formation of OA are unlikely to be widespread in Australian vineyards. This finding is further supported by the infrequent detection of OA in Australian wines (Fig. 1.2). A rapid increase in the population of black *Aspergillus* spp. occurred whenever berries were damaged. In vineyards, modes of berry damage directly associated with Aspergillus rot have included berry splitting due to summer rain (section 1.5; Leong et al. (2004)) and insect damage (Fig. 5.11; Lataste et al. (2004); Cozzi et al. (2005)). Clearly, minimising damage to berries is the primary strategy for decreasing the risk of Aspergillus rot and OA formation. Data presented here and elsewhere (Battilani et al., 2003a; Roset, 2003; Lataste et al., 2004) suggest that it is critical to prevent these rots from early veraison until harvest, as this is the likely period for OA formation. A secondary strategy for decreasing the incidence of Aspergillus rot is related to the second ranking factor for rot development identified in this study, namely, spore coverage. As soil is the primary reservoir of black *Aspergillus* spp. in vineyards, reducing the concentration of these fungi in soil through mulching, minimal tillage and moisture management (Kazi et al., 2003a,b, 2004) should also reduce the number of spores blown from soil onto berries.

Insecticides and fungicides may have a role in both these strategies: first, by minimising damage to berries caused by insects or fungi such as *B. cinerea* and the mildew pathogens (Lataste et al., 2004) and, second, by potentially reducing the viability of black *Aspergillus* spores on berry surfaces even when applied before the critical period for OA formation (Bae et al., 2004; Tjamos et al., 2004). Application of certain fungicides, such as a combination of fludioxonil and cyprodinil, during the critical period after veraison was effective in reducing fungal infection (Tjamos et al., 2004) and OA (Lataste et al., 2004) in grapes; however, application during the period leading up to harvest is currently not permitted in Australian viticulture (Bell and Daniel, 2004).

These findings jointly support the concept that much can be achieved through careful vineyard management to mitigate the risk of OA formation in grapes. Brera et al. (2005) directly associated the decreased extent of OA contamination of Italian wines with vineyard management regulations introduced by the Italian government in 2000 to minimise OA.

6 Fate of ochratoxin A during vinification

6.1 Introduction

OA production in grapes ceases at the commencement of processing, typically a pasteurisation step in industrial juice production and sulphiting in wine production (Roset, 2003). Fernandes et al. (2005) also demonstrated that OA is not produced during vinification. Hence the concentration of OA in the final product is a function of the initial concentration in the grapes and the effect of processing. Processes which reduce OA can be classified into two groups, physical removal and degradation.

Physical removal of OA first involves removing the site where OA has been produced, for example, the removal of visibly mouldy berries from table grapes. It is not well understood if OA is associated primarily with the skin, pulp or juice of grape berries. However, a strong association with the skin or pulp would suggest that a relatively small proportion of OA remains in the finished beverage. The high water content of grape berries may lead to the migration of OA from the zone of fungal growth to other parts of the berry (Engelhardt et al., 1999).

A second aspect of physical removal of OA is the partitioning of the toxin between solid and liquid phases during processing. Fernandes et al. (2003, 2005) conducted microvinification trials with grapes artificially contaminated with OA, and observed that the greatest reductions resulted from solid:liquid separation steps, such as pressing the juice or wine from the skins, or decanting the wine from precipitated solids. Many of the solids present in grape juice have an affinity for OA and will loosely bind and precipitate the toxin from solution (Roset, 2003), as do some fining agents added during winemaking, such as activated charcoal (Dumeau and Trione, 2000; Castellari et al., 2001; Silva et al., 2003).

Little is known about the degradation of OA by wine yeasts during fermentation, though this has been demonstrated during beer fermentation (Baxter et al., 2001). Bejaoui et al. (2004) noted that decreases in OA during fermentation were affected by choice of yeast strain. They postulated that these decreases occurred due to binding of OA to yeast cells, rather than degradation by the yeasts, as no degradation products

were observed. The fate of radiolabelled OA during fermentation supports this hypothesis (Lataste et al., 2004). Silva et al. (2003) reported reduction in OA content by lactic acid bacteria during malolactic fermentation which may follow the completion of primary (yeast) fermentation. However, Fernandes et al. (2003) argued that this is not a true degradation, rather, bacterial biomass bound OA that later settled out of the wine. The addition of sulphur dioxide has little effect on OA (Roset, 2003; Ratola et al., 2005).

This chapter reports on the fate of OA during vinification of white and red grapes, using methods, where possible, based on current Australian oenological practice. Difficulties in obtaining sufficient naturally-contaminated grapes for vinification (Fernandes et al., 2003, 2005) were overcome by inoculation of grapes on the vine with *A. carbonarius*, in order to simulate natural contamination.

6.2 Methods

6.2.1 Inoculation of grapes, incubation and harvest

Berries were inoculated before harvest with a suspension of *A. carbonarius* spores (prepared as described in section 2.3) at approximately 10^7 cfu/mL. Strains selected for inoculation were local to the region of the experimental vineyard, and were strong producers of OA when screened on CCA as described in section 3.2.1.2. Several inoculation techniques were employed, all involving puncture damage to the berry skin and subsequent contact with the spore suspension. In addition to the primary inoculation, a supplementary inoculation of additional fruit was often performed towards harvest to ensure sufficient fruit for vinification. At harvest, inoculated and uninoculated fruit were mixed to simulate high, intermediate and low levels of or nil OA in fruit. The intermediate and low or nil OA were omitted in 2004. Table 6.1 summarises the details of inoculation, incubation and harvest. Although the initial vinification trial was conducted in Mildura, in the Sunraysia region of Victoria, in subsequent vintages, grapes were sourced from the Hunter Valley due to proximity to other trials in progress at that vineyard and to Food Science Australia. Substitution of vineyards necessitated the change from Chardonnay to Semillon grapes for white vinification. In this chapter, data from weighing grapes and products during vinification are reported as mass (kg), according to standard food processing terminology.

Table 6.1: Preparation of ochratoxin A-contaminated grapes for winemaking

Location	Research Vineyard, Mildura, Victoria		Vineyard D (see Table 5.1), Hunter Valley, New South Wales			
Aspergillus carbonarius isolates	FRR 5374[a], FRR 5573, FRR 5574		FRR 5682, FRR 5683			
Vintage	2002		2003		2004	
Grape cultivar	Chardonnay	Shiraz	Semillon	Shiraz	Semillon	Shiraz
Method of inoculation	Berries injected using syringe {berries injected using syringe}[b]	Berries injected using syringe {skin scored using grater and sprayed with spore suspension}	Berries punctured with a bed of pins dipped in spore suspension {berries injected using syringe}	Berries injected using syringe	Berries injected using syringe	Berries injected using syringe
Period from primary inoculation until harvest	21 d {4 d}[b]	14 d {13 d}	9 d {3 d}	8 d	9 d	15 d
High OA wine: mass of grapes	53 kg inoc.[c]	120 kg inoc.	25 kg inoc.	28 kg inoc.	42 kg inoc. + 6 kg uninoc.[c]	26 kg inoc. + 11 kg uninoc.
Intermediate OA wine: mass of grapes	34 kg inoc. + 23 kg uninoc.	46 kg inoc. + 73 kg uninoc.	15 kg inoc. + 13 kg uninoc.	11 kg inoc. + 16 kg uninoc.	not performed	
Control wine: mass of grapes	51 kg uninoc.	118 kg uninoc.	32 kg uninoc.	27 kg uninoc.	not performed	
Volume of ferment	4 L	16 L[d]	2 L	4 L[d]	2 L	4 L[d]

[a] FRR: Culture collection of Food Science Australia, North Ryde, NSW, Australia
[b] { } bracketed text refers to supplementary inoculation of additional fruit
[c] inoc.: grapes inoculated with *A. carbonarius*; uninoc.: control grapes, not inoculated with *A. carbonarius*
[d] including pomace (skins, pulp and seeds)

6.2.2 Vinification - 2002, 2003

Harvested bunches were chilled at 4 °C before crushing in a crusher / destemmer at 1 tonne/h (Winery Supplies, Knoxfield, Vic, Australia). In subsequent descriptions, "must" refers to crushed grapes including liquid, skins and seeds before or during fermentation. Musts were pressed with a basket press (160 L, ratchet mechanism, Winery Supplies, Knoxfield, Vic, Australia) in 2002 and through 50% shadecloth (Mitre 10, Gordon, NSW, Australia) in a hydraulic press (S. Stowe & Sons, Bristol, UK; Fig. 6.1) in 2003.

Figure 6.1: Pressing Shiraz must through 50% shadecloth in a hydraulic press

In white vinification, after pressing, potassium metabisulphite (PMS; Fermtech, Qld, Australia) was added to generate 50 ppm SO_2 in the juice. Pectinase was added in the form of Pomolase AC50 in 2002 (0.05 mL/L juice; Enzyme Solutions, Vic, Australia) or Pectinase in 2003 (0.5 g/L juice; Fermtech, Qld, Australia). The juice was overlaid with nitrogen or carbon dioxide, and refrigerated at 4 °C for at least 24 h to precipitate solids. In 2002, the juice was divided into four replicate ferments at each toxin level before clarification; this division occurred after clarification in 2003. Total soluble solids (measured in °Brix using an Atago PR-32 refractometer, Tokyo, Japan) and titratable acidity as assessed by titration to pH 8.2 using a pH meter (Sentron® 1001, Netherlands; Iland et al. (2000)) were noted. The pH was adjusted to approximately 3.3 by the addition of tartaric acid (Fermtech, Qld, Australia) to bring the titratable acidity to 6.5-7.0 g/L. The clarified juice was siphoned into glass vessels filled with nitrogen or carbon dioxide and fitted with rubber stoppers containing water traps (Fig. 6.2a). *Saccharomyces cerevisiae* QA23 (Lallemand, Plympton, SA, Australia) was rehydrated and added at a rate equivalent to 0.2 g dry yeast/L juice. Diammonium phosphate (DAP; Sigma, MO, USA) was added at 0.5 g/L juice. The fermentation temperature was 19 °C in 2002 and 15 °C in 2003. Additional DAP was introduced during fermentation as required and fermentation was said to be complete when the concentration of reducing sugars was below 0.1% (Clinitest® tablets; Bayer Australia Ltd, Pymble, NSW, Australia). The wine was racked and PMS was added at a rate equivalent to 50 ppm SO_2 to stabilise the wine and prevent further fermentation. Bentonite (0.5 g/L; Fermtech, Qld, Australia) and Liquifine (2002: 1 mL/L; 2003: 0.6 mL/L; Winery Supplies, Knoxfield, Vic, Australia) were added, and the bottles placed

at 19 °C (2002) or 15 °C (2003) to allow precipitation of solids. A second racking was performed for all bottles, and PMS was added to bring the free SO_2 to 20 ppm. Analysis of free and bound SO_2 was performed by the aspiration method (Iland et al., 2000). The bottles were held below 4 °C for cold stabilisation for at least 30 d. The wine was filtered through a 0.2 µm filter (Enolmatic tandem bottle-filler, Winery Supplies, Knoxfield, Vic, Australia) into 375 mL glass bottles with cork closures.

In red vinification, the must was divided into four replicate fermentations at each toxin level. Fermentation was performed in food-grade plastic buckets (The Bottle People, NSW, Australia) fitted with water traps (Fig. 6.2b). PMS was added to generate 50 ppm SO_2 in the must, DAP was added at 0.5 g/L must, and tartaric acid was added to bring the titratable acidity to 6.5 g/L. *S. cerevisiae* D254 (Lallemand, Plympton, SA, Australia) was rehydrated and added at approximately 0.3 g/L must. The cap was plunged 2-3 times daily. The must was pressed after 4 d of fermentation at room temperature in 2002 (*ca* 24 °C), and after 6 d of fermentation at approximately 20 °C in 2003. The pressed wine was held in bottles at *ca* 22 °C until completion of fermentation, as defined by a concentration of reducing sugars less than 0.25% (Clinitest® tablets, Bayer Australia Ltd, Pymble, NSW, Australia). During the first racking, 50 ppm SO_2 was added, after which the wine was held at 19 °C (2002) or 15 °C (2003) to precipitate yeast cells and other solids. At the second racking, SO_2 was added to maintain a final concentration of 50 ppm. The wine was held below 2 °C for cold stabilisation for at least 30 d, after which the pH was adjusted to 3.5 and the wine bottled through a filtration line as described above.

Bottles were cellared at *ca* 22 °C in 2002, and at 15 °C in 2003, in order to assess the OA content after approximately 1 year of storage.

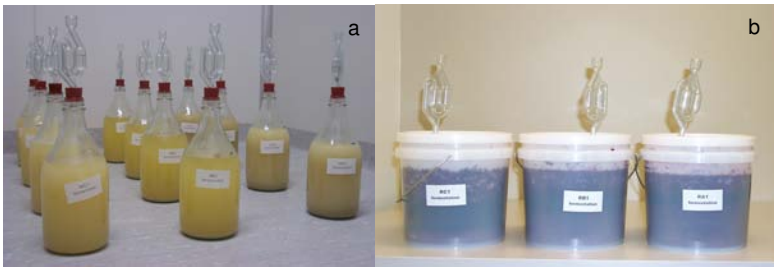


Figure 6.2: Fermentation vessels (a) for Semillon juice and (b) Shiraz must, 2003 and 2004

6.2.3 Sampling - 2002, 2003

After crushing, eight samples of must from each toxin level were collected in order to establish the initial total OA present in the berries. These samples were homogenised in a blender (Philips HR2835/AB) and assessed for the presence of *A. carbonarius* as described in section 2.1.

Juice or wine samples were collected for OA analysis after pressing, after the first and second racking, and after storage for approximately 1 year. These data were not amenable to statistical analysis, due to the much greater variability observed for non-homogenous must replicates than for homogeneous juice and wine samples. However, data regarding OA during storage were analysed as described in section 2.6, setting the OA concentration at bottling as 100%, with model: cultivar/(stage x level). High OA (2002), intermediate OA (2002), high OA (2003) and intermediate OA (2003) each constituted a different level according to initial OA concentration.

In white vinification, samples for OA analysis were also collected before and after juice clarification.

6.2.4 Effect of enzymes and bentonite during white juice clarification, 2004

As described in section 6.2.2 for white vinification in 2003, Semillon grapes were crushed and PMS added. The must was mixed and divided into 3.5 kg portions. A subsample of each portion was collected in order to establish the initial OA present. Portions were treated in triplicate with Ultrazyme CP-L (0.8 mL/L; Novozymes A/S, Dittingen, Switzerland) or Lallzyme HC (0.1 g/L; Lallemand, Plympton, SA, Australia) at the upper limit of rates recommended by the manufacturer, or were left untreated as controls. After incubation at room temperature for 2 h, the must was pressed, after which the compressed marc was mixed by hand, re-wrapped in the shadecloth, and pressed again. Samples of juice and marc were collected for OA analysis, and the remainder of the juice held at 1 °C for 4 d. The clarified juice was siphoned from the surface. Samples of clarified juice and lees were collected for OA analysis.

One additional control treatment (i.e. enzymes not added) was prepared to assess the efficacy of bentonite for the removal of OA during juice fining. After pressing, juice was divided into triplicate 450 g portions, with or without addition of bentonite at 0.1 g/L (Fermtech, Qld, Australia). Samples were collected before and after settling at 1 °C for 4 d.

Data were analysed according to the model treatment x stage, as described in section 2.6.

