UNIONE EUROPEA
Fondo Sociale Europeo

Università degli Studi
di Sassari

DOTTORATO DI RICERCA IN BIOTECNOLOGIE MICROBICHE AGRO-ALIMENTARI

Università degli studi di Sassari

DIPARTIMENTO DI SCIENZE AMBIENTALI AGRARIE E BIOTECNOLOGIE AGRO-ALIMENTARI

SEZIONE DI MICROBIOLOGIA GENERALE ED APPLICATA

PREVENTION AND BIOCONTROL OF OCHRATOXIN A IN WINE

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CICLO XXI

2005-2008

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INTRODUCTION

Mycotoxins

Mycotoxins are secondary fungal metabolites that exert toxic effects on animals and human beings. Secondary fungal metabolites are not necessary for the growth or reproduction of the fungus. Not all fungi are capable of producing mycotoxins; those that can are referred to as toxigenic. Many toxigenic fungi produce mycotoxins only under specific environmental conditions (Bennett and Klich, 2003). As a result, the occurrence of a toxigenic fungus on a suitable substrate does not necessarily mean that a mycotoxin is also present. Traditionally, mycotoxin-producing fungi have been divided into two groups: “field” (plant pathogenic) and “storage” (saprophytic) fungi. For some fungal species such as *Aspergillus flavus* or *Alternaria alternata*, however, contamination of crops can occur in the field or during subsequent storage under improper conditions. In general, many of the toxigenic species of *Fusarium* are pathogenic to plants and represent typical field fungi, although *Penicillium*, *Aspergillus,* and *Mucor* spp. are typically storage fungi. In addition, several mycotoxins may be found in spores of fungi, including *Alternaria* and *Stachybotrys* spp., where inhalation represents another route of exposure (Barkai-Golan and Paster, 2008).

There exist hundreds of mycotoxins (Bennett and Klich, 2003) but relatively few are frequently detected in foods and are considered relevant to human health. The significance of mycotoxins for human health is not easily assessed, as the effects are

often subtle. Toxicity is associated with continued ingestion of low doses, hence the designation “insidious poisons”.

Mycotoxins considered most relevant for human health by the Council of Agricultural Science and Technology (CAST) are aflatoxins, trichothecenes, fumonisins, zearalenone, ochratoxin A and ergot alkaloids (Table 1), although this list is continuously revised. Other mycotoxins are considered of less importance, due to limited occurrence or lack of evidence of their toxicity in humans. In some cases they are an important indicator of the use of poor quality raw materials, such as patulin in apple products (Pitt and Hocking, 1997).

Mycotoxin contamination of various foodstuffs and agricultural commodities is a major problem in the tropics and sub-tropics, where climatic conditions and agricultural and storage practices are conducive to fungal growth and toxin production. Different mycotoxins are likely to occur depending upon the food commodity under consideration. Mycotoxins have been reported to be carcinogenic, teratogenic, tremorogenic, haemorrhagic and dermatitic to a wide range of organisms, and known to cause hepatic carcinoma in man (Refai, 1988).

Tab.1 Mycotoxins considered to be most relevant for human health by CAST or for which official regulation limits in food commodities exist, and the main organisms responsible for their production in foods (CAST, 2003).

Mycotoxin	**Fungal species**
Aflatoxins	*Aspergillus flavus* and *A. parasiticus*
Ochratoxins	*A. ochraceus, A. alliaceus, A. niger* aggregate, *A. carbonarius*; *Penicillium verrucosum, Penicillium nordicum*
Trichothecenes	*Fusarium* species; *Trichothecium roseum*
Zearalenone	*F. culmorum*
Fumonisins	*F. verticillioides, F. proliferatum*
Patulin	*P. expansum*

There are many such compounds but only a few of them are regularly found in food and animal feedstuffs such as grains and seeds. Nevertheless, those that do occur in food have great significance in the health of humans and livestock. In a normal varied human diet, constant exposure to low levels of several toxins is possible. Very little is known about the effects of long-term low-level exposure, especially with regard to co-contamination with multiple mycotoxins. Since they are produced by fungi, mycotoxins are associated with diseased or mouldy crops, although the visible mould contamination can be superficial. The infection symptomatology of mycotoxin contamination is not obvious like other diseases where visible symptoms on plant parts are produced due to infection. With the increasing stringent regulations for mycotoxins, especially for aflatoxins imposed by the importing countries such as the European Union, the export industry of agricultural commodities is in jeopardy (Felicia, 2004).

Over the last two decades various international inquiries on worldwide limits and regulations for mycotoxins were published. A study by the United Nations' Food and Agriculture Organization (FAO) on worldwide regulations for mycotoxins revealed that at least 77 countries now have specific regulations for mycotoxins (FAO, 2004).

The risk of contamination by mycotoxins is an important food safety concern for grains and other field crops. Mycotoxins are produced by various fungal species with distinct ecological requirements affecting their worldwide distribution and incidence in foods. By elucidating the mycoflora of foods, mycotoxin hazards can be predicted

and the appropriate control measures undertaken, such as implementation of HACCP programs.

The concern about filamentous fungi in the vineyard has been traditionally linked to spoilage of grapes due to fungal growth. However, the discussion in the European Union concerning the establishment of a maximum limit for the presence of the mycotoxin ochratoxin A (OTA) in wines has increased concern about mycotoxin production.

Mycotoxin production can occur in the field and/or in post-harvest situations. It has been found that the synthesis of mycotoxins can occur in grapes before harvest, and thus they may be present in wine (Serra et al., 2004). Therefore, it is relevant to determine the mycoflora of grapes and the potential for mycotoxins to be present in wine (Serra et al., 2005).

The complex ecology of mould growth and mycotoxin production results in the production of mixtures of mycotoxins. Many of the developed countries have stringent regulations for many mycotoxins in food and feed and their ingredients. However, the risk of mycotoxin exposure continues in the developing countries in view of lack of food security, poverty and malnutrition (Williams et al., 2004). Decades of research have developed several individualized and comprehensive strategies for the removal of mycotoxins in food and feed. Mycotoxin decontamination by physical and chemical methods has been reviewed extensively elsewhere (Huwig et al., 2001). Although there are many different approaches available for mycotoxin decontamination, most of the approaches have not become

popular due to high cost or practical difficulties involved in detoxification process. Many physical adsorbents have been extensively studied and available as commercial preparations as animal feed additives. However, many of these adsorbents can bind to only a small group of toxins while show very little or no binding to others. (Huwig et al., 2001).

