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► **To cite this version:**

Angelo Visconti. Managing ochratoxin A risk in the grape-wine food chain. Food Additives and Contaminants, 2008, 25 (02), pp.193-202. 10.1080/02652030701744546 . hal-00577313

HAL Id: hal-00577313

<https://hal.science/hal-00577313>

Submitted on 17 Mar 2011

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Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2007-342.R1
Manuscript Type:	Special Issue
Methods/Techniques:	Mycology
Additives/Contaminants:	Mycotoxins
Food Types:	Wine, Fruit juices

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1 **Managing ochratoxin A risk in the grape-wine food chain**

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5

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For Peer Review Only

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3 1 **ABSTRACT**
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5 2 The main source of ochratoxin A (OTA) in the wine food chain is the infection of grapes by
6
7 3 “black aspergilli” occurring in the field. OTA producing black aspergilli include mainly
8
9 4 *Aspergillus carbonarius*, followed by *A. niger* and possibly *A. tubingensis*. They are
10
11 5 opportunistic fungi that may develop massively on damaged berries at ripening, although they
12
13 6 may occur and form OTA on grapes from veraison to harvest. Climatic conditions (high
14
15 7 humidity and temperature) and geographical location are important factors favouring OTA
16
17 8 accumulation in grape berries. The severity of aspergillus rot is influenced by excessive
18
19 9 irrigation and rainfall prior to harvest causing berry splitting. Berry wounds caused by insect
20
21 10 attacks provide preferential entries for black aspergilli. High OTA levels occur in grapes
22
23 11 severely damaged by the grape moth *Lobesia botrana*, particularly in the Mediterranean areas.
24
25 12 Some grape varieties display greater susceptibility to aspergillus rots due to intrinsic genetic
26
27 13 characteristics and bunch conformation (compact > sparse). Control measures for toxigenic
28
29 14 mycoflora in the vineyards must consider these critical control points. Proper fungicidal and
30
31 15 insecticidal treatments can reduce OTA contamination. Knowledge about the fate of OTA and
32
33 16 its distribution in wine and winery by-products is important to manage OTA risk in
34
35 17 contaminated stock. In our wine-making experiments, only 4% of the OTA present in grapes
36
37 18 remained in the wine whereas most is retained in pressed grape pomaces. OTA concentration
38
39 19 remained unchanged in wine after one year aging as well as in all liquid fractions collected
40
41 20 during vinification (must, free run wine, and wine after first and second decantation).
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43 21 Activated carbon can reduce OTA levels in wine but negatively affects wine quality.
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55 23 **KEYWORDS:** ochratoxin A, black aspergilli, mycotoxins, grape, wine, food safety
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1 INTRODUCTION

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8 Ochratoxin A (OTA) is a major mycotoxin produced by several species of *Aspergillus* and
9
10 *Penicillium* naturally occurring in a variety of food commodities prior to harvest or more
11
12 commonly during storage. Numerous animal studies have shown that OTA is a potent
13
14 nephrotoxin with the degree of renal injury depending on both toxin, dose and exposure time;
15
16 decreasing nephrotoxic sensitivity was observed from pig to rat, to mice. OTA is
17
18 immunotoxic, neurotoxic *in vitro* and *in vivo* in rats, teratogenic in mice, rats and rabbits
19
20 (JECFA 2001). Based on renal carcinogenicity shown in rats and mice, the IARC
21
22 (International Agency for Research on Cancer) has classified OTA as a Group 2B carcinogen,
23
24 i.e. carcinogenic to animals and possible carcinogenic to human (IARC 1993). Studies on the
25
26 genotoxicity of OTA remain controversial. Recent scientific evidence indicates that the site-
27
28 specific renal toxicity as well as the DNA damage and genotoxic effects of OTA, measured in
29
30 various *in vivo* and *in vitro* studies, are most likely attributable to cellular oxidative damage
31
32 (EFSA 2006). Various studies in humans have associated OTA with an endemic kidney
33
34 disease observed in the Balkans (Balkan Endemic Nephropathy and related Urinary Tract
35
36 Tumors), but convincing epidemiological evidence associated with OTA exposure is currently
37
38 lacking. It has been frequently found in human blood, urine and milk and has been
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40 demonstrated a widespread individual exposure to low levels of OTA in Europe and other
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42 continents (EFSA 2006).
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52 The widespread human exposure to OTA is well documented by a number of surveys
53
54 reporting the occurrence of OTA in a variety of food products. An assessment of the dietary
55
56 intake of OTA by the population of the European Community has been performed showing
57
58 that the main contributors to OTA exposure are cereals and cereal products (European
59
60

1 Commission 2002). Wine, coffee and beer were identified as significant contributors to
2 human OTA exposure. Dried vine fruit and grape juice contribute to a significant extent to the
3 OTA exposure of vulnerable groups of consumers such as children. The EFSA Scientific
4 Panel on contaminants in the food chain has recently adopted an updated scientific opinion
5 relating to OTA in food, taking into account new scientific informations and derived a
6 tolerable weekly intake (TWI) of 120 ng/kg body weight (EFSA 2006).