6.2.5 Ochratoxin A during fermentation, 2004

6.2.5.1 Semillon

Clarified juice from treatments described in section 6.2.4 was pooled, and fermentation was carried out as in 2003, described in section 6.2.2. At approximately 17, 15, 11, 8 and 6 °Brix (Atago PR-32 refractometer, Tokyo, Japan), vessels were swirled to resuspend flocculated yeasts, and samples collected for OA analysis. Samples of wine and lees after racking were also retained for OA analysis. Four replicate fermentations were conducted. Data were analysed as described in section 2.6.

6.2.5.2 Shiraz - static *vs* rotary fermentation

As described in section 6.2.2 for red vinification in 2003, Shiraz grapes were crushed and PMS added. Fermentation was modified from 2003 as follows: the fermentation temperature was monitored and maintained at 25-30 °C over 4 d. To mimic static fermentation, the cap was gently plunged three times daily, whereas to mimic rotary fermentation, must in the buckets was shaken vigorously at the same intervals. Four replicate fermentations of each treatment were conducted. Samples of must were collected before fermentation, to establish the amount of OA initially present. Juice samples were collected at various stages throughout fermentation and centrifuged at 3000 rpm for 15 min (Orbital 420, Clements Medical Equipment Pty Ltd, Somersby, NSW, Australia) before analysis for OA. After the first pressing, a sample of free run wine was collected. The compressed marc was mixed by hand, re-wrapped in the shadecloth, and pressed a second time; however, the mass of pressings (additional wine) was negligible. A sample of marc was retained for OA analysis. Free run wine and pressings were combined, and fermentation completed in glass bottles. Samples

of wine and lees were collected after the first racking. Data were analysed as described in section 2.6.

6.2.6 Recovery of juice and wine from lees, 2004

Samples (40 mL) of juice lees from white juice clarification and gross lees after the first racking stage of white and red vinifications were centrifuged at 3000 rpm for 5 min (Orbital 420, Clements Medical Equipment Pty Ltd, Somersby, NSW, Australia) to assess the OA potentially present in juice or wine recovered from lees by centrifugation. OA in the compacted lees was also assessed.

The OA concentrations in racked and centrifuged juice / wine were compared as described in section 2.6. Semillon juice lees, Semillon gross lees and Shiraz gross lees were each treated in a separate ANOVA.

6.2.7 Effect of fining agents on removal of ochratoxin A

Semillon wines from the trial described in section 6.2.5.1 were pooled after the first racking; likewise, Shiraz wines were pooled from the trial described in section 6.2.5.2. A sample of each pooled wine was collected for OA analysis. Wines (100 mL) were dispensed into glass vessels and fining agents added while the wine was mixed continuously with a magnetic stirrer to ensure even distribution. Wines were held at room temperature (*ca* 22 °C) for 2 d (Ruediger et al., 2004), after which a sample of clear wine was decanted and centrifuged at 3000 rpm for 15 min (Orbital 420, Clements Medical Equipment Pty Ltd, Somersby, NSW, Australia). A 10 g subsample of this centrifuged wine was weighed and analysed for OA as described in section 6.2.8.1. Fining agents were not added to the control wine. Treatments were conducted in triplicate. Fining agents were tested twice: first, at a rate representing the low to normal range of current Australian practice and, subsequently, at a higher rate representing the upper boundary (Rankine, 1989; Iland et al., 2000). Fining agents tested included bentonite (0.5 g/L, 2.5 g/L; Fermtech, Qld, Australia), potassium caseinate (K-caseinate; 0.1 g/L, 0.25 g/L; Winery Supplies, Knoxfield, Vic, Australia), gelatin (0.05 g/L, 0.15 g/L; Sigma, MO, USA), isinglass (0.016 g/L, 0.10 g/L; Biofine 1499, Deltagen, Boronia, Vic, Australia) and polyvinyl-polypyrrolidone (PVPP; 0.2 g/L, 0.8 g/L; Fluka, Switzerland) for white wine, and egg white (0.4 g/L, 0.6 g/L),

gelatin (0.05 g/L, 0.15 g/L) and yeast hulls (2.0 g/L, 5.0 g/L; Lallemand, Plympton, SA, Australia) for red wine.

Statistical analysis was performed by Colleen Hunt (BiometricsSA, Glen Osmond, SA, Australia). Data were analysed as described in section 2.6 according to the model cultivar/(fining agent x rate).

Proteins in the unfined wines were analysed at the Australian Wine Research Institute, Glen Osmond by the method described by Girbau et al. (2004) after centrifugation (4000 rpm, 15 min) and filtration through a 0.45 µm membrane.

Wines made in 2004 were not bottled for subsequent OA analysis after 1 year, as sampling would have extended beyond the completion date of this study.

6.2.8 Ochratoxin A extraction

6.2.8.1 Liquids

Samples (10 g) were mixed by vortexing with methanol (1.5 mL) and hydrochloric acid (10 N, *ca* 0.15 mL). The mixture was centrifuged at 2500 rpm for 15 min (Orbital 420, Clements Medical Equipment Pty Ltd, Somersby, NSW, Australia). A 900 mg C18 solid phase extraction cartridge (Maxi-Clean™, Alltech, Deerfield, USA) was conditioned with 5 mL acetonitrile followed by 5 mL water, and the supernatant was passed dropwise through this cartridge under vacuum (Vacuum manifold, Alltech, Deerfield, USA). The pellet was resuspended in 10% methanol (10 mL), then centrifuged at 2500 rpm for a further 15 min. This supernatant was also passed through the C18 cartridge.

A 200 mg aminopropyl cartridge (4 mL Extract-Clean™, Alltech, Deerfield, USA) was conditioned with 3 mL methanol. The C18 and aminopropyl cartridges were attached in series, and the sample was eluted from the C18 cartridge onto the aminopropyl cartridge with the addition of 10 mL methanol. The sample was eluted from the aminopropyl cartridge with 10 mL 35% ethyl acetate in cyclohexane containing 0.75% formic acid.

The eluate was dried under reduced pressure at 45 °C and was resuspended in 1 mL 35% acetonitrile containing 0.1% acetic acid for analysis by HPLC (section 6.2.8.3). The recovery for this method was > 98% for bottled wine (Giannikopoulos et al., 2004).

Certain liquid matrices containing substantial amounts of particulate matter thought to bind OA were analysed by the stable isotopic dilution method and LC-MS (section 2.5). Such matrices included white grape juice before clarification, white juice lees, white juice during fermentation containing yeast cells, white gross lees, red wine immediately after pressing, and red gross lees. In a modification to the method described in section 2.5.1, samples (10 g) were extracted with methanol (1.5 mL) and hydrochloric acid (10 N, *ca* 0.05 mL). White, unclarified grape juice was assessed during the 2002, 2003 and 2004 vintages; however, other matrices were only assessed during 2004 due to cost and time constraints.

6.2.8.2 Solids

OA in grape must and marc (skins and seeds after pressing) was extracted and analysed by LC-MS as described in section 2.5. Grape must was assessed during the 2002, 2003 and 2004 vintages; however, marc was assessed only during 2004 due to cost and time constraints, and the limited volume of isotopically-labelled internal standard available.

6.2.8.3 HPLC analysis

The HPLC method for analysis was similar to that reported in section 2.4.2, with the following modifications: the mobile phase consisted of acetonitrile:water:acetic acid (50:49:1, v/v) and was delivered at a flow of 1.3 mL/min, with post-column addition of ammonia (12.5% w/w, 0.2 mL/min). The injection volume was 3-30 µL. Samples which yielded OA concentrations that exceeded the scale on the detector were diluted in 35% acetonitrile containing 0.1% acetic acid and re-injected.

6.2.9 Other analyses

In 2003, a sample of Semillon juice obtained after crushing of high OA (inoculated) grapes was analysed for the presence of organic acids by the Analytical Services Group at the Australian Wine Research Institute, Glen Osmond, SA, Australia.

Likewise, Semillon and Shiraz juice samples from uninoculated and inoculated fruit were analysed in 2004.

In 2004, the moisture content of marc and lees was determined by drying a subsample at 90 °C overnight.

6.3 Results

6.3.1 Effect of *Aspergillus carbonarius* infection on appearance, total soluble solids and titratable acidity of wine grapes

A. carbonarius was isolated from uninoculated fruit at 2-130 cfu/g, and from inoculated fruit at 3.6 x 10^4-4.6 x 10^5 cfu/g. Counts of black *Aspergillus* spp. from musts made from mixtures of inoculated and uninoculated fruit (intermediate OA) were approximately half those from inoculated fruit. *A. niger* and *A. aculeatus* were also isolated from must.

Inoculated berries became discoloured starting at the point of inoculation, shrivelled, and often fell to the ground (Fig. 6.3). Upon crushing, it was noted that the berry pulp was macerated due to fungal growth. This was particularly noticeable in Shiraz must, where the juice of inoculated berries was heavily pigmented compared with that of uninoculated fruit (Fig. 6.4). Shrivelling of inoculated berries increased the total soluble solids compared with uninoculated fruit (Table 6.2). Inoculated fruit also displayed increased titratable acidity to an extent greater than that attributable to berry shrivelling alone. Data from 2004 demonstrated increases in malic and tartaric acid of approximately 60% and 14%, respectively; however citric acid in inoculated fruit increased to over 450% of the concentration in uninoculated fruit.

OA appeared to be higher in inoculated fruit during seasons when the time between inoculation and harvest was less than 10 d (Fig. 6.5).

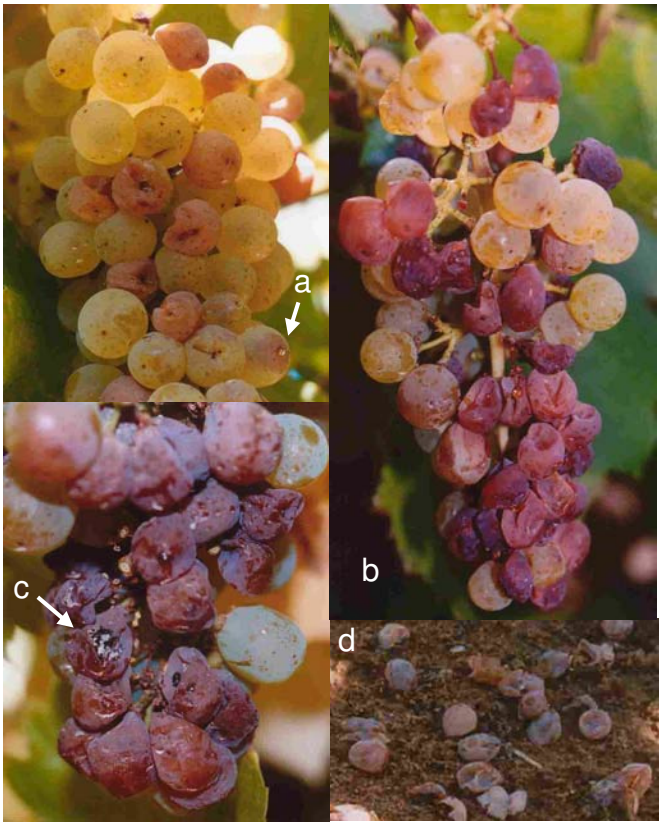


Figure 6.3: Shrivelling of Chardonnay berries inoculated with *Aspergillus carbonarius* before harvest, 2002, showing (a) berry discolouration at inoculation point, (b) shrivelling of inoculated berries, (c) sporulation and (d) bunch shatter

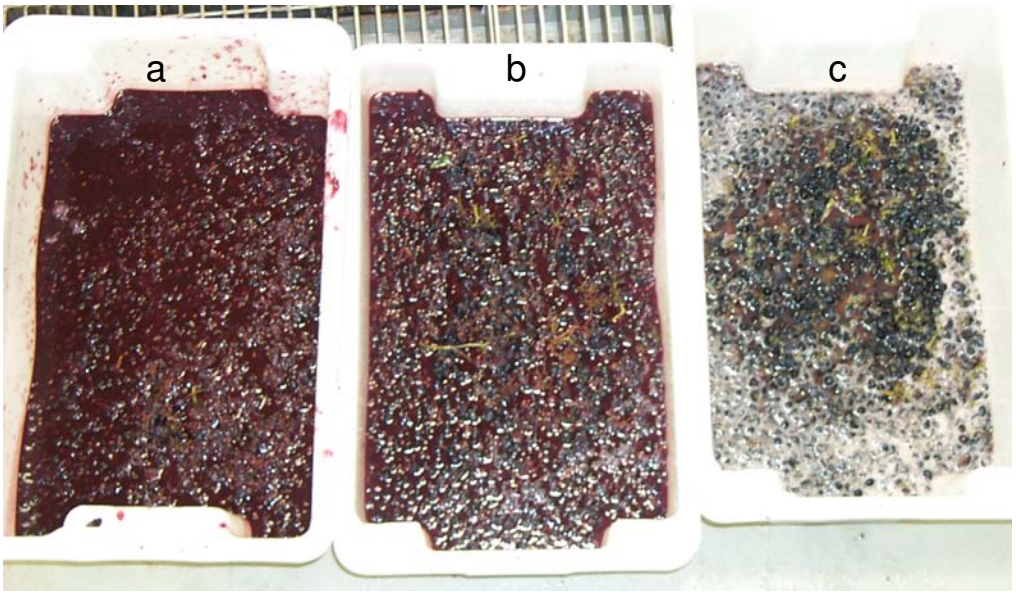


Figure 6.4: Shiraz must from (a) fruit inoculated with *Aspergillus carbonarius* before harvest, (b) a mixture of inoculated and uninoculated fruit (c) uninoculated fruit only, 2003

Table 6.2: Effect of *Aspergillus carbonarius* infection on total soluble solids and titratable acidity of wine grapes at harvest

Sample	Total soluble solids (°Brix)		Titratable acidity (g/L) *Organic acid concentration (g/L), where assessed*[a]	
	Uninoculated	Inoculated	Uninoculated	Inoculated
Chardonnay, 2002	22.3	24.1	4.8	6.0
Shiraz, 2002	24.1	26.2	not assessed	
Semillon, 2003	20.0	20.0	5.5 *2.4 malic*[a] *3.6 tartaric* *1.4 citric*	7.7
Shiraz, 2003	19.9	23.8	5.8	12.5
Semillon, 2004	15.8	19.0[b]	4.7 *1.1 malic* *3.6 tartaric* *0.2 citric*	7.3[b] *1.8 malic* *4.1 tartaric* *0.9 citric*
Shiraz, 2004	20.8	25.4[b]	5.1 *0.5 malic* *6.1 tartaric* *0.2 citric*	8.2[b] *0.8 malic* *7.2 tartaric* *1.5 citric*

[a] organic acid profile reported in *italics*
[b] total soluble solids and titratable acidity in inoculated fruit corrected for mixture of uninoculated and inoculated fruit in 2004