Biological decontamination of mycotoxins using microorganisms is one of the well-known strategies for the management of mycotoxins in foods and feeds. Among the different potential decontaminating microorganisms, *Saccharomyces cerevisiae* and lactic acid bacteria represent unique groups, which are widely used in food fermentation and preservation.

Many species of bacteria and fungi such as *Flavobacterium aurantiacum, Corynebacterium rubrum, Candida lipolitica, Aspergillus niger, Trichoderma viride, Armillariella tabescens, Neurospora* spp., *Rhizopus* spp., *Mucor* spp., etc. have been shown to enzymatically degrade mycotoxins (Bata & Lasztity, 1999). However, question remains on the toxicity of products of enzymatic degradation and undesired effects of fermentation with non-native microorganisms on quality of food. Considerable literature has accumulated in the field of decontamination of mycotoxins by microorganisms in the last two decades. Bata and Lasztity (1999) have reviewed detoxification by microbial fermentation. However, there are no reviews on decontamination of mycotoxins by microorganisms involved in food fermentation and its implications.

Ochratoxin A

Ochratoxin A

The mycotoxin ochratoxin A (OTA) (Fig.1), first obtained from a South African *Aspergillus ochraceus* Wilh. isolate (van der Merwe et al., 1965), consists of a dihydroisocoumarin moiety (the pentaketide-derived ochratoxin α) linked through the carboxyl group to phenylalanine.

Ochratoxins are a group of secondary metabolites produced by fungi of two genera: *Penicillium* and *Aspergillus*. Except ochratoxin-α (OTα), the ochratoxins comprise a polyketide-derived dihydroisocoumarin moiety linked via the 7-carboxy group to l-α-phenylalanine by an amide bond. Ochratoxins consist of ochratoxin A (OTA), its methyl ester, its ethyl ester also known as ochratoxin C (OTC), 4- hydroxyochratoxin A (4-OH OTA), ochratoxin B (OTB) and its methyl and ethyl esters and ochratoxin α (OTα), where the phenylalanine moiety is missing .

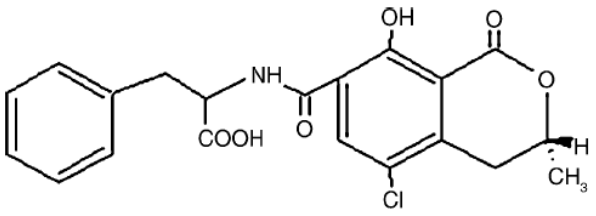


Fig. 1. Structure of ochratoxin A

OTA was first isolated in 1965 from *A. ochraceus* in a laboratory screening for toxigenic fungi (van der Merwe, 1965). Its configuration was determined using optical rotatory dispersion spectroscopy.

The IUPAC developed formula of OTA is l-phenylalanine-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7 yl)carbonyl]-(*R*)-isocoumarin .

It is a white, crystalline compound, highly soluble in polar organic solvents, slightly soluble in water and soluble in aqueous sodium hydrogen carbonate. OTA has weak acidic properties. The p*K*a values are in the ranges 4.2–4.4 and 7.0–7.3, respectively, for the carboxyl group of the phenylalanine moiety and the phenolic hydroxyl group of the isocoumarin part.

Fungal sources and biosynthesis

OTA production is dependent on different factors such as temperature, water activity (*a*w) and medium composition, which affect the physiology of fungal producers. In cool and temperate regions, OTA is mainly produced by *Penicillium verrucosum* or *P. nordicum* (Pitt and Hocking, 1997). *P. verrucosum* mainly contaminates plants such as cereal crops, whereas *P. nordicum* has been mainly detected in meat products and cheese. In tropical and semitropical regions, OTA is mainly produced by *A. ochraceus. A. ochraceus* is also referred to as *A. allutaceus* var *allutaceus* Berkely and Curtis (Pardo et al., 2005). *A. ochraceus* has been reported in a large variety of matters like nuts, dried peanuts, beans, spices, green coffee beans and dried fruits, but

also in processed meat and smoked and salted fish (WHO/FAO, 2001). Two other species of *Aspergillus* section *Nigri*, respectively, *A. niger* var. *niger* (Abarca et al., 1994; Belli et al., 2004) and *A. carbonarius* (Mitchell et al., 2004; Accensi et al., 2004) have been reported as OTA producers. The OTA contamination of substrata such as cereals, oilseeds and mixed feeds in warm zones is thought to be due to *A. niger* var *niger* in addition to *A. ochraceus* species (Accensi et al., 2004), whereas *A. carbonarius* seems to be more common on grapes, raisins and coffee (Sage et al., 2002; Cabanes et al., 2002).

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

The biosynthetic pathway for OTA has not yet been completely established in any fungal species.

Based on a mechanistical model according to the structure of OTA a biosynthetic pathway has been previously proposed, according to which the heterocyclic portion of OTA is structurally similar to mellein. Thus mellein has been proposed as a precursor of OTA. In contrary, Harris and Mantle, (2001) described in experiments with labelled precursors of OTA that mellein does not seem to play a role in OTA biosynthetic pathway.

However, the labelling experiments using both ^{14}C- and ^{13}C-labelled precursors showed that the phenylalanine moiety originates from the shikimate pathway and the dihydroisocoumarin moiety from the pentaketide pathway. The first step in the synthesis of the isocoumarin polyketide consists in the condensation of one acetate unit (acetyl-CoA) to four malonate units (Fig. 2).