7
8 Based on the available scientific toxicological and exposure data, the European Union
9 established maximum permitted limits for OTA in a variety of food products that have been
10 updated with the EC Regulation 1881/2006 (European Commission 2006). Maximum levels
11 have been set at 2 µg/kg for wine, fruit wine, grape juice, grape nectar and grape must
12 intended for direct human consumption, and at 10 µg/kg for dried vine fruit (currants, raisins
13 and sultanas). OTA was detected in wine for the first time in 1996 (Zimmerli and Dick 1996).
14 Thereafter several surveys have been conducted, mainly in Europe, on the occurrence of the
15 toxin in wine and related products, showing it as a problem mainly for Southern Europe. The
16 results of several reports from different countries are reported in Table 1 for a total of 3512
17 samples. A high incidence of contamination (from 40% to 87%) was reported in all surveys
18 but the Australian one having an incidence of 15% (Hocking et al. 2003), with maximum
19 level at 15.6 µg/kg recorded in Italy (European Commission 2002). OTA levels showed a
20 decreasing gradient from red to rosé, to white wines, and the same trend was observed for the
21 grape juice. Wines from southern and warmer regions of Europe showed incidence and levels
22 of contamination (72.3%, mean value 0.64 µg/kg, n = 635) higher than those from northern
23 European areas (incidence 50.3%, average 0.18 µg/kg, n = 835). Wines produced in Southern
24 Italy, where the climatic conditions favour the growth of OTA producing fungi in grapes,
25 generally show incidence and levels of contamination higher than wines produced in Northern

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2
3 1 and Central Italy. Recently, a survey of retailed wine samples from Southern Italy showed a
4
5 2 positive correlation between high levels of OTA and resveratrol-related compounds (Perrone
6
7 3 et al. 2007b). Considering that a significant reduction of hepatic and renal damage caused by
8
9 4 OTA was reported in mice fed with grape juice contaminated with OTA (Jeswal 1998), there
10
11 5 may be some possibility that toxic levels of OTA in red wine are, at some extent,
12
13 6 counterbalanced by the beneficial effects of resveratrol derivatives.
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20 8 In the following headings different aspects of OTA formation in grapes and strategies for
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22 9 prevention and control in the field are reported together with OTA distribution and possible
23
24 10 corrective actions during wine processing. Good agriculture and manufacturing practices are
25
26 11 currently under discussion by *Codex Alimentarius* for the adoption of a code of practice for
27
28 12 the prevention and reduction of OTA contamination in wine, based on a code of sound
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30 13 viticultural practices adopted by the Organisation Internationale de la Vigne et du Vin (OIV)
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32 14 in 2005 (FAO/WHO 2007).
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39 16 **Fungi responsible for OTA accumulation in grapes: taxonomy and ecology.**

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41 17 Fungi responsible for OTA accumulation in cereals, i.e., *Aspergillus ochraceus* and
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43 18 *Penicillium verrucosum*, were firstly thought to be involved also in OTA formation in grapes.
44
45 19 However, a number of studies performed during the last decade provide evidence of a quite
46
47 20 different situation showing that all fungi responsible for OTA in grapes belong to *Aspergillus*
48
49 21 section *Nigri*, the so called “black aspergilli” (Battilani et al. 2003). Most epidemiological
50
51 22 surveys, performed in Mediterranean, Australian and South American countries, have shown
52
53 23 that within the black aspergilli the biseriata species, *Aspergillus carbonarius* and *A. niger*
54
55 24 “aggregate”, and the uniseriate species, *A. aculeatus* and *A. japonicus*, are the prevalent ones
56
57 25 occurring on grapes (Battilani et al. 2003; Da Rocha Rosa et al. 2002; Leong et al. 2006a).
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5 2 The taxonomy of this Section is still not completely resolved, especially within the *A. niger*
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8 3 “aggregate” (a group of morphologically indistinguishable species), leading often to
9
10 4 misidentification of the species distribution in food. A comprehensive molecular
11
12 5 characterization of the black aspergilli occurring in grape in Europe was performed within the
13
14 6 EU project Wine-Ochra Risk (QLK1-CT-2001-01761) using representative strains isolated
15
16 7 from 107 vineyards of the Mediterranean basin (Bau et al 2006; Perrone et al 2006a,b). These
17
18 8 studies led to the identification of four main populations, namely *A. carbonarius*, *A.*
19
20 9 *tubingensis*, *A. niger*, and a group of *Aspergillus* “uniseriate”, that could be separated by using
21
22 10 molecular methods including AFLP, RFLP and sequences analysis. The *Aspergillus*
23
24 11 “uniseriate” group was clearly separated from *A. japonicus* and *A. aculeatus* by molecular
25
26 12 techniques but was morphologically indistinguishable (Perrone et al. 2006a,b). Ecological and
27
28 13 morphological differences between these species are summarized below.
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36 15 *A. carbonarius* was easily distinguished from other biseriate species due to its big and spiny
37
38 16 conidia; a high percentage of strains of this species (98-100%) have been shown to produce
39
40 17 OTA. Spore germination of *A. carbonarius* is very rapid, and occurs within 24 hours with
41
42 18 water activity (a_w) 0.90-0.99 and temperature 25-35°C. Optimal growth is at 32-35°C and a_w
43
44 19 0.95-0.98 (min 10°C and max 45°C). Optimal conditions for OTA production by *A.*
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46 20 *carbonarius* are at 20-25 °C and a_w 0.95/0.98 (Belli et al. 2005).
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51 21
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53 22 The *A. niger* “aggregate” comprised four different species indistinguishable by morphological
54
55 23 characteristics. The most frequent species isolated from grapes were *A. niger* and *A.*
56
57 24 *tubingensis*, while *A. foetidus* and *A. brasiliensis* were detected at minor extent (Perrone et al.
58
59 25 2007c). *A. niger* aggregate optimal growth conditions were 35–37 °C and a_w 0.93-0.98 (min
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3 1 6–8 °C and max 47 °C). This is one of the most common group of species in a wide range of
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5
6 2 fresh and dry fruits, cereals, etc., and is used in food processing as “GRAS” (generally
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8 3 regarded as safe). OTA production by *A. niger* “aggregate” normally occurs at 20-25 °C, and
9
10 4 a_w 0.95/0.98 (Esteban et al. 2004). A low percentage of OTA producing strains (5-10%) were
11
12 5 detected among the *A. niger* “aggregate” (Perrone 2006a).
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17 7 Among the uniseriate group, *A. aculeatus* and *A. japonicus* were often isolated from grapes,
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19 8 but were not proven to produce OTA although they grow at similar conditions as for *A.*
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21 9 *carbonarius*. Recently the *Aspergillus* “uniseriate” population from grapes in Europe were
22
23 10 characterized as a population molecularly quite different from *A. japonicus* and *A. aculeatus*
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25 11 (Perrone et al. 2006b). This population is being described as a new species to be called *A.*
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27 12 *uvarum* (Perrone et al. 2007 submitted).
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34 14 Several reports from South America claiming the production of OTA by strains of *A.*
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36 15 *japonicus* or that *A. niger* is the major responsible for OTA accumulation in grapes are based
37
38 16 on morphological identification of the producing strains (Dalcero et al. 2002; Chulze et al.
39
40 17 2006; Ponsone et al. 2007). These data have not been confirmed by molecular identification
41
42 18 of the species and should be regarded with ultimate care to avoid confusion in the literature.
43
44 19 In our survey of about 600 strains of black aspergilli, representative of 3-year sampling, 5%
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46 20 of *A. niger* “aggregate” strains (360) resulted OTA producers, while all *A. carbonarius* strains
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48 21 (200) and none of the *A.* “uniseriate” strains (50) were positive to OTA production (Cozzi et
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50 22 al. 2007).
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24 **Black aspergilli and OTA occurrence in the vineyards: role of environmental, ecological**
25 **and agronomical factors.**