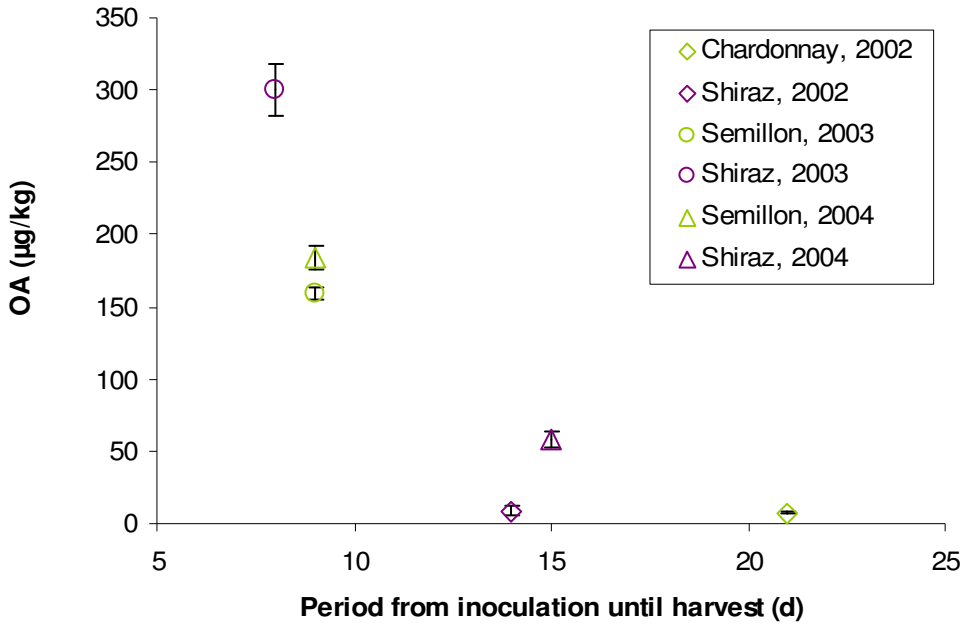


Figure 6.5: Ochratoxin A in must from grapes puncture-inoculated before harvest with a suspension of *Aspergillus carbonarius* spores. Error bars denote the standard error of the mean of three to ten subsamples of must

6.3.2 Ochratoxin A during vinification

Quantification of OA in must yielded a range of values, due to difficulties in obtaining representative subsamples of a non-homogeneous matrix. Nevertheless, similar trends were observed for white and red vinifications over the three vintages, namely, that reductions occurred at all the solid:liquid separation stages, with the greatest reduction in OA concentration at pressing (68-84%), additional reductions at racking and slight reductions upon storage (Fig. 6.6). Despite initial OA concentrations in must differing over a wide range, the final concentration of OA in wine after the first racking was remarkably similar among the white wines (2-7% of that in must, mean 4%). Likewise, red wines displayed similarities among the vintages (6-13% of that in must, mean 9%). Thus, the relative OA concentration in red wine after racking was approximately twice that of white wine at the equivalent stage. Whereas reductions in OA during storage were insignificant on the scale of the initial OA in must (1-3% of the initial must concentration), over 10-14 months of storage, significant reductions ($P < 0.01$) of 22% and 29% of the OA concentration at bottling were observed for white and red wines, respectively, from vintages 2002 and 2003.

OA was seldom detected in any samples collected during vinification of uninoculated grapes in 2002 and 2003.

In 2004, the extent of pressing and racking mirrored commercial practice as closely as possible. Semillon and Shiraz marc contained 57% and 52% moisture, respectively. Semillon juice lees contained 85% moisture, and Semillon and Shiraz gross lees contained 94% and 87% moisture, respectively. Data regarding the amount of OA in the marc and lees are presented in Figs 6.7a and 6.8a, expressed as vinification of 1 kg must. OA was preferentially partitioned into the solid phases (marc and lees), although these phases represented only a small proportion by mass (Figs 6.7b, 6.8b). For example, during clarification of white juice, the juice lees contained 85% of the total OA but comprised only 37% of the mass. Generally, the amount of OA entering a particular stage was conserved and divided between the solid and liquid phases at the next separation step. An obvious exception to this trend was observed at pressing, when the sum of OA in marc and juice / wine was greater than that initially present in must. The reasons for this are discussed in section 6.4.

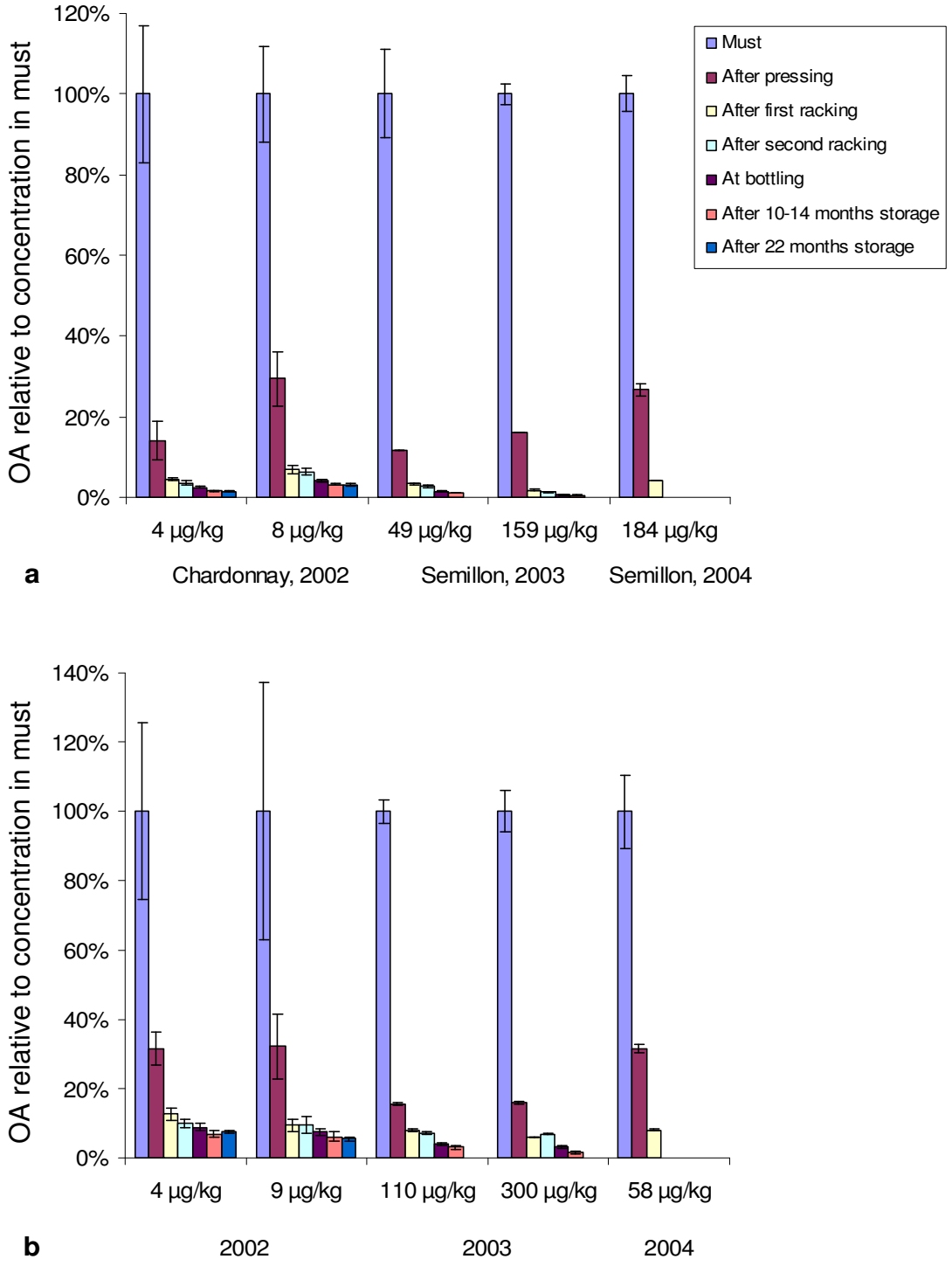


Figure 6.6: Summary of the fate of ochratoxin A during (a) white (Chardonnay and Semillon) and (b) red (Shiraz) vinification over three vintages. The concentration of OA initially present in must, designated as 100%, is given on the *x*-axis. OA in subsequent samples (µg/kg) is expressed relative to this initial concentration. Error bars denote the standard error of the mean of two to ten (typically four) subsamples or replicate fermentations. Samples after the first racking were not collected in 2004. Samples collected "after pressing" during red vinification in 2002 and 2003 contained much particulate matter, thus data presented here were corrected for a recovery of 54% by HPLC analysis. Equivalent samples in 2004 were analysed by stable isotopic dilution and LC-MS, hence no correction factor was necessary

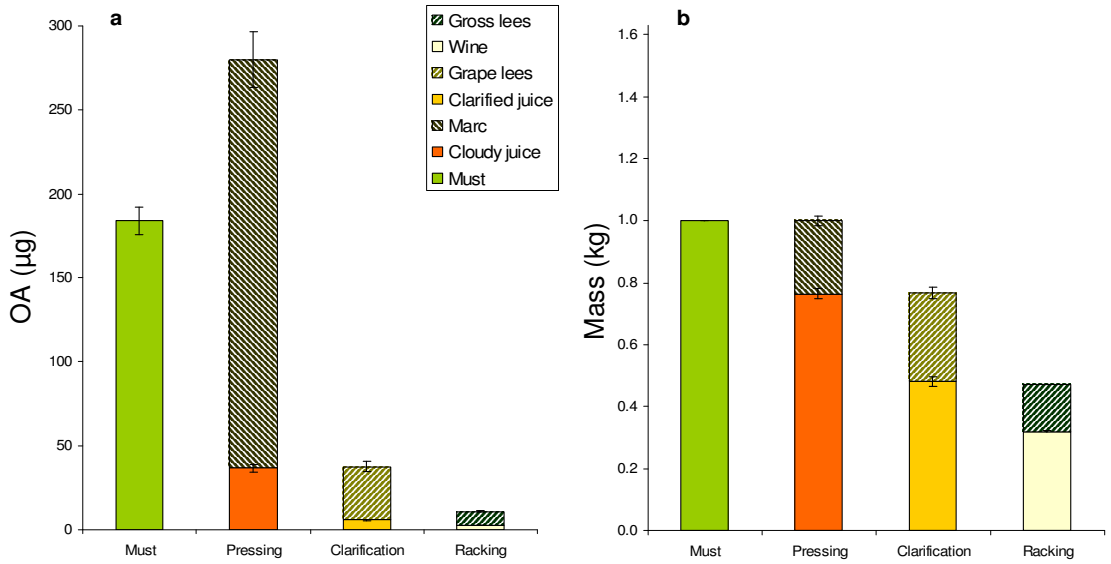


Figure 6.7: Partitioning of (a) ochratoxin A and (b) mass at solid:liquid separation steps during vinification of Semillon must (1 kg), 2004. Error bars denote the standard error of the mean of a minimum of four replicate vessels

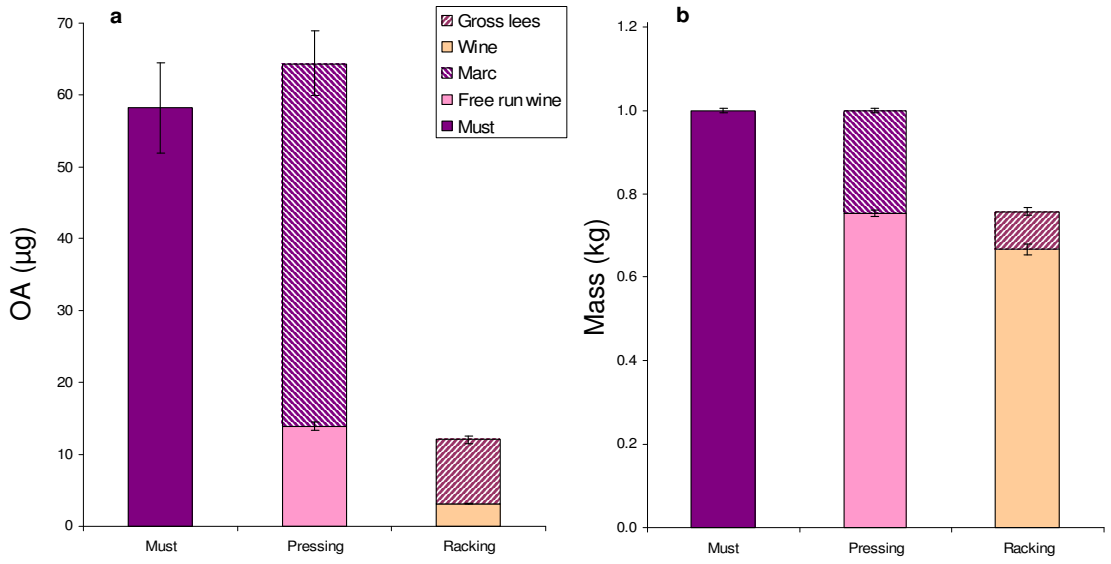


Figure 6.8: Partitioning of (a) ochratoxin A and (b) mass at solid:liquid separation steps during vinification of Shiraz must (1 kg), 2004. Error bars denote the standard error of the mean of eight replicate fermentations

6.3.3 Ochratoxin A during clarification

Although the concentration of OA in the cloudy (unclarified) white juice appeared to vary with treatment and vintage, the concentration of OA in clarified juice relative to the concentration in must was consistent (4-9%, mean 6%) across vintages and initial must concentrations (Fig. 6.9). ANOVA of the data from 2004 demonstrated no significant interactions between stage (cloudy or clarified juice) and enzyme; however, significant differences between the stages were observed, that is, cloudy juice contained more OA than clarified juice. Most importantly, differences among enzyme treatments were not significant, indicating that addition of pectolytic enzymes neither enhanced nor disrupted the binding of OA to juice lees. Likewise, ANOVA of data from the bentonite juice fining trial did not demonstrate significant differences in OA between juice fined with and without bentonite.

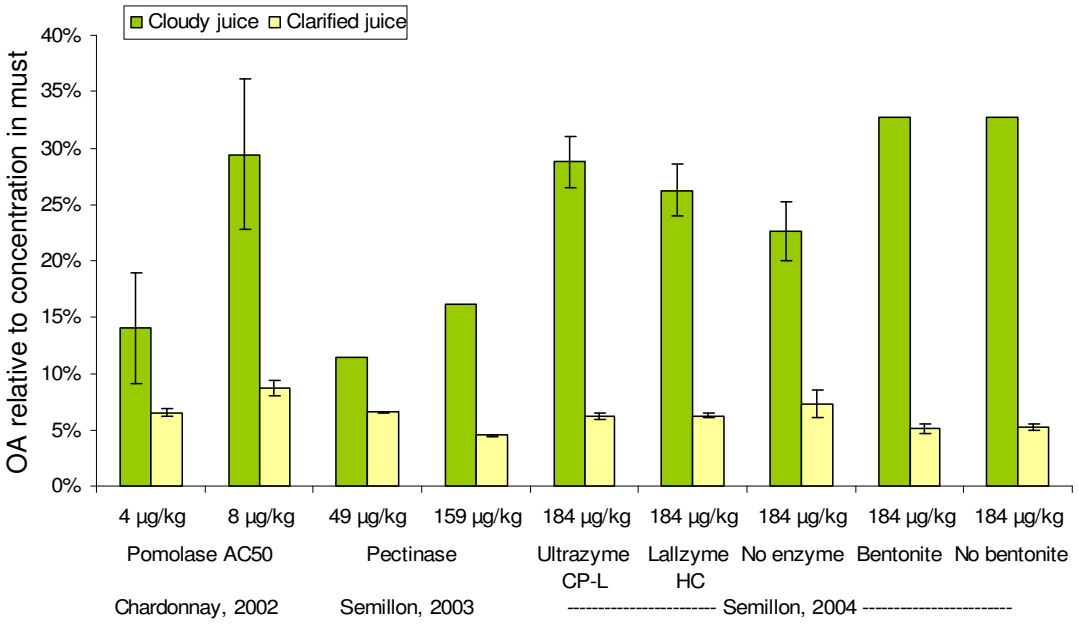


Figure 6.9: Summary of the reduction in ochratoxin A achieved during clarification of white grape juice over three vintages. The concentration of OA initially present in must, designated as 100%, is given on the *x*-axis. Error bars, where shown, denote the standard error of the mean of three to four subsamples or replicate settling vessels. See sections 6.2.2 and 6.2.4 for greater detail

6.3.4 Ochratoxin A during fermentation

6.3.4.1 Semillon, 2004

OA concentration appeared to be consistent during Semillon juice fermentation (Fig. 6.10), and was significantly reduced ($P < 0.05$) upon racking from the gross (yeast)

lees, even taking into account potential underestimation of OA due to differing analytical techniques for the samples taken at 18.6 °Brix and at racking.