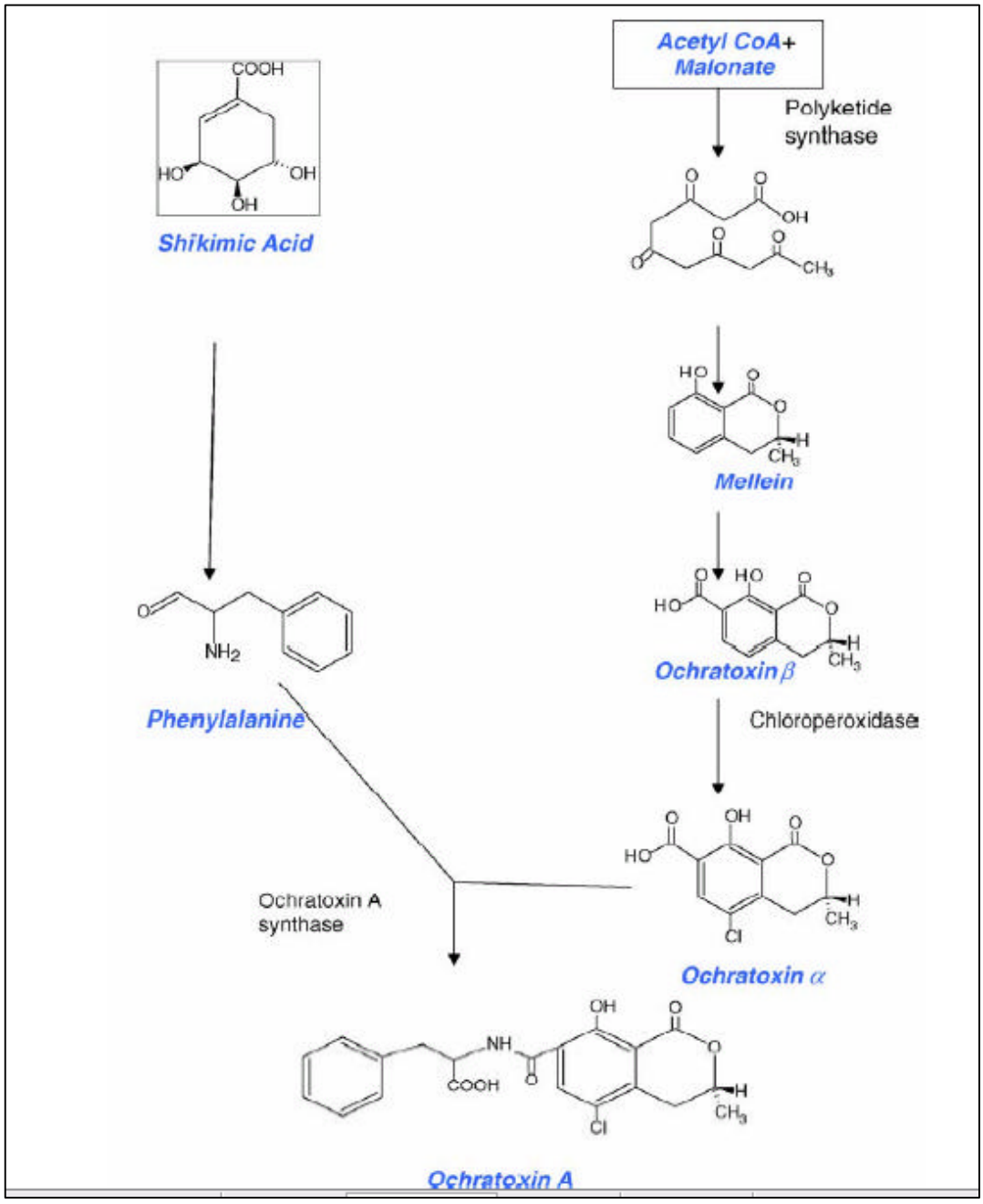


Fig 2. Possibile biosynthesis of OTA.

Recent data showed that this step requires the activity of a polyketide synthase (O Callaghan et al., 2003). Moreover, the gene encoding polyketide synthase appears to

be very different between *Penicillium* and *Aspergillus* species (O' Callaghan et al., 2003; Geisen et al., 2004).

In spite of a remarkable variety of end products, the individual polyketide biosynthetic pathways apparently follow a common basic reaction scheme. The key chain-building step of this reaction scheme is a decarboxylative condensation analogous to the chain elongation step of classical fatty acid biosynthesis (Kao et al., 1994). In the biosynthesis of most polyketide metabolites, the successive condensation step of small carbon precursor acid is catalyzed by a group of multifunction enzyme system called polyketide synthases (PKSs), (Metz et al., 2001). A typical fungal PKS (Fig. 3) is composed of principal domains including ketosynthetase (KS), acyltransferase (AT) and acyl carrier protein (ACP) and optional domains including dehydratase (DH), enoyl reductase (ER), ketoreductase (KR) and thioesterase (TE) (Graziani et al., 2004). Presence or absence of optional domains in a PKS decides about the type of polyketide formed. PKSs producing highly reduced polyketides contain KR, DH and ER optional domains; PKSs producing partially reduced polyketides contain KR and DH optional domains, while PKSs producing non-reduced polyketides contain none of these domains (Fujii et al., 2001).

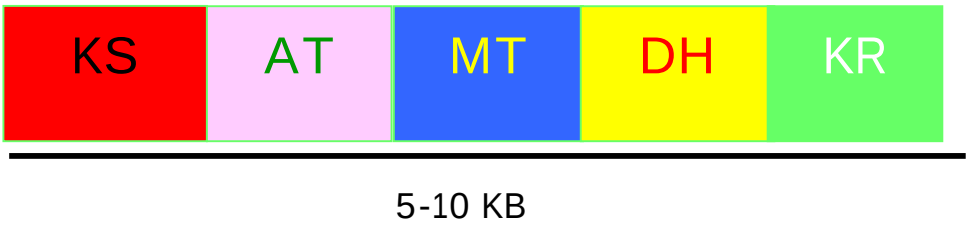


Fig. 3. General architecture of PKS.