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3 1 Black aspergilli cause a black rot disease of grape due to high fungal sporulation on berries
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5 2 which renders them completely deprived and dry. The incidence of colonised berries is related
6
7 3 more closely to seasonal conditions during the year of cultivation than to the grape growth
8
9 4 stage (Battilani et al. 2003; Cozzi et al. 2007; Leong et al. 2006a). Fungal conidia are usually
10
11 5 present on berry skin from setting and increase in number from early veraison to harvest, with
12
13 6 a peak at ripening. Black aspergilli overwinter in soil, and frequent soil cultivation can favour
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15 7 fungal infection in vineyard. The severity of aspergillus rot is influenced by excessive
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17 8 irrigation prior to ripening causing berry splitting. Rain prior to harvest is a common cause of
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19 9 berry damage favouring *Aspergillus* infection (Cozzi et al. 2007; Leong et al. 2006a). Berry
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21 10 damage caused by insects, birds or other fungal infection, is the primary factor affecting the
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23 11 disease development and OTA accumulation in berries.
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32 13 OTA is produced in vineyards and is normally absent up to early veraison. Bunches without
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34 14 visible symptoms can also contain OTA although berries with visible black moulds normally
35
36 15 show higher contamination levels. The absence of OTA at early growth stages can be
37
38 16 explained by major difficulties encountered by fungi in berry penetration (Cozzi et al. 2007).
39
40 17 The distribution of black aspergilli in vineyards can be summarized as follows: i) *A. niger*
41
42 18 “aggregate” is the principal group at all growth stages; ii) *A. carbonarius* incidence is 2-3
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44 19 times less than *A. niger* “aggregate” and increases from ripening to harvest; iii) *Aspergillus*
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46 20 uniseriate is the least represented group, sporadically occurring in Portugal, Greece and Spain,
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48 21 and more frequently in Italy, France and Israel (Battilani et al. 2006).
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55 23 Based on the results of the Wine-Ochra Risk project carried out in six Mediterranean
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57 24 countries, the incidence of berries infected by black aspergilli at harvesting is significantly
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59 25 correlated with the latitude and longitude, with a positive West – East and North – South
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3 1 gradient (Battilani et al. 2006). Following the geostatistical approach described by Battilani et
4
5 al. (2006), data on incidence of *A. carbonarius* were run with ArcView and a predictive map
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7 was drawn. The incidence of *A. carbonarius* was significantly correlated with geographic
8
9 coordinates showing a positive gradient going towards the South of Europe. Based on the
10
11 combination of the degree-day and rainfall parameters in late August - early September in
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13 several countries of the Mediterranean basin, discriminant analysis gave promising
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15 perspectives for predicting OTA presence in vineyards by the development of thermo-wetness
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17 maps (Battilani et al. 2006).
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25 10 Meteorological conditions as well as closeness to the sea have been shown to play a major
26
27 11 role in determining OTA occurrence in grapes (Cozzi et al. 2007). A 3-year survey (2004-
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29 12 2006) performed on 8 vineyards located in the Salento peninsula of Southern Italy, showed a
30
31 13 wide variability of OTA levels between different cultivation years. In particular, the 2005
32
33 14 crop was the most conducive to black aspergilli contamination due to the higher relative
34
35 15 humidity and rainfall levels, that were associated with hot temperatures at ripening and
36
37 16 harvest time (late August-September). Figure 1 shows the occurrence of black aspergilli and
38
39 17 OTA in 8 vineyards of the two major varieties (Negroamaro and Primitivo) cultivated in the
40
41 18 area during the three grape-harvest seasons (Cozzi et al. 2007). *Aspergillus niger* aggregate
42
43 19 was predominant from early veraison to ripening representing 80-85% of contamination. *A.*
44
45 20 *carbonarius* increased from veraison to ripening. OTA contamination of processed berries
46
47 21 was assessed and results were correlated with the incidence of black aspergilli population, in
48
49 22 particular with the increasing CFU values of *A. carbonarius*. The incidence of *A. carbonarius*
50
51 23 increased from ripening to harvest when vineyard relative humidity usually increased, with
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53 24 high risk of OTA accumulation always associated with hot temperature (Cozzi et al. 2007).
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3 1 Despite the widespread occurrence of OTA in various types of wine, there is limited
4
5 2 information on the ability of black aspergilli to infect berries and produce OTA in different
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7 3 grape varieties (Battilani et al. 2004). Grape varieties were shown to affect the incidence of
8
9 4 black aspergilli and the level of OTA contamination in *in vitro* experiments. Three out of the
10
11 5 twelve tested varieties, namely “Bianco di Alessano”, “Pampanuto” and “Uva di Troia”,
12
13 6 showed low OTA contamination after artificial infection with a mixture of 5 OTA producing
14
15 7 strains, whereas the most susceptible variety (Cabernet Sauvignon) contained over 200 µg/kg
16
17 8 OTA and about 80% incidence of colonized berries (Battilani et al. 2004).
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25 10 The role of the cropping system was monitored in a 2-year survey carried out on four different
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27 11 cropping systems, namely spur-pruned cordon, bower system, head (or small tree) system
28
29 12 and espalier, in eight vineyards in Apulia. In both years the espalier cropping system
30
31 13 produced the most contaminated grapes in terms of *A. carbonarius* infection and OTA
32
33 14 accumulation, as shown in Figure 2. This can be explained by the closeness of bunches to the
34
35 15 soil, which is the most important source of inoculum of *A. carbonarius*, as compared to spur
36
37 16 pruned cordon and bower system. The higher humidity occurring in the espalier cropping
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39 17 system with respect to the head system can explain the different contamination level despite
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41 18 the similar distance of bunches from the soil for both systems. (Cozzi et al. 2007).
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49 20 All the above ecoagronomical factors play individual roles in developing *A. carbonarius* on
50
51 21 grapes and the consequent OTA accumulation although the final result in relation to OTA risk
52
53 22 is better represented by a combination of all these factors. Developing of risk maps based on
54
55 23 critical control points can help to prevent and control OTA accumulation in grapes.
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60 25 ***Lobesia botrana* - an OTA risk factor in grapevine management**