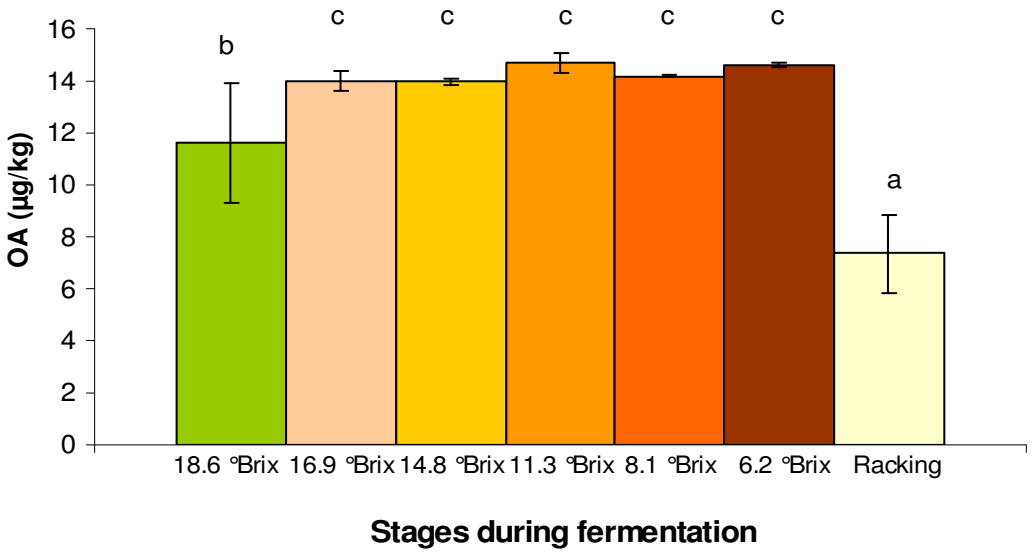


Figure 6.10: Ochratoxin A during Semillon fermentation and racking, 2004. Samples at 18.6 °Brix and at racking were analysed by HPLC and have been assigned an approximate error of 20%. Other samples taken during fermentation contained much yeast, and were analysed by LC-MS. Error bars for such samples denote the standard error of the mean of four replicate fermentations. Different letters denote values which differ according to Tukey's test of honestly significant difference at $P < 0.05$

6.3.4.2 Shiraz, 2004

Replicate fermentation vessels contained subsamples of the same must, hence the OA content of the juice was similar in both static and rotary ferments (Fig. 6.11). OA in the liquid portion of must reached a maximum part way through static fermentation, at *ca* 10 °Brix, after which it decreased. This trend was not apparent during rotary fermentation, a key feature of which was the decrease in OA in the liquid portion soon after commencement of fermentation. A finding common to both fermentation styles was increased OA in the liquid portion of free run wine compared with the centrifuged juice before fermentation, although this difference was not deemed to be statistically significant. ANOVA demonstrated that OA in the free run liquid did not differ significantly between static and rotary ferments.

Free run wine from the rotary style ferments was observed to contain more solid matter than free run wine from static ferments. Nevertheless, when analysed in these

matrices by LC-MS, the mean OA concentrations in free run wines from static and rotary ferments were 18.3 µg/kg and 18.5 µg/kg, respectively.

It is of note that OA in free run wine containing particulate matter (mean 18.4 µg/kg) was greater than OA in the liquid portion of this free run wine, separated by centrifugation (mean 2.8 µg/kg). OA in wine at racking (4.7 µg/kg) was also greater than in centrifuged wine.

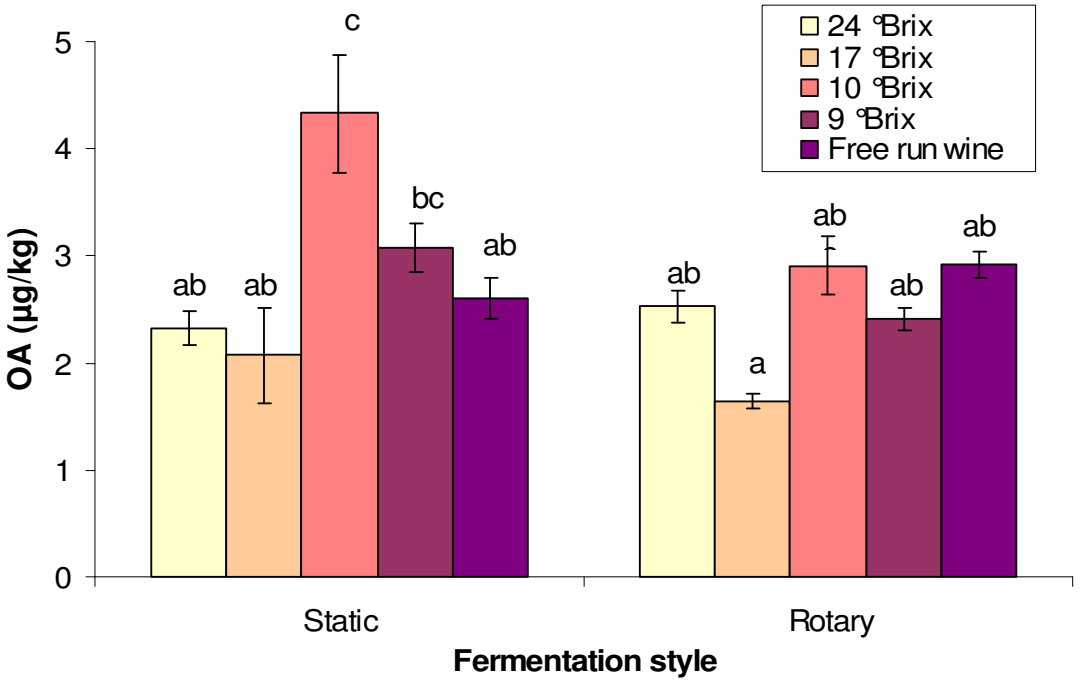


Figure 6.11: Ochratoxin A in the liquid portion of Shiraz must during fermentation until pressing, 2004. Error bars denote the standard error of the mean of three replicate fermentations, and different letters denote values which differ according to Tukey's test of honestly significant difference at $P < 0.05$

6.3.5 Ochratoxin A in juice and wine from lees

Centrifugation of lees recovered a substantial amount of juice and wine, particularly from Semillon (Table 6.3). Centrifugation compacted and concentrated the lees, which, in turn, concentrated OA to an equivalent extent. For example, during centrifugation, Semillon juice lees were concentrated approximately five fold by mass, and OA concentration also increased approximately five fold. ANOVA of OA concentrations in juice / wine from racking and juice / wine recovered by centrifugation did not yield consistent trends for the three lees matrices examined. OA in recovered (centrifuged) Semillon juice was significantly less than that in racked

juice ($P < 0.01$); the interaction between enzyme treatment and juice type (racked or centrifuged) was not significant, neither was the effect of enzyme treatment. Likewise, OA in Shiraz wine recovered from the lees was significantly less than OA in racked wine ($P < 0.001$); the interaction between fermentation style and wine type (racked or centrifuged) was not significant, neither was fermentation style. In contrast, the OA concentration of Semillon wine recovered from lees was approximately 11% greater than that of racked wine ($P < 0.01$).

Table 6.3: Ochratoxin A concentration (µg/kg) in juice and wine recovered from lees by centrifugation, 2004

Stage / Treatment		Juice / wine after racking		Juice / wine recovered from lees		OA in lees	OA in compacted lees after centrifugation
		OA	% by mass	OA	Additional % by mass recovered		
Semillon juice clarification	Ultrazyme CP-L	11.4 ± 0.6[a]	60 ± 3	10.0 ± 0.3	33 ± 3	109.7 ± 3.8	502.3 ± 28.0
	Lallzyme HC	11.5 ± 0.4	61 ± 2	10.6 ± 0.1	31 ± 4		
	No enzyme	11.2 ± 0.3	68 ± 5	8.9 ± 0.9	27 ± 1		
Semillon racking 1		7.3 ± 0.0	68 ± 0	8.1 ± 0.1	23 ± 1	55.4 ± 1.9	175.8 ± 13.4
Shiraz racking 1	Static ferment	4.6 ± 0.1	91 ± 1	3.0 ± 0.1	5 ± 0	103.8 ± 7.7	230.0 ± 15.4
	Rotary ferment	4.8 ± 0.1	86 ± 1	3.0 ± 0.0	9 ± 1		

[a] standard error of the mean of three to four replicate vessels

6.3.6 Removal of ochratoxin A by fining agents

Bentonite was the fining agent most effective for the removal of OA from Semillon wine (initial concentration *ca* 8 µg/kg), reducing OA significantly ($P < 0.05$) at both low and high rates of addition (Fig. 6.12). K-caseinate also reduced OA but only at the high rate of addition. For the Shiraz wine (initial OA concentration *ca* 5 µg/kg), yeast hulls reduced OA significantly at both low and high rates of addition, whereas gelatin was only effective at the high rate of addition. Other agents did not significantly reduce OA.

The unfined Semillon contained 56 mg/L total proteins, comprising 33 mg/L PR4 proteins, 20 mg/L thaumatin-like proteins and 3 mg/L chitinases. In contrast, the unfined Shiraz did not contain detectable proteins.

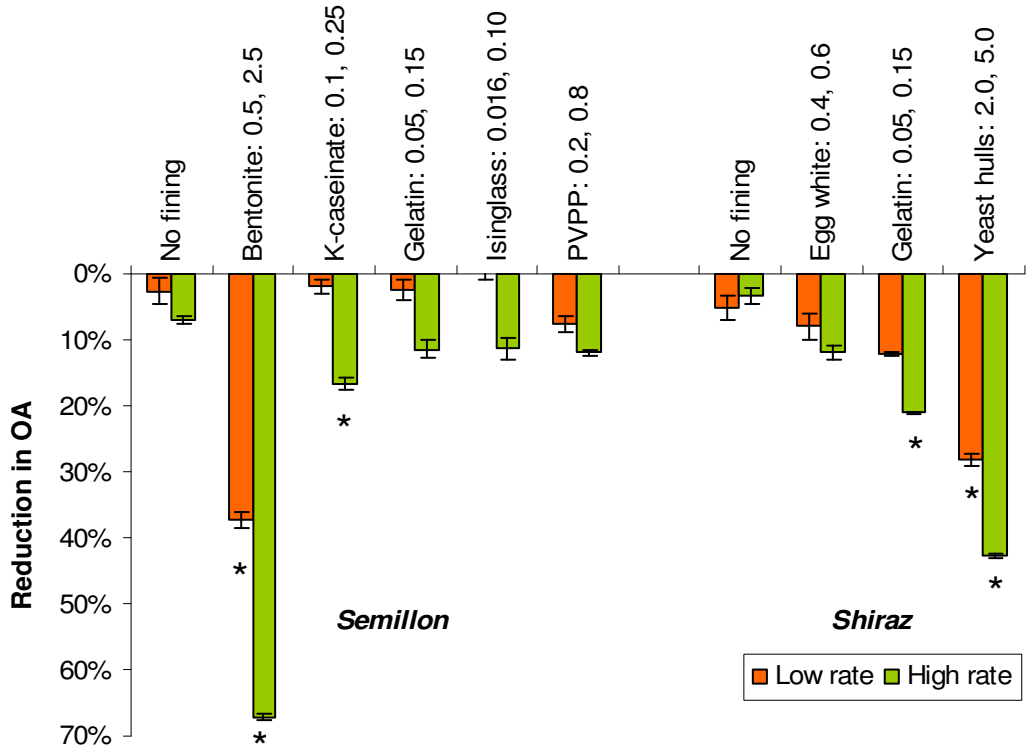


Figure 6.12: Effect of fining agents (g/L) added at two rates to wine containing ochratoxin A; Semillon *ca* 8 µg/kg, Shiraz *ca* 5 µg/kg. K-caseinate: potassium caseinate; PVPP: polyvinyl-polypyrrolidone. Error bars denote the standard error of the mean of three replicates. Asterisks denote treatments which conferred a reduction in OA significantly greater than that observed in the controls with no fining agent added, when analysed by Tukey's test of honestly significant difference ($P < 0.05$)

6.4 Discussion

Some of the effects of berry infection with black *Aspergillus* spp., such as discolouration and shrivelling, have been discussed in chapter 5. A notable feature observed during winemaking was the maceration of the pulp of infected berries, resulting in a greater extraction of grape pigments (Fig. 6.4). As members of *Aspergillus* section *Nigri* are potent producers of pectinase (Raper and Fennell, 1965), pectolytic activity could explain this observation. The increase in titratable acidity in infected fruit was primarily due to the increase in citric acid and, to a lesser extent, to berry shrivelling. The fungus, rather than the berry, was the likely source of this additional citric acid. Strains of *A. niger* are used commercially in the production of citric acid (Raper and Fennell, 1965), and it is probable that *A. carbonarius*, a closely

related species, also produces this acid. The observation that grapes harvested less than 10 d after inoculation often contained more OA than grapes incubated for longer periods may reflect fungal remetabolism of the toxin (section 4.4.3). However, seasonal variation is likely to be important, as conflicting trends were noted in the Semillon trial described in section 5.5, in which OA in bunches typically increased markedly 10 d after inoculation.

Data on reductions in OA achieved during vinification emphasised the importance of solid:liquid separation steps, first at pressing and, subsequently, after fermentation and racking from the gross lees; extent of reduction at these stages was in broad agreement with that reported by Fernandes et al. (2003, 2005) (Table 6.4). This study demonstrated that, during storage for 10-14 months, OA decreased by 22% and 29% of the concentration at bottling for white and red wines, respectively. Likewise, Garcia Moruno et al. (2005) reported an 8% reduction in OA added to red wine, mixed, and held at room temperature for 55 d, and a 10% reduction after 80 d. In contrast, Lopez de Cerain et al. (2002) did not report a decrease in OA concentration after storage of wine for 12 months. Perhaps these conflicting data arose due to bottling at a later stage of the wine maturation process, after which some precipitation had already occurred. The apparent greater reduction observed upon storage of red wines than of white wines in this study, could be associated with the more complex matrix in red wines, representing a greater potential for binding OA during polymerisation of various molecules and settling of those aggregates.

