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

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Statement of originality

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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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

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