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3 1 Black aspergilli are opportunistic fungi (saprophytes) mainly responsible for secondary rot of
4
5 2 grape berries developing through entry sites favoured by berry wounds or splitting caused by
6
7 3 either biotic or abiotic factors (insects, fungi, birds, rainfall, hail). *Lobesia botrana*
8
9 4 (*Lepidoptera: Tortricidae*) is the major grape berry moth in vineyards of Southern Europe,
10
11 5 and can complete three to four generations a year, depending on weather conditions during
12
13 6 late summer. Generally the first generation larvae of *L. botrana* damage flowers, while the
14
15 7 following generations damage berries at different ripening stages. A good correlation between
16
17 8 pest damage and OTA content has been found in grape berries, due to the contribution of *L.*
18
19 9 *botrana* to berry wounds and fungal spore dissemination (Cozzi et al. 2006). Larvae can either
20
21 10 contribute to spore dispersal or act as spore vectors, by trapping conidia in the cuticle
22
23 11 ornamentation, then facilitating a rapid fungal penetration by tunnelling into berries as
24
25 12 demonstrated for *Botrytis cinerea* (Fermaud et al. 1989; Cozzi et al. 2006). Grape berry
26
27 13 damage by *L. botrana* has been shown to increase considerably the contamination level of
28
29 14 black aspergilli and the consequent OTA accumulation in grapes. In Figure 3 a comparison
30
31 15 between groups of intact berries, black rot berries with and without *L. botrana* damages is
32
33 16 reported. All samples of berries damaged by *L. botrana* showed considerable levels of OTA
34
35 17 contamination (up to 1000 µg/kg OTA) and black aspergilli infection higher than 10⁶ CFU.
36
37 18 OTA was not detected in the group of intact berries, while it was found at levels below 1
38
39 19 µg/kg in ca. 42 % of black rot berries without grape moth damages (Cozzi et al. 2006). Field
40
41 20 trials, performed in 2004 and 2005 using both biological and conventional insecticidal
42
43 21 treatments, confirmed that a successful control of the third generation of *L. botrana* reduces
44
45 22 the inoculum of *Aspergilli* and the formation of OTA in grapes (Perrone et al. 2007a; Kappes
46
47 23 et al. 2005). It is therefore important to ensure an adequate insect control in combination with
48
49 24 fungicide treatment in order to obtain an effective pest management.
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1 **Chemical and biological control of OTA producing fungi.**