In *A. ochraceus*, the gene of polyketide synthase is expressed only under OTA permissive conditions and only during the early stages of the mycotoxin synthesis. Moreover, insertional mutagenesis leading to a distruption of the *pks* gene impaired OTA production (O Callaghan et al., 2003). No such data are presently available on *Penicillium*. In *Penicillium* species, Geisen et al. observed that *P. nordicum* and *P. verrucosum* use two different polyketide synthases for OTA synthesis. This difference is probably related to the *P. verrucosum* ability to produce CIT, also a polyketide-based mycotoxin, in addition to OTA. Once formed, the polyketide chain is modified through the formation of a lactone ring (synthesis of mellein) and the addition of a carboxyl group derived from the C1 pool such as *S*-methylmethionine and sodium formate (synthesis of ochratoxin-α). Subsequently, the chlorine atom is incorporated through the action of chloroperoxidase (synthesis of Ochratoxin-α , OTα). Ultimately, ochratoxin A synthetase catalyzes the linking of OTα to phenylalanine (synthesis of OTA) (Harris et al., 2001). Inhibition of OTA production can be achieved with various natural and synthetic compounds, in a in a species-dependent manner. This may indicate that the biosynthesis of this mycotoxin is not conserved in all the producing fungi (Bayman and Baker, 2006).

Toxicology

The International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). OTA is nephrotoxic and is suspected of being the main etiological agent responsible for human Balkan endemic nephropathy (BEN) and associated urinary tract tumours. Striking similarities between OTA-induced porcine nephropathy in pigs and BEN in humans are observed. Currently, the mode of carcinogenic action by OTA is unknown. OTA is genotoxic following oxidative metabolism (Leszkowicz and Manderville, 2007).

No epidemiological studies have yet adequately evaluated the cancer risk of OTA in human populations. Studies have shown OTA to be genotoxic as well as immunotoxic, although its mode of action is not fully understood. Organizations and agencies in many countries are currently promulgating standards for OTA in foods and beverages. Increased efforts in farm management and food safety are being made to mitigate the risks to public health posed by OTA (Clarck and Snedeker, 2006)

Both toxicokinetic (the changes of concentrations of a compound in the organism over time) and toxicodynamic (the dynamic interactions of a compound with biological targets and their downstream biological effects) factors determine the toxicity of OTA. Upon absorption from the gastrointestinal tract, OTA binds to serum proteins. Considerable variations in serum half-lives across species are known to be dependent on the affinity and degree of protein binding. Reabsorption of OTA from the intestine back to the circulation, as a consequence of biliary recycling, favours the systemic redistribution of OTA towards the different tissues. In addition, reabsorption of OTA occurs in the kidney proximal and distal tubules. Accumulation occurs in

blood, liver and kidney. Liver and kidney are also the major organs of OTA biotransformation. Metabolism of OTA has not been elucidated in details and at present, data regarding OTA biotransformation are controversial. In all species, both faecal and urinary excretions play important roles in plasma clearance of the toxin. In addition, the mammalian milk excretion appears to be relatively effective (Ringot et al., 2006).

Regulation of ochratoxins in foods

The European Union (EU) limits ochratoxin A in imported foods, with a maximum of 5 μg/kg (=ppb) in raw cereal grains, 3 μg/kg in processed cereal foods, and 10 μg/kg in dried vine fruits (raisins). As of April 2005, the EU imposed limits for ochratoxins in wine, grape juice and coffee. The limits are 2.0 μg /kg for wine and grape juice, 5.0 μg/kg for roasted coffee, and 10.0 μg/kg for instant coffee. In USA, in contrast, the FDA has not set advisory limits or action levels for ochratoxins in any commodity.

Ochratoxin A and wine

The occurrence of mycotoxin contamination has been reported in many raw and processed agricultural products (FAO, 2001). Contamination of cereals and legume grains has been investigated for a long time. However, research on vine products only started after 1996, with the first detection of ochratoxin A (OTA) in wine by Zimmerli and Dick (1996). Wine is considered the major source of OTA intake after cereals (Miraglia and Brera, 2002).

OTA in wine is a problem that originates in the vineyard (Battilani et al., 2006a). Of the black aspergilli fungi capable of synthesizing OTA, *A. carbonarius* has been identified as the major cause of contamination in berries (Cabanes et al., 2002). *A. carbonarius* exists as a saprophyte in the top layer of soil beneath vines. The fungus, thought to be blown onto bunches, develops in damaged berries (Leong et al., 2006). Lesions in berries may be caused by biotic agents, pests and diseases, and also by abiotic factors. Natural splitting of ripe berry skin after rainfall was reported by Leong et al. (2006) in Australia. Because of its active role in transporting spores into injured berries, the grape berry moth (*Lobesia botrana*) is a major cause of field colonization by black aspergilli (Cozzi et al., 2006).

However, damage on berries does not necessarily lead to OTA contamination in wine. Meteorological conditions and plant phenology also determine contamination (Battilani et al., 2004). Under *in vitro* conditions, Mitchell et al. (2004) and Belli et

al. (2005a) observed a high response of *A. carbonarius* growth and OTA production to substrate water activity (a_w). The optimum growth rate was found for a value of a_w 0.95–0.98, corresponding to the grape chemical composition at early veraison. Veraison is the stage when berries begin to soften and sugar content increases. Under field conditions, Leong et al. (2006) observed that veraison marked the onset of potential for *A. carbonarius* rot development and reported a significant increase in damaged berry susceptibility to rot development with ripening during the 20 days before harvest.

In terms of climatic conditions, a relationship is usually found between the temperature and contamination severity depending on the years and field locations (Belli et al., 2005a; Belli et al., 2005b; Battilani et al.; 2006a, Battilani et al., 2006b; *In vitro* investigations on synthetic grape juice medium backed the hypothesis of a temperature dependency for *A. carbonarius* development and OTA synthesis. Generally, most *A. carbonarius* strains grow optimally at 30–35 °C with no growth below 15 °C. However, optimum OTA production was observed at 15–20 C (Mitchell et al., 2004; Belli et al., 2005a; Belli et al., 2005b). While fungus development and OTA production are regularly found to be temperature-dependent, the effects of rainfall and humidity appear to be more erratic, depending on the studies and the authors. Battilani et al. (2006b) reported a significant effect of rainfall on fungal colonisation and OTA content in bunches. Conversely, Belli et al. (2005b) did not find any relationship. In Greece, Tjamos et al. (2006) observed higher OTA content in wines from wet island conditions than from dry continental conditions.