Table 6.4: Comparison of reduction in ochratoxin A during vinification achieved in this and two related studies

Citation	Vinification	Initial OA concentration in grapes (µg/kg)	OA relative to concentration in must (crushed grapes)	
			After pressing	After first racking
This study	White	4-184	mean 20%	mean 4%
	Red	4-300	mean 25%	mean 9%
Fernandes et al. (2003)	White with maceration	7	15%	11%
	White without maceration	7	45%	30-33%
	Red	9	8%	6%
Fernandes et al. (2005)	Red	0.4-7	mean 42%	mean 17%
	Port	0.1-5 (in juice)	mean 8% relative to juice	not reported

Regarding mass transfer of OA throughout white and red vinification (Figs 6.7, 6.8), the amount of OA in marc was probably overestimated, which led to the sum of OA in marc and juice / wine being greater than that initially present in must. The reason for overestimation lay in the difficulty of obtaining homogeneous samples of marc and, more specifically, in homogenisation of this marc subsample with sufficient quantities of water to bring the OA concentration within a range suitable for analysis by stable isotopic dilution. Despite mixing, heavier seed particles settled immediately, whereas lighter skin particles remained suspended in the homogenate. Thus a 15 g sample of homogenate was biased towards sampling the skins, which often contain a greater concentration of OA than pulp (Battilani et al., 2001), and biased against sampling the seeds, which are sterile (Bau et al., 2005a) and thus unlikely to contain OA. Data for mass transfer at subsequent stages demonstrated a clear passage of OA with no substantial losses, suggesting that OA was not degraded during vinification; rather, it was partitioned between solid and liquid phases, and thus discarded with the solids. During red vinification, 24% of the total amount of OA in grapes was present in red wine after pressing, and 5% after the first racking. Fernandes et al. (2005) reported similar values (32% and 11% after pressing and racking, respectively). The lower values observed for white vinification in this study (20% and 1% after pressing and racking, respectively) could be attributed to two fundamental differences between red and white vinification. First, pressing of white grapes occurred before fermentation, hence, in the absence of alcohol, OA was poorly soluble in the liquid phase. Second, white juice clarification before fermentation reduced OA to 3% of the amount in must, and this removal of grape proteins to which OA was bound may have enhanced the competitive binding of OA to yeast during fermentation. OA bound efficiently to white grape lees and red gross lees after fermentation, hence it was possible to concentrate these lees by centrifugation and recover additional juice or wine, without liberating OA into solution (Table 6.3). In fact, the OA concentration in recovered (centrifuged) Semillon juice and Shiraz wine was lower than in racked juice / wine, possibly as centrifugation, in addition to concentrating the lees, removed particulate matter from the juice / wine. The binding to white gross lees did not appear to be as strong, as OA in the recovered (centrifuged) wine was 11% greater than in the racked wine. Garcia Moruno et al. (2005) mentioned that binding of OA to yeast during white fermentation did not appear to be as strong as during red fermentation. Given

the small number of replicates (n = 3) in this study and the similarities in concentration between racked and recovered juice / wine, further investigation is required to determine if the practice of recovering juice and wine from lees, which may be beneficial economically, increases the risk of OA contamination. Of greater concern is the potential use of waste streams, such as rachides after crushing (section 5.5.2), marc and lees, for production of grape alcohol, or extraction of tartaric acid, tannins or grapeseed oil. OA is fairly heat stable (Roset, 2003; Arici et al., 2004) and little is known about the fate of OA during these processes, although OA has not been detected to date in grape alcohol distilled from such streams (Tarac Technologies, pers. comm. 28/07/04).

The linear decrease in OA observed by Lataste et al. (2004) during juice fermentation appears to be due to binding of OA to the increasing yeast biomass, rather than degradation. When this biomass was included in the analysis (Fig. 6.10), OA concentration was constant throughout white juice fermentation, and only decreased after the yeast cells precipitated and the wine was decanted from the gross lees. These data are supported by the findings of Bejaoui et al. (2004) and Lataste et al. (2004) discussed in section 6.1. It is, likewise, likely that OA is not degraded during red must fermentation; however, meaningful repeated sampling of homogeneous must from small-scale fermentation vessels is difficult. Nevertheless, sampling the liquid portion alone indicated the complex partitioning of OA during red must fermentation, as potentially two competing effects existed. As for juice fermentation, the increase in yeast biomass would bind OA; however, increasing alcohol concentration may have increased extraction of OA from the skins and pulp into the liquid phase. The fluctuations in OA in the liquid phase during static and rotary fermentations of red must (Fig. 6.11) were likely to result from these competing effects. For example, the maximum OA concentration observed partway through static fermentation could hypothetically be due to alcohol increasing the solubility of the toxin; however at later phases, yeast biomass increased to bind this OA again. Greater maceration of pulp by rotary fermentation may have bound more OA during the early to middle stages of fermentation, most notably at 17 °Brix. Given these differences in the amount of particulate matter generated by these fermentation styles, it is of note that the OA concentration in free run wine at pressing did not differ significantly between styles, when considering either the liquid portion of wine, or the total wine including

suspended particulate matter. Perhaps an equilibrium of OA between the solid and liquid phases was reached upon completion of must fermentation, as the amount of OA, alcohol, yeast, and grape solids would be the same for both fermentation styles, even though the grape solids from rotary fermentation would be, on average, of smaller particle size than those from static fermentation. The process of draining and then pressing the free run juice through the marc would allow the solid and liquid phases to equilibrate rapidly. OA was strongly associated with solids in free run wine and even wine after racking, as the centrifuged liquid portion of free run wine contained much less OA than the former substrates (2.8 µg/kg *cf* 18.4 µg/kg and 4.7 µg/kg in free run wine and racked wine, respectively)

Two stages other than fermentation during which OA was reduced were clarification and racking, and these were examined to ascertain the effect of commonly used adjuvants on removal of OA. Pectolytic enzymes are commonly added to grapes at crushing to enhance the recovery of juice during pressing (Rankine, 1989). No clear trends emerged regarding the effect of enzymes on OA in cloudy juice compared with that in crushed grapes (must) (Fig. 6.9), possibly due to slight differences in pressing between the different vintages and treatments. However, regardless of the presence and type of enzyme added, the concentration of OA in clarified juice (4-9% of initial concentration in must) was remarkably consistent over the three vintages with initial concentrations 4-184 µg/kg OA in must. Addition of pectolytic enzymes, thus, did not appear to enhance or disrupt the binding of OA to grape solids. It should be noted that fungal pectinases potentially present in infected fruit (activity postulated in Fig. 6.4 for Shiraz must) may have contributed substantial pectolytic activity above that of the enzymes added. These data suggested that the nature and amount of grape solids to which OA was initially bound in white juice, were the primary determinant of the extent of reduction in OA and, furthermore, that the presence of these grape solids was fairly consistent across cultivar and vintage. Whereas the addition of bentonite to cloudy juice may have compacted lees, it did not, itself, bind additional OA; neither did it increase the net amount of lees, on a wet or dry weight basis (data not shown), to which OA was bound. Hence, as observed for pectinases, addition of bentonite also did not enhance precipitation of OA with grape solids beyond that which occurred due to natural settling. The relatively poor ability of bentonite directly to bind and precipitate OA was reported by Castellari et al. (2001) during a wine fining trial.

These authors found that bentonite was an efficient adsorbent of OA per unit surface area; however, at concentrations used in wine fining, the active surface area was small and limited its efficacy compared with agents such as activated charcoal.

Data from the trial of various fining agents used in Australia are compared with published results for those agents in Table 6.5. It should be noted that the wine matrix after racking from the gross lees is markedly different from juice, in that OA is more soluble in wine than juice, due to the presence of alcohol and, furthermore, the molecules to which OA may already be bound are also likely to be soluble in wine. Thus removal of OA at this stage involves more than simple precipitation or filtration of solids. Rather, it requires either direct binding and precipitation of OA, or binding and precipitation of molecules to which OA is already bound. The significant reduction in OA achieved through addition of bentonite to Semillon wine (Fig. 6.12) was similar to that reported by Fernandes et al. (2005), and stands in marked contrast to its lack of efficacy as a juice fining agent (section 6.3.3) or as a wine fining agent reported by Castellari et al. (2001). Castellari et al. (2001) noted that bentonite was typically used to bind and precipitate proteins in wine, and postulated that proteins would compete with OA for binding sites on bentonite particles. The data in this study suggested that "sandwich" rather than competitive binding occurred; that is, OA was bound by ionic interactions (Valenta, 1998) to grape-derived proteins present at 56 mg/L wine, which, in turn, were bound to bentonite and precipitated from the wine. Indirect support for the hypothesis that OA was already bound to proteins in wine was present in the form of relatively poor reductions in OA achieved with gelatin and K-caseinate, both proteinaceous agents which were reported by Castellari et al. (2001) to be more effective than bentonite for the removal of OA. These proteins would have competed with proteins already present for binding OA, while only slightly enhancing the precipitation of various protein aggregates. This model is supported by data from Lataste et al. (2004), who demonstrated that a combination of gelatin (to bind OA) and bentonite (to bind and precipitate the gelatin) was more effective for reducing OA than combinations of gelatin and silica gel or gelatin and gallotannins. These authors also demonstrated that filtration after fining to remove fine particles and flocs further reduced OA. PVPP was not an effective fining agent for removal of OA, as previously demonstrated (Table 6.5); neither was isinglass, in this, the first test of its efficacy for toxin reduction.

The Shiraz wine in this trial did not contain detectable proteins; hence, proteinaceous fining agents such as gelatin and egg white both reduced OA slightly more than observed in the control wine with no agent added. This reduction was significant for gelatin at the higher fining rate. Gelatin achieved a greater OA reduction in the Shiraz than the Semillon wine, in terms of both amount and proportion of toxin removed. In the absence of proteins in the Shiraz wine, OA was likely to be present in wine in a freely-soluble form, thus readily bound to the gelatin, whereas in the Semillon wine, other proteins already present may have competed with gelatin for the binding of OA. These suggestions contradict the hypothesis of Castellari et al. (2001), who proposed that polyphenols in red wine would compete with OA for binding sites on gelatin, hence predicting that gelatin would be less effective in red than in white wine. The competitive interactions between OA, fining agent, proteins in wine and polyphenols, are evidently complex. The efficacy of yeast hulls added as a fining agent to bind OA has been demonstrated in this study, and is supported by findings on the binding properties of yeast material during fermentation as discussed above, or when added as a preparation of live or inactivated yeasts, or even as yeast lees after fermentation (Table 6.5; Bejaoui et al. (2004), Garcia Moruno et al. (2005)). Data from this and other fining trials have highlighted that no single fining agent, apart from, perhaps, activated charcoal (as reported in section 6.1), uniformly delivers the maximum reduction in OA in every type of wine. Rather, in the future it will be necessary to identify which constituents of wine, such as proteins or polyphenols, interact critically with OA and with the fining agents. After measuring these key parameters, it may then be possible to select the fining agent or combination of agents, and rates of addition, most likely to reduce OA while minimising impact on the wine sensory attributes.

This study has demonstrated that the majority of OA present in grapes is removed during vinification, regardless of the initial severity of contamination of the grapes, brought about, in this instance, by direct inoculation with *A. carbonarius*. Natural OA contamination of grapes at harvest appears be relatively infrequent (Sage et al., 2002; Bellí et al., 2004c; Sage et al., 2004). Battilani et al. (2005b) and Serra et al. (2005b) reported maximum OA concentrations in grapes at harvest of only 0.65 µg/kg in 2003, and 0.06 µg/kg in 2002, respectively. The highest concentration of OA occurring

naturally in grapes at harvest reported to date is 13 µg/kg, from an Italian vineyard in 1999 (Battilani et al., 2003a). An 85% reduction in OA concentration during vinification would be required for wine made from such grapes to meet the European limit of 2 µg/L (European Commission, 2005), and this extent of reduction seems achievable based on vinification data (Table 6.4). Infrequent or low levels of OA contamination, coupled with removal of OA during winemaking, are likely to have led to the relatively infrequent incidence of OA contamination of wine (Fig. 1.2).

Table 6.5: Efficacy of fining agents in this study for the reduction of ochratoxin A, compared with related studies

Citation	This study		Castellari et al. (2001)	Dumeau and Trione (2000)	Silva et al. (2003)	Fernandes et al. (2005)	
Wine type	White (Semillon)	Red (Shiraz)	Red	Red	Red	White	Red
OA source	wine made from OA contaminated grapes		OA naturally occurring in unclarified wine	OA added to wine	OA naturally occurring in wine	OA added to crushed grapes before vinification	
Initial OA concentration in wine	7.6 µg/kg 8.0 µg/kg	5 µg/kg	3.78 µg/L 1.5 µg/L	2 µg/L	1.9 µg/L	1.68 µg/L 0.64 µg/L	not stated > 0.56 µg/L[a] > 0.73 µg/L[a]
Volume	100 mL	100 mL	50 mL	not stated	500 mL	not stated	not stated
Fining incubation	> 48 h at room temperature	> 48 h at room temperature	12 h at 4 °C	48 h	25 °C, time not stated	not stated	not stated
Removal of fining agent	centrifugation, 3000 rpm x 15 min	centrifugation, 3000 rpm x 15 min	2900 *g* x 15 min	filtration	centrifugation, 5000 rpm x 15 min, then filtration	not stated	not stated
Fining agent addition rate; reduction in OA compared with initial concentration in wine							
No agent	3-7%	3-5%	not tested	not tested	not tested	29%	not stated
Gelatin white, 0.015-0.12 g/L[b] red, 0.03-0.3 g/L[b]	0.05 g/L; 2% 0.15 g/L; 11%	0.05 g/L; 12% 0.15 g/L; 21%	1 g/L; 17-30%	not tested	not tested	not tested	0.1 mL/L; 8-21% more than control[c]
Bentonite 0.5-3.0 g/L[b]	0.5 g/L; 37% 2.5 g/L; 67%	not tested	1 g/L; 8%	not tested	not tested	0.5 g/L; 40% (7-17% more than control[c])	not tested
Potassium caseinate 0.05-0.25 g/L[b]	0.1 g/L; 2% 0.25 g/L; 17%	not tested	1 g/L; 24-35%	not tested	not tested	*Casein,* 0.4 g/L; 0-13% more than control[c]	not tested

PVPP 0.1-0.8 g/L[b]	0.2 g/L; 8% 0.8 g/L; 12%	not tested	1 g/L; < 1-6%	not tested	not tested	0.16 g/L; 0-27% more than control[c]	not tested
Egg white 0.3-0.6 g egg white/L[b]	not tested	0.4 g/L (*ca* 0.16 g protein/L); 8% 0.6 g/L; 12%	1 g/L; 40-42%	not tested	not tested	not tested	0.1 g/L; 22-34% more than control[c]
Yeast hulls 1-5 g/L[d]	not tested	2 g/L; 28% 5 g/L; 43%	not tested	0.5 g/L; 13%	0.5 g/L; 13% 1.0 g/L; 32%	not tested	not tested

[a] concentration after settling with no fining agent
[b] ranges for rates of addition in common use in Australian viticulture, taken from Iland et al. (2000)
[c] reduction in OA reported relative to control, to which no fining agent was added
[d] range in common use in Australian viticulture (A. Markides, pers. comm. 21/01/04)

7 General Discussion

7.1 Strategies to minimise ochratoxin A in grapes and wine

7.1.1 Viticulture

Strategies to minimise OA in grapes can be classified into two categories: those that can be implemented as part of general vineyard management, and those more specifically targeted towards grapes at risk of OA contamination.