2 The following chemicals have been shown to be active in reducing at different extent both
3 fungal growth and OTA levels in grape bunches: mepanipyrim, pyrimethanil, fluazinam,
4 iprodione and the mixture cyprodinil and fludioxonil. The latter mixture was confirmed as
5 effective in several field trials carried out in several Mediterranean countries including
6 France, Spain, Greece and Italy (Bellì et al. 2007; Kappes et al. 2005; Tjamos et al. 2004).
7 The most effective treatment was observed at 21 days before harvesting and a previous
8 treatment at veraison was suggested in high risk conditions. This mixture of active ingredients
9 applied against black aspergilli with the same combination and schedule, both in dosage and
10 timing, is effectively used against grey mould, caused by *Botrytis cinerea*. Moreover, the
11 insecticide treatment against *L. botrana* in combination with the fungicide contributes
12 significantly to reduction of OTA level in the field particularly in crop years at high
13 contamination risk (Kappes et al. 2005). Promising results were also obtained by using yeast
14 as biological control agents which were isolated from grapes in Greece and in Italy. In
15 particular, good results were obtained with two strains of *Cryptococcus laurentii* and
16 *Aureobasidium pullulans*, respectively, in Greece (Dimakopoulou et al. 2005), and with a
17 strain of *Hanseniaspora uvarum* in Italy using weekly or two-weekly treatments.

19 **Distribution of OTA in wine and winery by-products and its fate during vinification of** 20 **red grapes.**

21 OTA occurring in grapes is transferred to wine and relevant by-products during vinification.
22 Therefore, the availability of reliable analytical methods for OTA determination in must, wine
23 and relevant by-products is important for an adequate risk management of OTA
24 contamination in the wine food chain. The availability of rapid methods would be necessary
25 in winery for screening the whole production in order to take prompt corrective actions.

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3 1 Several rapid methods are available for OTA analysis in food products that need to be adapted
4
5 2 for their application to wine and by-products (Visconti and De Girolamo 2005). The AOAC
6
7 3 official method 2001.01 for OTA determination by HPLC in wine (Visconti et al. 2001) can
8
9 4 also be used for must providing that solid fraction is previously separated by centrifugation
10
11 5 (Solfrizzo et al. 2006). The fate of OTA during vinification of grape has been studied by
12
13 6 different authors with contrasting results. Fernandes et al. (2003) observed an increase of
14
15 7 OTA concentration in must during maceration of crushed grapes and a consistent reduction of
16
17 8 OTA during pomaces and lees separations. Grazioli et al. (2006) found little or no reduction
18
19 9 of OTA concentration in wine after the first racking (separation of lees) while a significant
20
21 10 reduction of OTA concentration was observed after spontaneous malo-lactic fermentation
22
23 11 occurring between the first and the second racking. In contrast, Rousseau (2004) reported that
24
25 12 OTA content in must increases after crushing grapes, and reaches maximum levels during
26
27 13 malo-lactic fermentation. Leong et al. (2006b) reported that 24% of OTA originally present in
28
29 14 crushed red grape passed into free run wine (must) and a 72% OTA reduction was recorded in
30
31 15 wine after the first racking.
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41 17 The different approaches used by these authors to conduct these studies could explain the
42
43 18 controversial results. Due to unavailability of naturally contaminated grape some of these
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45 19 studies were performed either by artificially inoculating grape with toxigenic *A. carbonarius*
46
47 20 (Leong et al. 2006b) or by spiking uncontaminated grapes with OTA (Fernandes et al. 2003).
48
49 21 These materials are different from naturally contaminated grapes which comprise both
50
51 22 contaminated and uncontaminated berries. Spiking uncontaminated grapes with OTA
52
53 23 produces an apparent reduction of OTA concentration in the resulting wine since most of the
54
55 24 spiked OTA is adsorbed by the grape pomaces and biomass (grape skins, pulp, yeasts released
56
57 25 in must) (Fernandes et al. 2003). On the contrary, when using naturally contaminated berries
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3 1 for vinification, grape pomaces and solid biomass have high OTA concentration and represent
4
5 2 the source of OTA in wine (Solfrizzo et al. 2007). Moreover, in most of these studies OTA
6
7 3 was only monitored in liquid fractions and no measurements were performed on pressed
8
9 4 pomaces and lees thus the distribution of OTA between solid and liquid fractions obtained
10
11 5 during vinification was not measured (Grazioli et al. 2006; Fernandes et al. 2003; Rousseau
12
13 6 2004). Another critical point is the sample preparation of liquid fractions (must and wines
14
15 7 before racking), which contain suspended biomass, before OTA analysis. The separation or
16
17 8 inclusion of the biomass in the sample to be analysed has a significant effect on the measured
18
19 9 OTA concentration due to the high amount of OTA reversibly bound to the biomass. Indeed
20
21 10 Leong et al. (2006b) included the biomass when unseparated liquid fractions (must and
22
23 11 wines before racking) were analysed for OTA. Consequently the OTA concentrations found
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25 12 in these fractions were much higher than that found in the same liquid fraction analysed after
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27 13 spontaneous sedimentation of biomass (first racking).
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36 15 The fate of OTA and its distribution in wine and winery by-products during vinification of
37
38 16 naturally contaminated Negroamaro and Primitivo grapes has been recently reinvestigated at
39
40 17 laboratory (microvinification) and industrial level by Solfrizzo et al. (2007). Samples of must
41
42 18 (before and after maceration), grape pomaces, wine and lees (after the first and second
43
44 19 racking) were analysed for OTA in order to evaluate its levels at each step of vinification.
45
46 20 Before analysis the liquid fractions were centrifuged in order to separate the biomass and
47
48 21 measure the soluble OTA. Results of microvinification experiments showed that only 4% of
49
50 22 the OTA present in grapes remains in the wine whereas 95% of OTA originally present is
51
52 23 retained on pressed grape pomaces (98% in the skin and 2% in the seeds) and 1% is retained
53
54 24 on the lees. Leong et al (2006b) found that 9% of OTA originally present in grapes passed
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56 25 into wine. The use of these wine by-products as food ingredients should be therefore avoided
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3 1 or checked for OTA contamination. OTA concentration in must remained nearly constant
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6 2 after maceration, pressing, juice clarification, alcoholic fermentation and lees separations
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8 3 (after first and second racking). The same OTA concentrations were found in wine samples
9
10 4 analysed after one year. The results obtained with the microvinification were also confirmed
11
12
13 5 at industrial level. An increase of OTA concentration in must was observed during maceration
14
15 6 of Primitivo crushed grapes highly contaminated with OTA. This increase could be explained
16
17 7 by the high concentration of OTA in the grapes that required a longer time for toxin to
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19
20 8 equilibrate between must and grape pomaces.
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22 9