Since 2006, a new EC regulation has imposed 2 μg/l as the upper limit for OTA concentration in wines (CR 1881/2006). The current literature provides the basis for modelling OTA production on a berry level. Yet, on a vineyard level OTA production also results from the number of colonized berries. In France, Sage et al. (2004) and Bejaoui et al. (2006) discovered that OTA mainly concerns Mediterranean vineyards. In fact, the level of *A. carbonarius* in soil may be reduced by temperatures below the optimum survival temperature (25 C), in the upper layer of soil (Leong et al., 2006).

Occurrence of OTA in wine

Wine is an important beverage in the world trade. France, Italy, Spain and the US are the main producing countries followed by Argentina, China and Australia. The first three countries are the main wine exporters. Ochratoxin A was first detected in wines by Zimmerli and Dick, 1995 and 1996. Ochratoxin A is a frequent contaminant of wine, with an apparent increase in levels in wines originating from southern areas of Europe with their warmer climates (Otteneder and Majerus, 2000). The amount of OTA was also found to be dependent on the latitude of the vineyard: at lower latitudes, OTA contamination is usually higher. Indeed, Domijan and Peraica (2005) observed that among Croatian wine samples all wines produced in the south (Adriatic coast) were OTA-positive, while white wines came from the north of Croatia were not contaminated by OTA. The climatic and geographic differences influence mould

contamination and OTA contamination of grapes. Contamination levels as high as 15.6 μg l^{-1} have been reported in Southern Europe (Miraglia and Brera, 2002) with red wines frequently more contaminated than white wines from the same wine-growing region (Otteneder and Majerus, 2000). Majerus et al., (2000) suggested that the reason for the different levels of OTA concentration of white, rose and red wines is the different wine-making technique, since longer mash standings in red wine could lead to higher OTA content in wine. The maximum levels of OTA detected were 15.6, 6.32 and 8.86 μg l^{-1} for red, rose and white wines, respectively (Miraglia and Brera, 2002).

After the first report on the occurrence of OTA in wine (Zimmerli and Dick, 1996) several surveys were conducted to assess the relevance of the presence of this mycotoxin in wine and grape products (Battilani et al., 2006). The EU has agreed a maximum admitted level of 2.0 μg OTA/kg for wine (European Commission, 2005). The first data on OTA occurrence in wine marketed in Spain are due to Burdaspal and Legarda (1999). Most of their samples were domestic, but some of them were imported. Dessert wines showed the highest incidence level (about 73%) followed by rosé, red and white wines, in that order. Further surveys have been reported (López de Cerain et al.; 2002, Bellí et al.; 2004, Blesa et al.; 2004; Mateo et al., 2006 ;Hernández et al., 2006). The overall % of contaminated samples was 51.5%. The highest OTA concentration was 15.25 ng/ml in dessert wine. Except for dessert wines, the highest levels were <4.5ng/ml. The EU limit is not applicable to liquor or dessert wines with >15% alcohol content. The high levels found in dessert wines are

probably due to raising. In Italy, wines have been extensively surveyed for this toxin. OTA incidence was higher in red wines (78.4%), followed by rosé and dessert and white wines. The highest level (7.63 ng/ml) was found in red wine. Dessert wines are quite prone to be contaminated with OTA as in the case of Spanish wines (Visconti et al., 1999; Pietri et al., 2001: Brera et al., 2005; Bacaloni et al., 2005). In Germany, a value of 7.0 ng/ml was found in Italian red wine exported to Germany (Majerus and Otteneder, 1996; Majerus et al., 2000). In Greece, more than 66% of samples showed detectable OTA levels and both red and sweet wines showed the highest levels (Markaki et al., 2001; Soufleros et al.; 2003; Stefanaki et al., 2003). More than 50% and 100% of samples analyzed in Cyprus and Turkey, respectively, had detectable levels of the toxin (Ioannou-Kakouri et al., 2004 and Anli et al., 2005). In other European countries, the problem concerns mainly imported wines (Zimmerli and Dick, 1996). Eder et al., (2002) detected OTA in only 1/116 Austrian wines. Research performed by the German Federal Ministry of Health between 1995 and 1998 showed that total OTA incidence was 40% but contamination was considerably higher in red wines and wines from southern Europe regions. In France, Ospital et al., (1998) found OTA in 29 wines (0.01–0.27 ng/ml) but a value of 0.78 ng/ml was found in a French red wine exported to Germany. In Portugal, Festas et al., (2000) did not find OTA in 64 domestic wines but Soleas et al., (2001) detected it in 5/37 samples of Portuguese wine. A survey on 340 Portuguese wine revealed that OTA was detectable in 20.3% of the samples and the highest level was 2.1 ng/ml (Ratola et al., 2004). According to the SCOOP task 3.2.7 report (European Commission, 2002), after

performing a survey on 1470 wine samples, the OTA mean level was 0.36 ng/g. However, a level of 15.6 ng/g was reported in red wine from southern Europe (Miraglia and Brera, 2002).

Siantar et al., (2003) found that 69/84 US wines contained <0.01 ng OTA/ml and the remaining contained <1 ng/ml. Soleas et al. (2001) analyzed 71 samples of US red wine and 40 samples of US white wine and found that only 8 red wine samples exceeded 0.05 ng/ml. Ng et al., (2004) report that US wines had no quantifiable OTA levels. Soleas et al. (2001) found OTA in 16.6% of 580 red wine samples and in 3.9% of 362 white wine samples marketed in Canada but were unable to detect the toxin in their US samples. Canadian wines, when compared with imported products, showed both lower OTA occurrence and lower contamination level. Wines from North America have lower OTA levels than European wines (Ng et al., 2004). Rosa et al., (2004) detected OTA in 24% of 42 wine samples from Brazil, Argentina and Chile (0.0283–0.0707 ng/ml). There the problem of OTA contamination in wines is not as concerning as in Europe (Chulze et al., 2006). However, Soleas et al. (2001) found OTA in Argentinean wines. Australian wines were included in several surveys. Most samples contained <0.05 ng/ml and the highest level was 0.62 ng/ml (Leong et al., 2006). Thus, Australian wine does not seem to pose a serious problem for consumers' health. Sugita-Konishi et al. (2006) studied wines commercialized in Japan and found that 6/10 wines had detectable OTA levels (0.07–0.72 ng/g). In South Africa, Shephard et al., (2003) detected the toxin in 24 local samples. There the highest level

(2.67 ng/ml) was found in noble wine by Stander and Steyn (2002). Filali et al. (2001) found OTA in 30 wine samples from Morocco.