Damage to grapes and, to a lesser extent, spore coverage, were shown to affect the severity of Aspergillus rot. These risk factors may be reduced through vigilant vineyard management. The importance of prophylactic application of insecticides to minimise damage to grapes and subsequent OA formation was highlighted by Lataste et al. (2004). Application of both fungicidal and insecticidal sprays to minimise berry damage and bunch rot already forms the basis of most Australian vineyard management programs. Manipulation of irrigation, canopy density and bunch architecture, to reduce the risk of berry splitting and subsequent rot development associated with large, compact bunches within dense canopies, further reduces the risk of OA formation. As soil is the main reservoir of black *Aspergillus* spp. in vineyards (Kazi et al., 2004), reducing such species in soil, in turn, reduces the number of spores that may be blown onto bunches, that is, the extent of spore coverage. Practices such as minimal soil tillage, irrigation to increase soil moisture and certain types of mulch, were shown to decrease *A. carbonarius* in soils (Kazi et al., 2004; Leong et al., 2005a). Some conflicting observations between those studies and the simple water activity x temperature model examined in chapter 4 serve to highlight the complex interactions in soil which determine the long-term viability of fungal propagules. Perhaps the wide implementation of such vineyard and soil management practices in the large-scale, technology-driven viticulture associated with "new world" wine-producers, contributes to infrequent OA contamination of those wines.

In the long term, alternative management strategies could involve genetic manipulation of grapevines to increase production of pathogenesis-related (PR) proteins and phytoalexins, which confer resistance to a range of fungal pathogens. Genetic manipulation may involve plant breeding or genetic modification, the latter

requiring consumer acceptance to proceed. PR proteins and phytoalexins are produced constitutively at well defined stages of berry development, and are also induced in response to fungal infection (Jacobs et al., 1999; Tattersall et al., 2001; Jeandet et al., 2002). However, a conundrum arises. PR proteins may protect the vine from fungal infection, but these molecules resist degradation during vinification and thus cause the development of haze in white wines upon storage; methods for their removal often detrimentally alter wine quality (Ferreira et al., 2004). In contrast, phytoalexins appear to be ideal candidates for increased production through genetic manipulation, as, in addition to limiting fungal growth, the phytoalexin, resveratrol, may have positive effects on human health (Jeandet et al., 2002). Infection by *A. carbonarius* stimulated production of certain phytoalexins in berries and, in turn, these compounds incorporated into culture media restricted growth of this species (Bavaresco et al., 2003). Whereas growth was restricted by phytoalexins in those media, OA production was stimulated. Further study of the interaction of these compounds with a wide range of fungi is essential.

Targeted management strategies require prior identification of vineyards or regions in which grapes and wine are at risk of OA contamination. Several researchers in Europe report increased isolation of black *Aspergillus* spp. in warm climates, either resulting from geographic or seasonal variation (section 5.1). Increased rainfall and humidity were associated with decreasing isolation of *Aspergillus* in Italy and Portugal (Serra et al., 2003; Battilani et al., 2005b) but had no obvious effect in Spain (Bellí et al., 2005a). These and other data were incorporated into a model for incidence of black *Aspergillus* contamination of grapes in southern Europe (Battilani et al., 2005a). The critical parameters for this model were determined to be the summation of daily temperatures (positive term) and rainfall (negative term) during August; that is, the period from early veraison to berry ripeness. Increased contamination by *Aspergillus* in warm conditions is likely to be associated with optimum temperatures for growth above 35 °C for *A. niger*, the most commonly isolated species, and around 30 °C for *A. carbonarius.* The optima appear to be similar for European isolates and those from Australia examined in this study. Increased *Aspergillus* contamination in dry conditions could be associated with prolonged survival, particularly in soil (Kazi et al., 2004), as well as the general ability of black *Aspergillus* species to compete with other fungi at reduced water activities, hence their frequent isolation from dried foods (Pitt

and Hocking, 1997). Temperature was observed to exert a greater influence than water activity on growth rate in this study; likewise, the model of Battilani et al. (2005a) demonstrated that the effect of temperature on incidence of *Aspergillus* was stronger than the effect of rainfall. Those authors also noted that humidity was influenced by proximity to the sea, as well as rainfall. Most importantly, they strongly cautioned against assuming a direct correlation between incidence of *Aspergillus* spp. and OA contamination, in keeping with the findings of Battilani et al. (2005b) and Bellí et al. (2005a). This is apparent when considering that *Aspergillus* spp. increased in dry conditions, as discussed above, but OA in grapes increased in humid (Roset, 2003) and wet conditions (Battilani et al., 2003a). Humid and wet conditions may facilitate OA formation through increase in damage to berries and thus Aspergillus rot. However, such damage may involve a number of related mechanisms, such as increased canopy density, increased bunch weights, increased susceptibility to mildews or primary *Botrytis* rots, or even direct berry splitting following summer rain.

Predictive modelling of OA contamination is complex, due to several competing effects (Fig. 7.1). For example, warm temperatures increase the incidence of *Aspergillus*; however, cool temperatures extend ripening periods, and thus increase the potential for berry damage through infection by *B. cinerea* or insect infestation, both of which can lead to subsequent development of Aspergillus rot and OA formation. Likewise, black *Aspergillus* spp. infecting berries from veraison onwards may produce more OA below 20 °C, but grow faster and may remetabolise OA more rapidly above 30 °C. It seems unlikely that warm regions with a high initial incidence of *Aspergillus* contamination would experience the extended periods below 20 °C before harvest that are favourable for OA production; thus, the significance of cooler temperatures overnight requires further examination. It is possible that climate and related effects influence OA formation even more than cultivar (Battilani et al., 2004; Battilani et al., 2005b). Current models to predict incidence of *Aspergillus* could identify particular regions and seasons in which grapes were at risk for OA contamination. However, sophisticated monitoring would then be necessary to implement measures specifically to minimise OA formation in those vineyards.

Tools for monitoring the incidence of toxigenic black *Aspergillus* spp. include the simple lateral flow immuno-assay (Danks et al., 2003) to test for OA production by

fungi grown on standard culture media. This technique does not require identification to the species level. Alternatively, PCR-based methods can identify species such as *A. carbonarius* (Perrone et al., 2004), in which the majority of strains are toxigenic. Development of PCR-based methods for the detection of toxigenic *A. niger* strains is likely to be hampered by the close genetic relationship between non-toxigenic and toxigenic strains, as demonstrated in this study. In addition, such a method may not be useful for vineyard monitoring, as only a small proportion of *A. niger* strains are toxigenic and, thus, such strains probably contribute infrequently to OA contamination.

If toxigenic black *Aspergillus* spp. were frequently identified in a vineyard in the months before harvest, application of fungicidal sprays, such as those containing the combination, cyprodinil and fludioxonil, may reduce the incidence of Aspergillus rot and OA contamination (Lataste et al., 2004; Tjamos et al., 2004; Battilani et al., 2005b). In this study, fungicidal sprays did not appear to hinder development of Aspergillus rot in bunches which were artificially inoculated; however, Lataste et al. (2004) noted that fungicides which had no apparent effect on artificially inoculated bunches were, nevertheless, useful in reducing naturally occurring infection. It is not clear if data from fungicide trials in one region would be applicable to other regions or countries, given differences in factors such as climate, cultivar, canopy management and general microbiota. However, black *Aspergillus* strains characterised in chapter 3 from various regions and, indeed, substrates, appear to be interrelated at the molecular level, thus may respond to fungicides in a similar manner. The only exception to these data was the slight clustering of *A. carbonarius* strains from Queensland, one of which (FRR 5692) also grew faster and produced more OA than strains from other regions. If these physiological traits are shared by other strains in the cluster, this association between strain relatedness, physiology and location would warrant further investigation and, in turn, may require regionally-specific management strategies.

Approaches other than fungicidal sprays, which may reduce the potential for Aspergillus rot, include leaf removal (Duncan et al., 1995), which improves aeration within the canopy. It should be noted that these management techniques target formation of OA during visible infection with black *Aspergillus* spp. after veraison. The relative importance of OA production resulting from asymptomatic infection and

OA production in immature, pea size berries, the latter observed in both natural and inoculated settings (Battilani et al., 2001; Serra et al., 2005b), is yet to be established. Such modes of OA formation, if significant, could require alternative measures to minimise OA.

Vineyards identified as at risk of OA contamination would require careful monitoring during the period for Aspergillus rot development and OA formation between veraison and harvest, when berries are at a_w 0.98-0.95. In a vineyard with a high proportion of toxigenic isolates, bunches or clusters displaying severe Aspergillus rot could, if necessary, be removed by hand, as OA appears to be concentrated in severely diseased berries. Such visual inspection typically occurs during harvest of table grapes, possibly leading to the infrequent OA contamination in that commodity. However, mouldy grapes are usually discarded onto the vineyard floor, thus potentially contributing to the reservoir of *Aspergillus* spp. in the soil, as observed for table grape vineyards in Queensland.

Rapid methods for the detection of OA in grapes are required for monitoring OA contamination in vineyards and also at receival at the winery. Opportunities and limitations for PCR-based methods in this setting are discussed in chapter 3. Current methods for quantification of OA in grapes and wine rely on HPLC, which is relatively time-consuming. Immunological assays have been hampered by compounds in grapes and wine which inhibit antibody binding (Ngundi et al., 2005). A system for representative sampling of grapes both in the vineyard and at receival, coupled with a semi-quantitative analytical method to detect OA in grape juice on site, would be of immense value. The tube immunoassay format (Dewey and Meyer, 2004) described for screening fungal antigens in grape juice at receival shows some promise. Detection of OA in the weeks before harvest could prompt implementation of some of the strategies described above, whereas detection of OA at receival would provide wineries with vital information for rejecting the load.

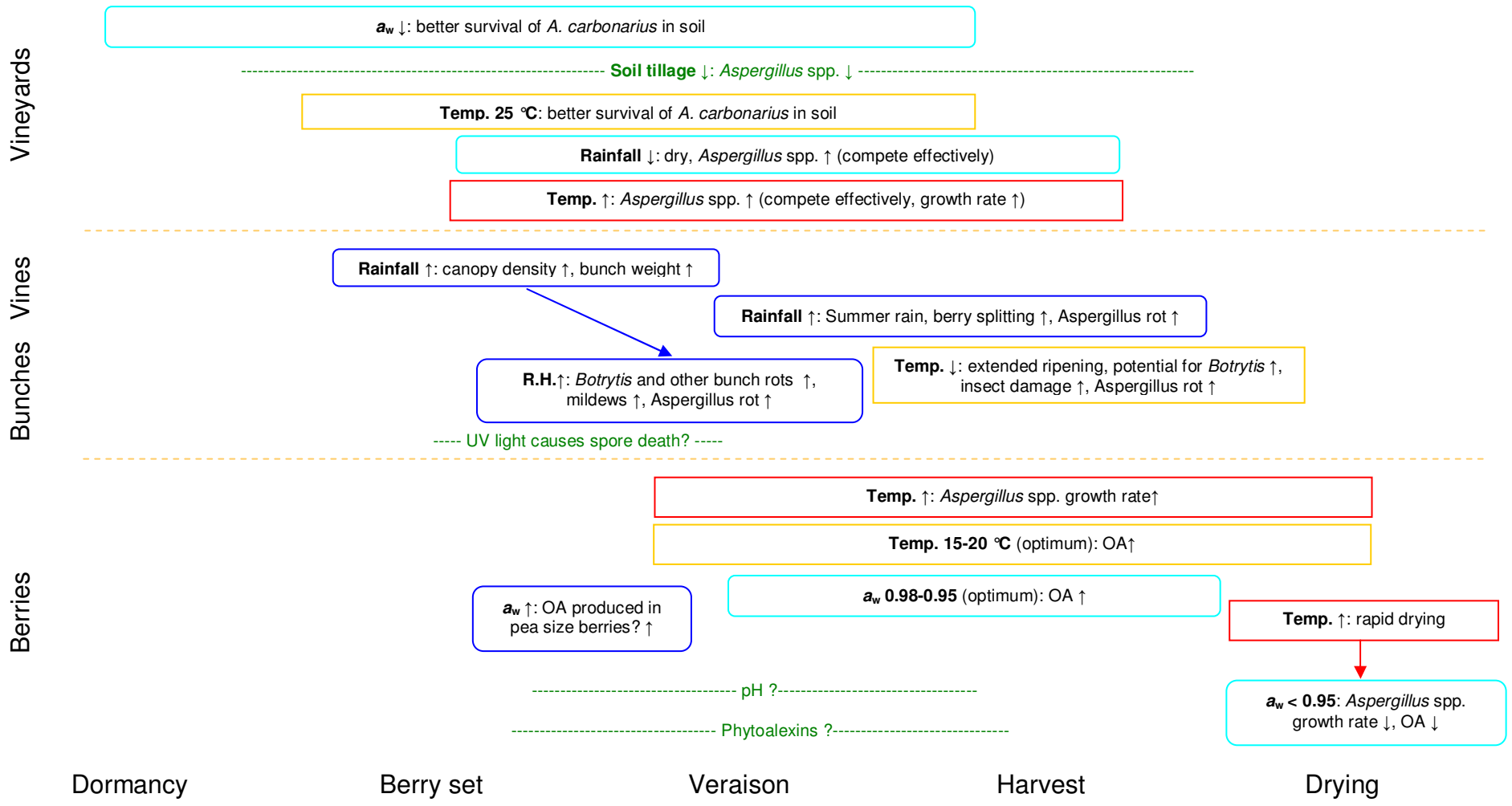


Figure 7.1: Factors which may affect the incidence and growth of black *Aspergillus* spp. in vineyards and formation of ochratoxin A in grapes, in particular, changes in temperature (Temp.) and water activity (a_w) or relative humidity (R.H.). ↑ = increase; ↓ = decrease

7.1.2 Dried vine fruit production

In addition to the strategies proposed for viticulture, rapid drying of fruit to below 0.92 a_w would minimise the risk for OA formation, given that toxin production decreases markedly between 0.95 and 0.92 a_w, especially at 25 °C and above. The finding that severely infected, discoloured berries were the primary source of OA in Semillon bunches inoculated with *A. carbonarius* supports a previous report that dried berries, darkened as a result of berry splitting and/or natural fungal infection, displayed greater black *Aspergillus* counts and OA contamination than golden berries (Leong et al., 2005a). Further refinements in laser and colour sorting technology may prove useful for removing dark amber berries and thus reducing the overall OA concentration in contaminated loads.

7.1.3 Oenology

The greatest reduction in OA was achieved during pressing of grapes, and additional reductions may be achieved through manipulation of subsequent solid:liquid separation stages. However, trials reported in chapter 6 demonstrated that certain variations in common Australian oenological practice had neither detrimental nor positive effects on OA reduction. Such practices included pectolytic enzyme treatment of white must, bentonite fining of white juice, static or rotary fermentation of red must, and recovery of juice from white grape lees or wine from red gross lees by centrifugation.

The primary mode of removal of OA during vinification was adsorption to solids and proteins, rather than degradation. In particular, binding of OA to yeast cells during fermentation appeared to play an important role in OA reduction, one that is not possible in grape juice production, leading to the observation in chapter 1 that contamination in juice was often greater than in wine in the same studies. Bejaoui et al. (2004) proposed that this adsorptive capacity of yeasts could be exploited to bind OA in synthetic and commercial grape juices. In those trials, heat- or acid-treated yeast cells removed more OA than viable cells, as the treatments denatured yeast proteins and created more binding sites; acidic conditions also enhanced binding of OA to yeast cells via protonation of the molecule. Heat-treated yeast cells bound OA more rapidly than commercially available yeast hulls, though both completely

removed OA from red grape juice (10 µg/L) within 15 min. In vinification, treated yeast cells could be added to juice or must at the start of fermentation to bind OA, in keeping with the occasional practice of adding yeast hulls to activate fermentation through the release of essential fatty acids and vitamins. This would increase the amount of OA removed by increasing the adsorptive biomass. Furthermore, fermentative yeast strains could be chosen based on efficiency in adsorbing OA (Bejaoui et al., 2004). Differences among strains of lactic acid bacteria, in terms of OA removal during malolactic fermentation (Silva et al., 2003), may also be exploited, although it is unclear whether these differences arise from differing efficiencies in converting malic acid into biomass to bind OA or if strains possess enzymes which degrade OA. Nearly equivalent amounts of OA present in wine before, and in wine and lees after, malolactic fermentation (Fernandes et al., 2005) would suggest that removal of OA occurred due to adsorption rather than degradation.