10 **Removal of OTA**

11 Several fining agents have been tested for their ability to remove OTA from contaminated
12 must/wines (Castellari et al. 2001; Leong et al. 2006c). Oenological decolourising carbon has
13 been reported to remove the highest amount of OTA, although carbon also removes
14 anthocyanins and other coloured polyphenols from wine. The effectiveness of the treatment
15 with oak wood fragments depended upon the quantity of wood chips and powder used
16 (Savino et al. 2007). Removal of OTA from grape juice, must and wine by using oenological
17 yeast strains has been reported (Bejaoui et al. 2004; Garcia Moruno et al. 2005; Cecchini et al.
18 2006). The removal of OTA during fermentation is based on adsorption mechanism other than
19 degradation. However, the efficacy of yeasts for OTA reduction at industrial level as well as
20 their impact on wine quality parameters (phenol compounds) has not been shown. In our
21 laboratory we have confirmed OTA reduction by yeasts or inactivated yeast walls while a
22 consistent reduction of colour index (expressed in terms of Folin Ciocalteu index) occurred.
23
24 The results obtained in our laboratory on the efficacy of selected adsorbent materials to
25 remove OTA from contaminated red wine are reported in Table 2. The best results in terms of

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2
3 1 OTA removal were obtained with carbon or commercial preparations containing carbon
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5 2 (Mikofree, Myco AD A-Z, Standard Q/FIS). On the other hand the efficacy in removing OTA
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7 3 was proportional to the reduction of polyphenol content of treated wines.
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11 12 5 **Conclusions**

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15 6 The main source of OTA in the wine production chain is the infection by “black aspergilli”
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17 7 occurring in the field. *A. carbonarius* is the main species responsible for OTA accumulation
18
19 8 in grape berries occurring from early veraison to ripening. OTA production is influenced by:
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21 9 climatic conditions/geographic areas; grape varieties/crop systems; berries damage caused by
22
23 10 insects, fungal infection or excessive irrigation/rainfall. Fungicidal and insecticidal treatments
24
25 11 reduce OTA contamination. Susceptibility to infection can vary from different years and
26
27 12 regions. Developing of risk maps based on critical control points can help to prevent and
28
29 13 control OTA accumulation in grapes. Availability of rapid methods for OTA analysis is also
30
31 14 important for preventive and corrective intervention at some critical control points. After
32
33 15 maceration of (red) grapes OTA remains stable during vinification and after one year aging.
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35 16 During vinification of (red) grapes only 4% OTA remains dissolved in the wine, while 96% is
36
37 17 retained by solid winery by-products (grape pomace and lees). Carbon reduces OTA
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39 18 concentrations in wines, but negatively affects wine quality. Good Agriculture Practices
40
41 19 (balanced soil tillage, irrigation, nitrogen fertilization, pruning) and Good Manufacturing
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43 20 Practices (reduced harvest to vinification time, segregation of rot bunches) help considerably
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45 21 to reduce OTA contamination risk. Main critical control points as well as preventive and
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47 22 corrective actions are summarized in Table 3.
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3 1 Legenda to Figures
4