Regulation of ochratoxin levels in grape-derived products

Maximum levels for ochratoxin A in cereals have been set by Commission Regulation (EC) No. 472/2002 and the sampling method has been regulated by Commission Directives 2002/26/EC and 2002/27/EC. For dried vine fruits (raisins, currants and sultanas) the maximum tolerable level of OTA is 10 μg/Kg according to Directive 2002/27/EC (Commission of the European Communities, 2002). Currently maximum permitted levels of 2 μg/Kg have been established for OTA in wines and grape must based drinks in the European Union (Commission regulation No 123/2005 amending Regulation No 446/2001 as regards ochratoxin A). Furthermore, there are also national laws and regulations in the Member States covering other foodstuffs not regulated by European law or other mycotoxins. Some countries and buyers (e.g. Finland, some British supermarkets) also carry out OTA controls and apply their own limits (sometimes as low as 0.5 μg/Kg).

Fungi responsible for ochratoxin A contamination

Recent studies have focused on identifying the source of OTA in wines in Mediterranean countries. Data suggests that OTA contamination in these countries as well as in subtropical parts of Brazil, Argentina and Australia is caused by black

aspergilli. Although the main source of black aspergilli is soil, members of this section (Aspergillus section Nigri) have been isolated from various other sources (Kozakiewicz, 1989). Black aspergilli are the causal agents of several plant diseases, and considered as opportunistic pathogens of grape and may cause bunch rot (sour rot) or berry rots, and raisin mould (Varga et al., 2004). These species usually attack damaged berries. A recent study indicated that black aspergilli are also responsible for vine canker of grapes (Michailides et al., 2002).

Among black aspergilli, *A. carbonarius* is the most important as OTA producing isolates are observed more frequently (41–100% of the examined isolates; Abarca et al., 2003; Battilani et al., 2003; Te´ren et al., 1996). Apart from *A. carbonarius*, other black aspergilli including the *A. niger* aggregate and *A. aculeatu*s have also been found to produce OTA on grapes (Battilani et al., 2003). Recenlty, Medina et al., (2005) observed OTA production in *A. tubingensis* isolates originating from grapes. This is in agreement with Varga et al. 2005 findings. Due to their ability to produce OTA at a wide range of temperatures, OTA can be continuously produced in the field. This fact has to be taken into account in commodities such as grapes, raisins and wine, where *A. carbonarius* and members of the *A. niger* aggregate are considered to be the main sources of the OTA contamination.

Ochratoxin A contamination of dried vine fruits was also found to be due to the action of black aspergilli in Europe including Spain (Abarca et al., 2003), the Czech Republic (Ostry et al., 2002), Hungary (Varga et al., 2005) and in other parts of the

world including Argentina (Magnoli et al., 2004; Romero et al., 2005) and Australia (Leong et al., 2004).

In countries with colder temperate climates such as Germany, Northern Hungary, the Czech Republic or northern parts of Portugal, France and Italy, black aspergilli have not been isolated from grape berries in spite of the presence of OTA in wines (Abrunhosa et al., 2001; Ostry et al., 2005; Torelli et al., 2003; Varga et al., 2005; Zimmerli and Dick, 1996). In colder climates, *Penicillium* species were found to be responsible for OTA contamination of several agricultural products including cereals (Pitt, 2000). Although *Penicillium* species are able to grow and produce mycotoxins in must and wine (Moller et al., 1997), OTA producing penicillia have rarely been found on grapes. However, Battilani et al., (2001), and Rousseau (2004) identified OTA producing *Penicillium* species from grapes in Northern Italy and France suggesting that *Penicillium* species could be responsible for OTA contamination of grapes in these regions.

Fig 4. *A. carbonarius* infection on grape berries

Factors affecting OTA contamination of grapes

Several factors influence fungal colonization of grapes. Climatic conditions were found to have a significant effect. OTA contamination of grapes and wines were found to vary from year to year even in the same vineyard (Rousseau, 2004). Location of the vineyard is also important; the Mediterranean basin is particularly affected, including Southern regions of France and Italy, Greece, and certain regions of Portugal and Spain (Rousseau, 2004). In a recent study, ochratoxin producing black aspergilli were mainly isolated from vineyards located in Southern parts of

Portugal characterized by hot and dry summers, and hardly any black aspergilli were recovered from vineyards located in Northern parts of Portugal where temperatures are moderate during summer (Abrunhosa et al. 2001; Serra et al., 2003). Similarly, OTA producing black aspergilli were only isolated from southern parts of Hungary (Varga et al., 2005).

Soil was found to be the main source of inoculum of OTA producing black aspergilli in Australian vineyards (Clarke et al., 2004). Black aspergilli were more frequently isolated from frequently cultivated soil than from soil of minimally cultivated vineyards. Additionally, mould counts were higher in soil under vines than in soil between vine rows (Clarke et al., 2004). Health of the grapes is of prime importance in view of OTA contamination. Rotted or damaged berries were found to contain more OTA than healthy berries (Rousseau, 2004). Damage can be caused by larvae of grape moth and other insects (*Eudemis, Cochylis sp*.), fungal pathogens, and by excessive irrigation or rain damage. Research carried out at the Interprofessionnel de la Vigne et du Vin France (ITV France) indicated that larvae of the grape moth (*Eudemis* and *Cochylis* sp.) act as vectors for conidial dispersal of OTA-producing fungi (Rousseau, 2003). Strict correlation was observed between the number of perforations caused by these larvae and OTA concentrations in grapes. Consequently, researchers at the Institut Cooperatif du Vin (ICV) successfully used the insecticides Lufox (carbamate type insecticide containing luferunon and fenoxycarb), Decis (a pyrethroid insecticide containing delthametrin) and Bt (*Bacillus thuringiensis*) for lowering OTA content of wines (Merrien, 2003). *B. thuringiensis* was also found to

significantly inhibit the growth of OTA-producing fungi on grapes in another study (Bae et al., 2004). Battilani et al. (2004) found that different grape varieties differ in their susceptibility to *A. carbonarius* colonization and OTA accumulation. Skin thickness also affects OTA contamination (Rousseau, 2004). These observations are extremely important, since they open the possibility for breeding grape varieties resistant to *Aspergillus* colonization and OTA accumulation.