During post-fermentation fining, the adsorptive properties of yeast hulls may again be useful for reducing OA in red wines. Analysis of the initial protein content in wine may also guide the selection of a suitable fining agent or combination of agents. Agents such as bentonite may remove proteins to which OA is already bound, whereas agents such as gelatin may provide proteinaceous material to which OA can bind; these may also be used in combination. A novel technique to exploit the excellent OA-binding properties of activated charcoal (Dumeau and Trione, 2000; Castellari et al., 2001; Silva et al., 2003) has been proposed (Summary Year 3; http://www.ochra-wine.com, accessed 24/03/05), which also takes into account the tendency of charcoal to remove most of the wine pigments. A proportion of red juice was set aside before fermentation, fined with charcoal to remove OA, then added back into the fermentation vessel to extract pigments from the pomace. However, given current OA contamination levels in Australian wines, such a technique is probably not warranted in this country.

Multiple methods exist for the screening of finished wines for OA before release to the domestic market or for export; some methods are amenable to automation (Brera et al., 2003). Of note is the development, for this study, of a stable isotopic dilution method for OA analysis, suitable for a wide range of substrates because the internal

standard corrects for variations in recovery. Such a method will be useful to study the suitability of marc and lees, in which is OA concentrated, for subsequent processing.

7.2 Concluding remarks

Much progress has been made over the past 10 years since the first reports of OA in wine, in terms of identifying the source of contamination, conditions associated with the development of Aspergillus rot and toxin production, and the binding of OA to solids during vinification. Strategies applicable to both wine and dried vine fruit vineyards are being formulated for reducing the incidence of toxigenic *Aspergillus* spp. in vineyards, predicting regions in which grapes are at risk of OA contamination and controlling rots using fungicide application programs. Research to clarify whether the primary mode of infection is opportunistic, latent, or otherwise, is still required, as is information on toxin production and degradation with regard to berry maturity. Identifying the genes for OA production by black *Aspergillus* spp. and elucidating their regulation at a molecular level may yield tools for understanding the complex interactions between the fungus, environment and substrate. Extensive surveys of wines produced worldwide have demonstrated that OA is seldom present at concentrations above the 2 µg/L limit recently introduced by the European Union (European Commission, 2005); thus, it appears unlikely that this limit will restrict trade in the international wine export market, worth over US$17 billion in 2003. However, a better understanding of factors affecting OA contamination in vineyards and during winemaking will provide the grape industry with tools to manage future challenges. Such challenges may arise due to planting grapes in new regions, introduction of new cultivars, moves towards organic viticulture, or even climate change. Continued vigilance is necessary to minimise OA contamination of grapes and wine.

Appendices

A Mycological media

Inorganic reagents were of analytical grade or better and were obtained from APS (Seven Hills, NSW, Australia), BDH (Merck, Kilsyth, Vic, Australia) or Sigma (St Louis, MO, USA). Unless otherwise stated, glucose was obtained from Scalzo Food Industries (as dextrose monohydrate from wheat; Kensington, Vic, Australia), sucrose from Woolworths (Homebrand white sugar; Yennora, NSW, Australia); yeast extract and peptone from Amyl Media (Dandenong, Vic, Australia) and agar from Gelita (Beaudesert, Qld, Australia).

Coconut Cream Agar (Dyer and McCammon, 1994)

Coconut Cream (Trident, Thailand)	500 mL
Agar	15 g
RO water	500 mL

Sterilise by autoclaving at 115 °C for 15 min.

Czapek Yeast extract Agar (Pitt and Hocking, 1997)

KH_2PO_4	1 g
Czapek concentrate[a]	10 mL
Trace metal solution[b]	1 mL
Yeast extract, powdered	5 g
Sucrose	30 g
Agar	15 g
RO water	1 L

Sterilise by autoclaving at 121 °C for 15 min.

[a]*Czapek concentrate*

KCl	25 g
$NaNO_3$	150 g
$MgSO_4.7H_2O$	25 g
$FeSO_4.7H_2O$	0.5 g
RO water	500 mL

[b]*Trace metal solution*

$CuSO_4.5H_2O$	0.5 g
$ZnSO_4.7H_2O$	1 g
RO water	100 mL

Dichloran Rose Bengal Chloramphenicol agar (Pitt and Hocking, 1997)

Glucose	10 g
Peptone, bacteriological	5 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Agar	15 g
Rose Bengal	25 mg (5% w/v in water, 0.5 mL)
Dichloran	2 mg (0.2% w/v in ethanol, 1 mL)
RO water	1 L

Sterilise by autoclaving at 121 °C for 15 min.

Isotopically-labelled glucose medium

KCl	0.5 g
$NaNO_3$	3 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
$CuSO_4.5H_2O$	0.005 g
$ZnSO_4.7H_2O$	0.01 g
K_2HPO_4	1 g
Yeast extract	2 g
Glucose	20 g
RO water	1 L

pH adjusted to 6 with 10 M HCl.

All the constituents apart from the glucose were sterilised by autoclaving for 121 °C for 15 min.

U13-C D-glucose was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). The glucose was made up as a 20% w/v solution in RO water and sterilised by filtration through a 0.2 µm membrane (Millipore, Billerica, MA, USA). It was added to the medium prior to inoculation (see Appendix B).

Spezielle Nahstoffarmer Agar medium (modified from Nirenberg (1976))

KH_2PO_4	1.0 g
KNO_3	1.0 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
Glucose	2.0 g
Sucrose	2.0 g
Yeast extract	1.0 g
RO water	1 L

Sterilise by autoclaving at 121 °C for 15 min.

Synthetic Grapejuice Medium (modified from Mitchell et al. (2003); Bellí et al. (2004b))

D(+) Glucose[a]	70 g
D(-) Fructose[b]	30 g
L(+) Tartaric Acid[a]	7 g
L(-) Malic acid[a]	10 g
$(NH_4)_2SO_4$	0.67 g
$(NH_4)_2HPO_4$	0.67 g
KH_2PO_4	1.5 g
$MgSO_4.7H_2O$	0.75 g
NaCl	0.15 g
$CaCl_2$	0.15 g
$CuCl_2$	0.0015 g
$FeSO_4.7H_2O$	0.021 g
$ZnSO_4$	0.0075 g
(+) Catechin[a]	0.05 g

Made up to 1 L with RO water.
pH adjusted to 4.2 with 10 M NaOH.

Agar	25 g

Sterilise by autoclaving at 100 °C for 30 min.

[a] Sigma, St Louis, MO, USA
[b] Univar, APS, Seven Hills, NSW, Australia

Yeast Extract Sucrose agar (modified from Samson et al. (2000))

Yeast extract	20 g
Sucrose	150 g
Trace metal solution[b]	1 mL (as for Czapek Yeast Agar)
Agar	15 g
RO water	1 L

Sterilise by autoclaving at 121 °C for 15 min.

B Preparation of isotopically-labelled ochratoxin A

A few spores from a culture of *Aspergillus ochraceus* FRR 3846, isolated from Australian soybeans, grown on CYA at 25 °C for 7 d, were gently collected on the point of a sterile inoculating wire under a stereomicroscope, and carefully inoculated into isotopically-labelled glucose medium (10 mL x 16; Appendix A) in 100 mL conical flasks, with minimal disturbance to the liquid. Flasks were stoppered with cotton wool bungs, and incubated at 25 °C in the dark for 7 d, after which the liquid was harvested with a pasteur pipette.

Purification and characterisation of the isotopically-labelled standard were performed by Peter Varelis and Georgina Giannikopoulos of the Analytical Chemistry section, Food Science Australia. Briefly, the liquid and biomass from the flasks were combined and OA quantitatively extracted first with 75 mL diethyl ether, then with 75 mL 1% sodium bicarbonate. For purification, the aqueous extract was extracted into 50 mL ether, then 50 mL cyclohexane. This layer was then acidified using HCl (10 N) before repeated extraction into ether (3 x 75 mL). The aqueous layer was purified using 900 mg C18 solid phase extraction cartridges (Maxi-Clean™, Alltech, Deerfield, USA). The cartridges were washed with methanol and then eluted with acetonitrile. All organic extracts were combined and dried under vacuum at 40 °C to yield an orange solid with a caramelised odour. The solid was dissolved in 1.5 mL 50% ethyl acetate in cyclohexane and the analyte was purified from other metabolites using a Chromatotron® (under argon; 55% ethyl acetate in cyclohexane, 0.1% formic acid, 1 ml/min; Harrison Research, Palo Alto, CA, USA). All extracts containing a high content of OA were combined, and the flask dried under vacuum at room temperature. The analyte was reconstituted in methanol and analysed by LC-MS (Figure B.1). The chromatogram demonstrates the presence of pure OA and the mass spectrum shows the presence of $^{13}C_{11}$-OA (70% yield) and $^{13}C_{20}$-OA (30% yield) and their corresponding sodium adducts. The effective concentration of isotopically-labelled OA was approximately 1.6 µg/mL.

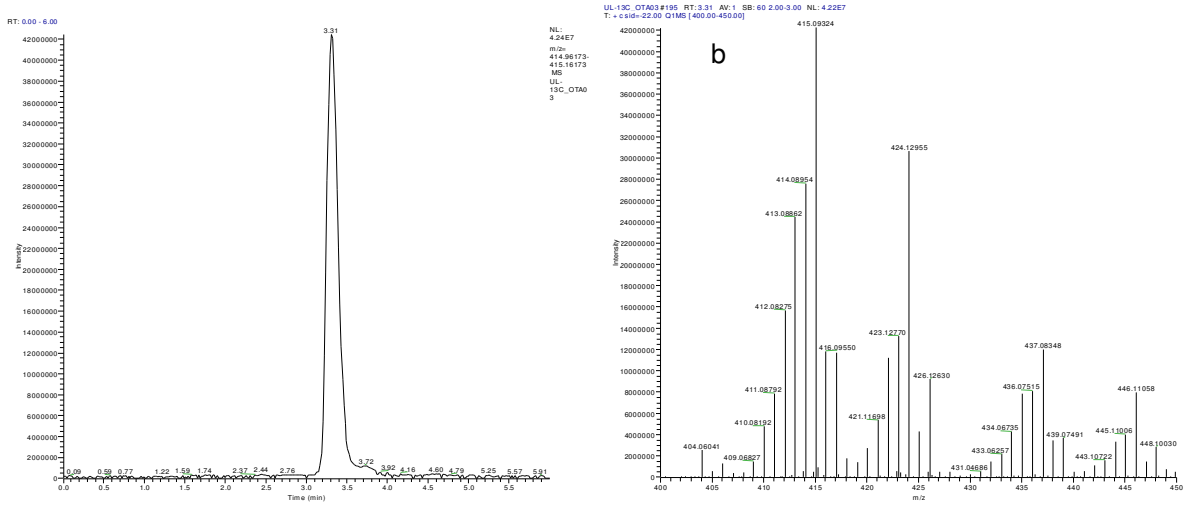


Figure 0.1: Chromatogram of (a) ^{13}C-ochratoxin A and (b) the corresponding mass spectrum

C Molecular biology reagents

Unless otherwise stated, reagents were obtained from Promega (Madison, WI, USA) or Sigma (St Louis, MO, USA).

DNA extraction buffer

Tris-HCl	100 mM, pH 8.0
Ethylenediamine tetraacetic acid (EDTA)	20 mM
NaCl	0.5 M
Sodium dodecyl sulfate (SDS)	1 % w/v

Dissolve 2.42 g Tris in 100 mL Milli-Q water, checking that the pH is 8.0. Then add 8.0 mL of 0.5 M EDTA, 5.8 g NaCl and 2.0 g SDS. Make up to 200 mL with water, autoclave. Warm the solution in the microwave for a few seconds to dissolve the precipitate before use.

Protein Precipitating Solution

Potassium acetate	3 M
Glacial Acetic Acid	4 % (approximately)

Slowly add 29.45 g potassium acetate to 60 mL Milli-Q water while stirring. Adjust to pH 5.5 with glacial acetic acid. Make up to 100 mL with water, autoclave.

Binding matrix (1/5 strength)

Binding matrix	66 mL (BIO 101, Inc., Carlsbad, CA, USA, ordered from Integrated Sciences, Willoughby, NSW, Australia)
Guanidine isothiocyanate	6 M

Dissolve 187.2 g guanidine isothiocyanate in 264 mL sterile Milli-Q water. Add 66 mL Binding Matrix (one bottle) and mix.

Salt Ethanol Wash Solution

Sodium acetate	100 mM
Ethanol	70 % v/v

Dissolve 4.1 g sodium acetate in 130 mL Milli-Q water, add 370 mL 95% ethanol.

Tris-EDTA buffer, pH 8

Tris	10 mM
EDTA	1 mM

Tris-Borate EDTA buffer, pH 8.3

Tris-Borate	45 mM
EDTA	1 mM

Agarose gels

Agarose	2 % w/v

Make up in TBE buffer.

D Molecular data

Table D.1: Presence or absence of bands scored in the analysis of ERIC-PCR amplification of black *Aspergillus* spp.

Species	Accession no.	Order on gel, Fig. 3.4	Presence / absence[a] of bands at loci 1 - 16															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A. niger	A-75[b]	1	1	0	1	0	0	0	1	0	0	0	1	1	0	0	1	0
A. niger	A-136	2	1	1	1	0	0	0	1	0	0	0	1	1	0	0	1	0
A. niger	A-942	3	1	1	1	0	0	0	1	0	0	1	1	0	0	0	0	0
A. niger	A-943	4	1	1	0	1	0	0	1	0	1	0	0	1	0	0	1	1
A. niger	CBS 139.52[b]	5	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1
A. aculeatus	A-1122	6	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1
A. aculeatus	A-1325	7	0	1	1	0	0	0	0	1	1	0	1	1	0	0	0	0
A. aculeatus	A-1355	8	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1
A. carbonarius	NRRL67[b]	9	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0
A. carbonarius	CBS 127.49	10	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0
A. carbonarius	A-941	11	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	A-881	12	0	1	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. niger	FRR 2522[b]	13	0	1	1	0	0	0	1	0	0	0	1	0	1	1	0	0
A. niger	FRR 333	14	0	1	1	0	0	0	1	0	1	0	1	0	1	1	0	0
A. carbonarius	FRR 5374	15	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5573	16	0	0	0	1	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5574	17	0	0	0	1	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5682	18	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5683	19	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5696	20	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5693	21	0	0	1	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5697	22	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0
A. carbonarius	FRR 5698	23	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0
A. niger	CBS 618.78	24	1	1	0	1	1	0	1	0	0	0	1	1	0	0	1	0
A. niger	CECT 2088[b]	25	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	1

Table D.1 (cont.)