5 2 **Figure 1.** Occurrence of black aspergilli and OTA in 8 vineyards of Primitivo and
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7 Negroamaro varieties in Apulia during three grape-harvest seasons (2004-2006).
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12 5 **Figure 2.** Influence of the training systems on the epiphitic black aspergilli CFU/g of grape
13 berries samples and the OTA contamination in “Primitivo” during 2004/2005. OTA levels
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15 6 berries samples and the OTA contamination in “Primitivo” during 2004/2005. OTA levels
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17 7 with same letters are not significantly different according to the Duncan test ($P < 0.01$).
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22 9 **Figure 3.** Logarithmic graph of the distribution of OTA concentration in grape berries in
23 relation to black aspergilli contamination levels in groups of berries: In: intact berries, Ar:
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25 10 relation to black aspergilli contamination levels in groups of berries: In: intact berries, Ar:
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27 11 *Aspergillus* rotten berries, and Lb: *Aspergillus* rotten berries with *L. botrana* larvae damages.
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Table 1. Occurrence of ochratoxin A in wine

Reference	No. of analyzed samples	Main origin	Incidence of contamination	Range (µg/kg)
Zimmerli & Dick (1996)	118	Europe	70%	<0.005 - 0.4
Majerus & Otteneder (1996)	144	Europe	42%	<0.01 - 7.0
Ospital <i>et al.</i> (1996)	30	France	50%	<0.01 - 0.3
MAFF (1997, 1998)	60	Europe	56%	<0.01 - 0.8
Ueno (1998)	46	Japan	41%	<0.01 - 0.2
Burdaspal & Legarda (1999)	192	Spain	82%	<0.01 - 0.6
Visconti <i>et al.</i> (1999)	55	Italy	87%	<0.01 - 7.6
Otteneder & Majerus (2000)	420	Europe	48%	<0.01 - 3.3
Pietri <i>et al.</i> (2001)	111	Italy	82%	<0.001 - 3.8
European Commission (2002)	1470	Europe	59%	<0.01 - 15.6
Soufleros <i>et al.</i> (2003)	35	Greece	63%	<0.02 - 3.2
Hocking <i>et al.</i> (2003)	601	Australia	15%	<0.02 - 1.0
Tateo & Bonomi (2003)	80	Italy	85%	<0.01 - 2.9
Finoli <i>et al.</i> (2004)	150	Italy	80%	<0.01 - 5.2

Table 2. Percent removal of ochratoxin A (OTA) from red wine containing 10 µg/L OTA and treated with different amounts of adsorbent.

Adsorbent	Adsorbent dosage (g/L)					
	0.1	0.2	0.4	0.5	1.0	8.0
Oenological decolourising carbon (Esseco)	80 (9) ^a	92 (11)	- ^b	-	-	-
Activated carbon (SIGMA)	36	73	85	88	90	-
Mikofree (Perdomini)	-	-	-	63 (4)	83 (6)	-
Standard Q/FIS (Feed Industry Service)	9	28	31	-	68	-
Myco AD A-Z (Special Nutrients Inc.)	11	25	28	-	53	-
Cholestyramine (SIGMA)	6	10	16	-	35	-
Bentonite (Vason)	1	12	-	-	-	-
Polygel (AEB Group)	2	2	-	-	-	-
Oak wood chips (different types and sizes) ^c	-	-	-	-	-	26-42

^a Percent reduction of polyphenol content (Folin Ciocalteu index) is reported in parenthesis

^b not tested

^c used on red wine containing 8.5 OTA µg/L

Table 3 Main critical control points, preventive and corrective measures to reduce ochratoxin A contamination in grapes and wine.

Risk factors	Preventive and corrective measures
Field and pre-harvest	
<ul style="list-style-type: none"> • Mediterranean basin, closeness to the sea • High temperature and relative humidity from veraison to harvest • Rainfall during ripening period (berry splitting) • Berry damage (high risk with grape berry moth infestation) • Grape training system susceptibility (high risk with espalier) • High nitrogen fertilization, frequent tillage • Grape variety susceptibility 	<ul style="list-style-type: none"> • Monitoring climatic conditions from veraison to harvest • Monitoring with trap system the <i>Lobesia botrana</i> pressure in the vineyards • Monitoring black aspergilli rot berries from veraison to ripening • Avoid excess of vigour and vegetation favouring aeration of bunches • Avoid tillage from veraison to harvest • Combined fungicide/insecticide treatments (1 or 2) when favourable climatic conditions occur
Harvest – Wine making	
<ul style="list-style-type: none"> • Mechanical harvest without selection of bunches • High incidence of rot bunches • Long grape storage after harvesting (> 8 h) 	<ul style="list-style-type: none"> • Anticipate harvest time in high OTA risk areas when favouring conditions occur • Segregate rot bunches at harvesting • Minimize storage time before processing • Control OTA contamination in must • Use carbon preparations to reduce OTA contamination during fermentation