Fate of ochratoxin A during wine making

Data published to date are controversial regarding the fate of OTA during vinification. Fernandes et al., (2003) carried out a vinification trial using spiked grapes to follow the fate of OTA during wine making. Their data indicate that OTA content increases during maceration, while most OTA was removed during solid-liquid separation steps. Similarly, Leong et al. (2004); Ratola et al., (2004) observed that during wine making, most OTA is removed with the marc (pomace). OTA binds to grape proteins and yeast cell wall during fermentation. Kozakiewicz et al. (2003) found that most OTA was removed during malolactic fermentation possibly due to the action of lactic acid bacteria. In contrast, Rousseau (2004) reported that OTA content increases after crushing grapes, and reaches maximum levels during malolactic fermentation. OTA levels were found to decrease afterwards, and this decrease was suggested to be due to either the action of lactic acid bacteria, or to OTA adsorption to yeast cell walls. In another study, Solanet (2003) found that OTA

content of the liquid phase of fermentation broth decreases constantly during alcoholic fermentation.

Control measurement

Improvements in vitivinicultural and winemaking practices are required to reduce OTA in wine (Jørgensen, 2005 and OIV, 2005). Rousseau and Blateyron (2002) emphasized that the occurrence of OTA in wine may be decreased by about 80% using appropriate vineyard management. Battilani et al. (2004) suggested that management of black aspergilli in vineyards should focus on the status of berries between early veraison and ripening, and on decreasing the incidence of black aspergilli in vineyards. Emmett, et al. (2004) have proposed strategies for management of *A. carbonarius* in vineyards. Research has to be done to limit as much as possible the possible pathways that favour fungal infection of berry pulp, for example, by attacks of insects (Cozzi et al., 2006) and others pests, or action of fungi such as *Botrytis cinerea*. The preliminary strategies include producing small loose bunches that are well dispersed through well aerated canopies by the use of vineyard management, vine pruning and irrigation practices; preventing pest damage to berries and bunches, especially between veraison and harvest; minimising mechanical and environmental damage (e.g. sunburn and rain damages) to berries and bunches; and minimising incidence of *A. carbonarius* in bunches by vineyard floor management. Integration of these strategies were suggested to minimise the development of bunch rot caused by *A. carbonarius* in vineyards, reduce amounts of OTA produced by *A.*

carbonarius in grapes and minimise the incidence of OTA in wine. To decrease OTA content in wines removing rotten grapes prior to crushing and pressing should also be carried out. Since OTA content of damaged berries was higher than that of undamaged ones, selecting grapes seems to be the best and natural way to limit OTA occurrence in wine (Kozakiewicz et al., 2003). Chemical treatments with fungicides to control *A. carbonarius* and *A. niger* aggregate species have been studied (Varga and Kozakiewicz, 2006). The pyrimidine fungicide pyrimethanil and fosetyl-Al and the dicarboximide folpel were found to be the most effective for lowering fungal colonization and OTA content of wines. We should mention that some fungicides were found to stimulate OTA production in grapes (Battilani et al., 2003). Some fining agents have been studied to adsorb the mycotoxin and activated carbon has been shown to be quite efficient although other components that contribute to the wine aroma are also removed (Castellari et al., 2001 and Gambuti et al., 2005). The addition of bentonite could successfully be used to remove OTA binding proteins and decreased OTA content by 67% (Leong et al., 2004). The addition of these adsorbents to red wine by up to 0.2 g/l did not modify substantially the colour, but high amounts of adsorbent (≥50 g/l), even with the combination of oenological tannins, affected the color and organoleptic properties of wines Other treatments can occur during the winemaking process. OTA is removed by partition with the marc and lees in the usual process.). Bacteria, for example *Lactobacillus plantarum* and *Oenococcus oeni*, or application of absorbents such as charcoal, liquid gelatin or yeast cell wall preparations have been found to reduce OTA content of wine (Castellari, et al. 2001;

Kozakiewicz et al., 2003). Bejaoui et al., (2004) successfully used inactivated *Saccharomyces* strains to lower OTA content of grape juices. Fermentation reduces the OTA level, which may be due to adsorption by yeast cells (Silva et al., 2003 and Bejaoui et al., 2004). Measures to control the toxin in grape juice and in wine are needed combined with more research on detoxification.

AIM OF THE STUDY

Aim of the study

Biological control is the use of living agents to control pests or plant pathogens. This approach is being increasingly considered by the scientific community as a reliable alternative to pesticide utilization in field and in post-harvest. The biological approach is highly desirable for controlling fungal growth on grapes, helping to reduce the amount of agrichemical residues in grapes, wine and related products.

This research was aimed at developing natural biocontrol strategies, in order to reduce the presence of ochratoxigenic fungi in grapes.

Among the microorganisms considered for biological control, yeasts are particularly promising. They can colonize plant surfaces or wounds for long periods under dry conditions and it is suggested that their mechanisms of antagonism are mainly based on competition for space and nutrients (Kalogiannis and Spotts, 2006).

Yeasts possess many features which make them eligible as biocontrol agents in fruits and other foods. They have (i) simple nutritional requirements, (ii) the capacity to grow in fermenters on inexpensive media, (iii) the ability to survive in a wide range of environmental conditions, (iv) no production of anthropotoxic compounds.