Species	Accession no.	Order on gel, Fig. 3.4	Presence / absence[a] of bands at loci 1 - 16															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A. niger	CBS 554.65	26	1	0	1	0	0	0	0	1	0	0	1	1	0	0	0	1
A. niger	A-946	27	1	1	1	0	0	0	1	0	0	0	1	1	0	0	1	0
A. niger	CBS 134.48	28	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0
A. niger	CBS 121.55	29	0	0	1	0	0	0	1	1	0	0	1	0	0	0	1	1
A. niger	A-947	30	1	1	0	0	0	0	0	1	0	0	0	1	0	0	1	0
A. niger	A-1241	31	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0
A. niger	A-220	32	0	0	0	1	0	0	0	1	0	0	1	1	0	0	1	0
A. niger	w-148[b]	33	1	0	1	0	1	0	0	0	0	0	0	1	0	0	1	1
A. niger	A-655	34	1	0	1	0	1	0	0	0	0	0	0	1	0	0	1	1
A. niger	A-487	35	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0
A. niger	A-615	36	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1
A. niger	A-656	37	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
A. carbonarius	CBS 110.49	38	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0
A. carbonarius	A-642	39	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0
A. carbonarius	A-1040	40	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0
A. carbonarius	A-1070	41	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	A-1477	42	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	A-1500	43	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. aculeatus	A-1356	44	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1
A. carbonarius	FRR 5699	45	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5700	46	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5690	47	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5702	48	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5703	49	0	0	1	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5704	50	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0
A. carbonarius	FRR 5705	51	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0
A. carbonarius	FRR 5706	52	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0
A. carbonarius	FRR 5707	53	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0

A. carbonarius	FRR 5708	54	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5709	55	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0
A. carbonarius	FRR 5710	56	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5711	57	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5691	58	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0
A. carbonarius	FRR 5712	59	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5713	60	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5714	61	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5715	62	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5716	63	0	0	0	0	0	0	1	1	0	0	0	1	0	0	1	0
A. carbonarius	FRR 5717	64	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5718	65	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5692	66	1	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0
A. carbonarius	FRR 5719	67	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0
A. niger	FRR 5694	68	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0
A. niger	FRR 5695	69	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
A. niger	FRR 5720	70	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
A. niger	FRR 5721	71	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
A. niger	FRR 5722	72	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0

[a] presence of the band scored as 1, absence scored as 0

[b] A and w: isolates from the collection of the Autonomous University of Barcelona, Veterinary Faculty, Bellaterra, Barcelona, Spain. CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands. NRRL: Agricultural Research Service Culture Collection, Peoria, IL, USA. FRR: Food Science Australia, North Ryde, NSW, Australia. CECT: Coleccion Espanola de Cultivos Tipo, Burjassot, Valencia, Spain

Table D.2: Polymorphism in allele size at six microsatellite loci in black *Aspergillus* spp.

Species	Accession no.	Locus Allele size (bp)					
		ACNM1	ACNM2	ACNM3	ACNM5	ACNM6	ACNM7
A. niger	A-75[a]	475	428	195	164	452	402
A. niger	A-136	485	428	192	164	455	405
A. niger	A-220	485	428	192	164	455	405
A. niger	A-487	473	null	186	164	null	387
A. niger	A-615	475	418	181	164	null	378
A. niger	A-655	473	409	184	166	431	387
A. niger	A-656	474	418	181	163	null	399
A. niger	A-942	509	418	189	163	431	399
A. niger	A-943	null	409	184	163	431	387
A. niger	A-946	490	446	186-195	204	449	388
A. niger	A-947	474	null	181	164	null	382
A. niger	A-1241	483	428	195	164	446	388
A. niger	w-148[a]	473	418	189	166	431	387
A. niger	CECT 2088[a]	490	428	215	201	455	402
A. niger	CBS 134.48[a]	474	null	186	164	null	382
A. niger	CBS 121.55	477	416	184	163	450	387
A. niger	CBS 139.52	483	428	195	164	446	388
A. niger	CBS 554.65	485	428	192	190	452	388
A. niger	CBS 618.78	487	428	192	193	446	402
A. niger	FRR 2522[a]	490	418	184	163	429	390
A. niger	FRR 333	477	416	184	163	429	387
A. niger	FRR 5694	null	not analysed	190	163	431	387
A. niger	FRR 5695	467	not analysed	177	167	431	468
A. niger	FRR 5720	473	null	181	164	null	382
A. niger	FRR 5721	473	null	181	164	null	382
A. niger	FRR 5722	475	446	198	201	458	405

A. carbonarius	A-1040	null	413	189	null	null	null
A. carbonarius	A-1070	null	413	189-192	null	null	null
A. carbonarius	A-1477	null	413	189-192	null	null	null
A. carbonarius	A-1500	null	413	190	null	null	null
A. carbonarius	A-642	null	413	189-192	null	null	null
A. carbonarius	A-881	null	null	189	null	null	null
A. carbonarius	A-941	null	null	189-192	null	null	null
A. carbonarius	CBS 110.49	null	413	189-192	null	null	null
A. carbonarius	CBS 127.49	null	null	189-192	null	null	null
A. carbonarius	FRR 5374	466	null	189	164	null	399
A. carbonarius	FRR 5573	null	null	189-192	163	null	null
A. carbonarius	FRR 5574	null	null	189-192	null	null	null
A. carbonarius	FRR 5682	null	null	189-192	null	null	null
A. carbonarius	FRR 5683	null	413	189-192	null	null	null
A. carbonarius	FRR 5690	null	413	189-192	null	null	null
A. carbonarius	FRR 5691	null	413	189-192	null	null	null
A. carbonarius	FRR 5692	466	413	189-192	null	null	null
A. carbonarius	FRR 5693	null	413	189-192	null	null	null
A. carbonarius	FRR 5696	null	413	189-192	null	null	null
A. carbonarius	FRR 5697	466	413	189-192	null	null	null
A. carbonarius	FRR 5698	466	413	189-192	null	null	null
A. carbonarius	FRR 5699	null	null	189-192	null	null	null
A. carbonarius	FRR 5700	null	413	189,192,194	null	null	null
A. carbonarius	FRR 5702	null	413	192	null	null	null
A. carbonarius	FRR 5703	null	413	189-192	null	null	null
A. carbonarius	FRR 5704	null	413	189-192	null	null	null
A. carbonarius	FRR 5705	null	null	189	null	null	387
A. carbonarius	FRR 5706	466	null	189-192	null	null	null
A. carbonarius	FRR 5707	null	413	189-191	null	null	387
A. carbonarius	FRR 5708	null	null	189-192	null	null	null
A. carbonarius	FRR 5709	null	413	189-192	null	null	387

Table D.2 (cont.)

Species	Accession no.	Locus Allele size (bp)					
		ACNM1	ACNM2	ACNM3	ACNM5	ACNM6	ACNM7
A. carbonarius	FRR 5710	null	413	189-192	null	null	null
A. carbonarius	FRR 5711	null	413	189-192	null	null	null
A. carbonarius	FRR 5712	466	413	189-192	null	null	387
A. carbonarius	FRR 5713	null	413	189-192	null	null	null
A. carbonarius	FRR 5714	null	413	189	null	null	null
A. carbonarius	FRR 5715	466	413	189-192	163	null	399
A. carbonarius	FRR 5716	466	413	189-192	null	null	null
A. carbonarius	FRR 5717	466	413	189-192	null	null	null
A. carbonarius	FRR 5718	null	null	189-192	null	null	null
A. carbonarius	FRR 5719	null	413	151,181,190,192	null	null	null
A. carbonarius	NRRL67[a]	null	null	189-192	164	null	null
A. aculeatus	A-1122	464	null	189-192	null	null	null
A. aculeatus	A-1325	465	null	189-192	null	null	null
A. aculeatus	A-1355	465	null	189-192	null	null	null
A. aculeatus	A-1356	465	null	189-192	null	null	null

[a] A and w: isolates from the collection of the Autonomous University of Barcelona, Veterinary Faculty, Bellaterra, Barcelona, Spain. CECT: Coleccion Espanola de Cultivos Tipo, Burjassot, Valencia, Spain. CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands. FRR: Food Science Australia, North Ryde, NSW, Australia. NRRL: Agricultural Research Service Culture Collection, Peoria, IL, USA

E Spray application and sampling times in Vineyard D, Hunter Valley

Table E.1: Spray application, 2003

Spray dates for Semillon + Shiraz (approximate dates for Chardonnay in brackets)	Standard program (applied to Chardonnay, Semillon and Shiraz)		Syngenta program (applied to Semillon only)	
	Product[a]	Rate (g or mL/100L)	Product[a]	Rate (g or mL/100L)
16/09/2002: bud burst (Chardonnay, 9/09/02)	Thiovit Jet	500	Thiovit Jet	500
27/09/2002 (Chardonnay, 20/09/02)	Delan 700 WG Delfin WG Topas 100 EC Thiovit Jet	50 12.5 12.5 300	Delan 700 WG Delfin WG Topas 100 EC Thiovit Jet	50 12.5 12.5 300
18/10/2002: 10% flower (Chardonnay, 11/10/02)	Bravo	1L/ha		
21/10/2002: 10% flower			Amistar WG	50
28/10/2002: 80% flower (Chardonnay, 21/10/02)	Scala 400 EC Mancozeb Thiovit Jet	200 200 300	Switch Mancozeb Thiovit Jet	80 200 300
19/11/2002: bunch closure (Chardonnay, 12/11/02)	Fortress 500	75	Fortress 500	75
Shiraz, inoculation at pre-bunch closure (20/11/02)				
23/11/2002 (Chardonnay, 16/11/02)	Copper oxychloride Thiovit Jet Success Naturalyte	200 300 40	Copper oxychloride Thiovit Jet Success Naturalyte	200 300 40
Chardonnay, inoculation at pre-bunch closure (20/11/02) Semillon, inoculation at pre-bunch closure (5/12/02)				
16/12/2002 (Chardonnay, 9/12/02)	Thiovit Jet Copper oxychoride	300 200	Thiovit Jet Copper oxychloride	300 200
Chardonnay, inoculation at veraison (17/12/02) Shiraz, inoculation at veraison (17/12/02)				
28/12/2002: veraison (Chardonnay, 21/12/02)	Crop Care Captan WG Thiovit Jet	125 300	Thiovit Jet	300
Semillon, inoculation at pre-harvest (6/01/03)				
15/01/2003	Delfin WG	12.5	Delfin WG	12.5

(Chardonnay, 8/01/03)				
Chardonnay, inoculation at pre-harvest (13/01/03) Shiraz, inoculation at pre-harvest (21/01/03)				
Chardonnay, harvested 24/01/2003 Semillon, harvested 27/01/2003 Shiraz, harvested 6/02/2003				

[a] active constituent bracketed: Amistar WG (azoxystrobin), Bravo (chlorothalonil), Crop Care Captan WG (captan), Delan 700 WG (dithianon), Delfin WG (*Bacillus thuringiensis* var. *kurstaki*), Fortress 500 (procymidone), Mancozeb (mancozeb), Thiovit Jet (sulfur), Topas 100 EC (penconazole), Scala 400 EC (pyrimethanil), Success Naturalyte (spinosad), Switch (cyprodinil + fludioxonil)

Table E.2: Spray application, 2004

Cultivar, spray date	Standard program	
	Product[a]	Rate (g or mL/100L)
Chardonnay, 29/08/2003 Semillon + Shiraz, 2/09/2003	Thiovit Jet	500
Chardonnay, 8/09/2003 Semillon + Shiraz, 10/9/2003	Thiovit Jet Full-Bac WDG	250 12.5
Chardonnay, 22/09/2003 Semillon + Shiraz, 26/09/2003	Delan 700 WG Full-Bac WDG Topas 100 EC Thiovit Jet	50 12.5 12.5 250
Chardonnay, 29/09/2003	Dipel DF Dithane 750 DF Kumulus DF Topas 100 EC	12.5 200 200 12.5
Chardonnay, 15/10/2003 Semillon + Shiraz, 14/10/2003	Bugmaster Flowable Dithane 750 DF Kumulus DF (Chardonnay) Mancozeb (Semillon + Shiraz)	150 200 200 200
Chardonnay, 20/10/2003 Semillon + Shiraz, 21/10/2003	Barrack 720	180
Chardonnay, 24/10/2003 Semillon + Shiraz, 28/10/2003	Kumulus DF Legend Scala 400 EC	200 20 200
Chardonnay, 5/11/2003 Semillon + Shiraz, 19/11/2003	Dithane Rainshield Kumulus DF Full-Bac WDG (Semillon + Shiraz)	200 300 12.5
Shiraz, inoculation at pre-bunch closure (25/11/2003)		
Chardonnay, 19/11/2003 Semillon + Shiraz, 28/11/2003	Dithane Rainshield (Chardonnay) Kumulus DF Success Naturalyte Country Copper Oxychloride 500 WP (Semillon + Shiraz)	300 300 20 400
Chardonnay, inoculation at pre-bunch closure (25/11/2003)		

Chardonnay, 27/11/2003 Semillon + Shiraz, 9/12/2003	Dithane Rainshield Kumulus DF Switch	200 300 80
Chardonnay, 10/12/2003	Dithane Rainshield Kumulus DF	200 300
Semillon + Shiraz, 17/12/2003	Agri-fos 400 Country Copper Oxychloride 500 WP Kumulus DF	6 L / ha 400 300
Chardonnay, inoculation at veraison (22/12/2003)		
Chardonnay, 23/12/2003 Semillon + Shiraz, 23/12/2003	Country Copper Oxychloride 500 WP (Chardonnay) Sulfostar DF Kocide Blue (Semillon + Shiraz)	400 300 150
Chardonnay, 27/12/2003 Semillon + Shiraz, 2/01/2004	Crop Care Captan WG Sulfostar DF Sulfostar DF	125 300 (Chardonnay) 200 (Semillon + Shiraz)
Shiraz, inoculation at veraison (6/01/2004) Semillon, inoculation at 30 d pre-harvest (6/01/2004)		
Chardonnay, 6/01/2004	Country Copper Oxychloride 500 WP Sulfostar DF	400 300
Chardonnay, inoculation at pre-harvest (15/01/2004)		
Semillon + Shiraz, 8/01/2004	Delfin WG	12.5
Semillon, inoculation at 20 d pre-harvest (15/01/2004) Semillon, inoculation at 10 d pre-harvest (26/01/2004) Shiraz, inoculation at pre-harvest (26/01/2004)		
Chardonnay, 27/01/2004	Citric Acid Potassium metabisulphite	200 500
Semillon + Shiraz, 27/01/2004	Spiral Aquaflow	75
Chardonnay, harvested 4/02/2004 Semillon, harvested 4/02/2004 Shiraz, harvested 19/02/2004		

[a] active constituent bracketed: Agri-fos 400 (phosphorous acid), Barrack 720 (chlorothalonil), Bugmaster Flowable (carbaryl), Country Copper Oxychloride 500 WP (copper oxychloride), Crop Care Captan WG (captan), Delan 700 WG (dithianon), Delfin WG (*Bacillus thuringiensis* var. *kurstaki*), Dipel DF (*Bacillus thuringiensis* var. *kurstaki*), Dithane 750 DF (mancozeb), Dithane Rainshield (mancozeb), Full-Bac WDG (*Bacillus thuringiensis* var. *kurstaki*), Kocide Blue (copper hydroxide), Kumulus DF (sulfur), Mancozeb (mancozeb), Legend (quinoxyfen), Thiovit Jet (sulfur), Topas 100 EC (penconazole), Scala 400 EC (pyrimethanil), Spiral Aquaflow (procymidone), Success Naturalyte (spinosad), Sulfostar DF (sulfur), Switch (cyprodinil + fludioxonil)

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