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Figure 1

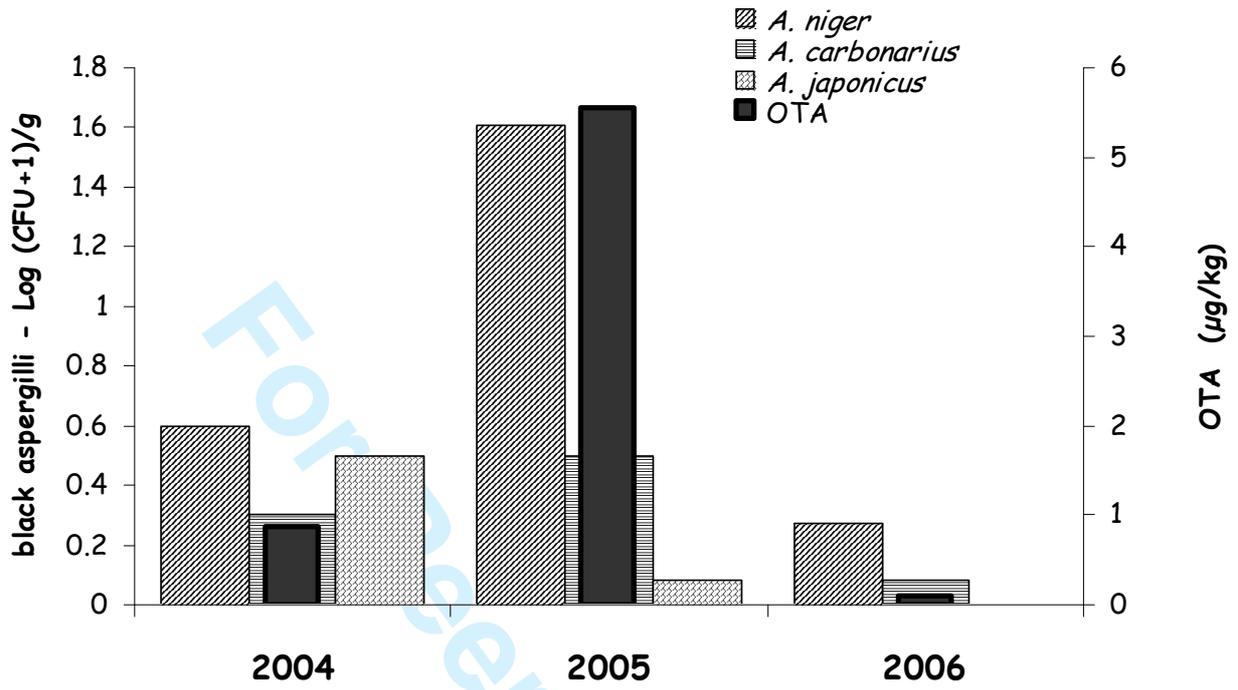
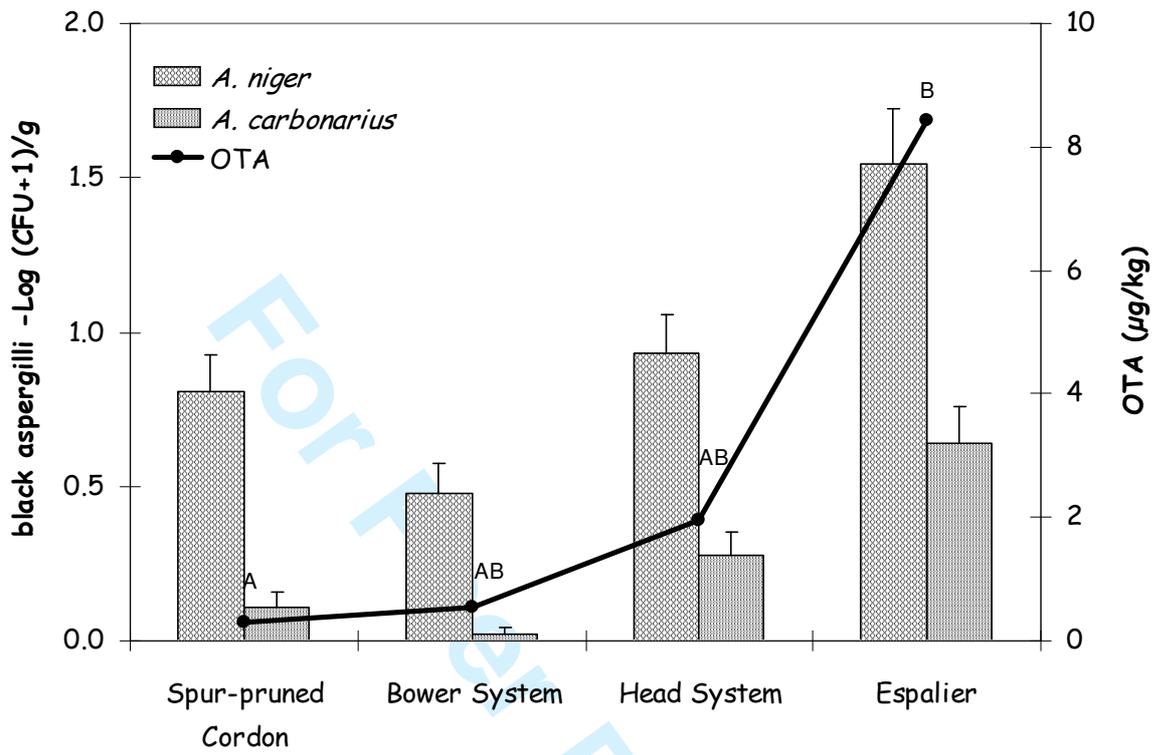


Figure 2



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Figure 3