Antagonistic yeasts were shown to reduce the growth of filamentous spoilage moulds both *in vitro* and *in vivo*. However, biocontrol microorganisms can inhibit the growth of the infecting fungi without reducing the metabolic activity of the active hyphae. Competition among microorganisms for essential environmental factors, such as nutrients and space, is expected to have a dramatic effect on the secondary

metabolism of spoilage moulds. In particular, nutritional competition has been reported to play a fundamental role in yeast–mould interactions. In fact, nutrient availability strongly affects secondary metabolism, including mycotoxin production (Chand-Goyal et al., 1996).

In wine making, OTA levels were found to decrease during fermentation, and this decrease was suggested to be due to the action of lactic acid bacteria and yeasts.

The aim of the present work was to develop a biological control tool as an alternative to the use of fungicides that would help the wine making industry becoming more environmental friendly and at the same time that would reduce yield losses due to *Aspergillus* spp., infection and the consequent OTA presence in wine.

The main objectives of this thesis were: 1) to test whether some wine yeasts may have a potential for biocontrol of *Aspergillus* spp., mainly responsible for the accumulation of ochratoxin A (OTA) in grape and wine such as *A. carbonarius* and *A. ochraceus* 2) to evaluate the effect of selected antagonistic yeasts on the expression of known OTA biosynthetic genes and on OTA production in culture.

MATHERIALS AND METHODS

Materials and methods

Effect of yeasts on ochratoxigenic fungi: *A. carbonarius* and *A. ochraceus* strains

Strains

In this work we analysed two fungal strains: one strain of *Aspergillus carbonarius* (MPVP A 0566) and one strain of *Aspergillus ochraceus* (MPVP A 0703), belonging to the Collection of the University of Piacenza (courtesy prof. P. Battilani). These strains are highly virulent and effective OTA producers. Spore suspensions of both MPVP A 0566 and MPVP A 0703 were prepared by collecting conidia from 5-day-old colonies (grown on PDA at 25 °C) in distilled water to assist the dispersal of conidia. The spore concentration was determined by a Thoma haemocytometer.

Yeast strains used in this study belong to Di.S.A.A.B.A Collection of the University of Sassari, Italy. We analysed 23 strains of *Saccharomyces cerevisiae* and 9 strains of *Kloeckera. apiculata*, isolated from grape, must and wine samples collected from Sardinia (tab.2). Yeasts cell suspensions were prepared by inoculating 50 ml of YPD with a loopful of cells and incubation on a rotary shaker (180 rpm) at 25 °C for 24 h.

Tab. 2. Yeasts strains tested for their biocontrol potential against ochratoxigenic moulds.

Genus species	Strain	Isolated from	Isolation year
Saccharomyces cerevisiae	BY4741		
Saccharomyces cerevisiae	1090	Must Oristano	1965
	1182	Must Cannonau Oliena	1988
	1189	Must Alghero	1968
	1153	Must Oliena	1967
	1161	Must liena	1967
	1226	Must Cannonau Alghero	1988
	1236	Must Cannonau Oliena	1988
	1237	Must Cannonau Oliena	1988
	1494	Must Oliena	1967
	1304	Must Oliena	1967
Kloeckera apiculata	3184	Must Cannonau Alghero	1986
	3187	Must Vernaccia Zeddiani	1986
	3188	Must Vernaccia Zeddiani	1986
	3189	Must Malvasia Bosa	1986
	3191	Mosto Malvasia Bosa	1986
	3197	Grapes Sangiovese Alghero	1986
	3198	Grapes Vernaccia Zeddiani	1986
	3199	Grapes Sangiovese Alghero	1986
	3200	Grapes Nuragus Cagliari	1986

S. cerevisiae flor	1043	Wine Malvasia Magomadas	1984
	1739	Wine Oristano	1965
	M25	Wine Malvasia	
	2D		
	1765	Wine Oristano	1965
	1768	Wine Oristano	1965
	1769	Wine Oristano	1965
	1770	Wine Oristano	1965
	910.1	Wine Malvasia Magomadas	1984
	910.2	Wine Malvasia Magomadas	1984
	910.3	Wine Malvasia Magomadas	1984

Saccharomyces cerevisiae BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)

Culture media

The culture media used were:

-Yeast Extract Sucrose (YES) agar and broth, which contained (per litre): yeast extract, 20 g; sucrose, 150 g; magnesium sulfate, 0.5 g; and for agar 15g Difco agar was added.

-Czapek Yeast Extract (CYA) agar and broth, which contained (per litre): K_2 HPO, 1 g; Czapek concentrate, 10 ml; trace metal solution, 1 ml; yeast extract, 5 g; sucrose, 30 g; agar, 15 g (Pitt and Hocking, 1997)

-Potato Dextrose Agar (PDA) which contained (per litre): 39 g of PDA with 0,062g tetracycline hydrochloride and 0,062g streptomycin sulphate.

-Yeast Peptone Dextrose (YPD) agar and broth which contained (per litre): bacto-yeast extract 10 g, bacto-peptone 20 g, bacto-agar 20 g.

Identification of yeast isolates

The yeast isolates were identified according to Esteve-Zarzoso et al. (1999) by amplifying the region spanning the 5.8S rRNA gene and flanking the internal transcribed spacers 1 and 2 5.8-ITS; (White et al., 1990), using ITS1 (5′ TCCGTAGGTGAAC CTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) primers. The amplified DNAs (0.5–10 μg) were digested without further purification with the restriction endonucleases *HinfI*, *HaeIII* and *CfoI* (New England

Biolabs, USA) according to the supplier's instructions. The PCR products and their restriction fragments were separated on 1,4% and 1,8% agarose gels, respectively, with 1X TAE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8). After electrophoresis, gels were stained with ethidium bromide (5 μg/ml) and visualized under UV light (300 nm). A 100-bp DNA ladder marker (Invitrogen, Carlsbad,USA) served as the size standard.

Characterisation of *S. cerevisiae* strains

The *S. cerevisiae* strains were characterised according to Marinangeli et al., (2004).
In a population of wild *S. cerevisiae* genes coding for cell wall proteins contain minisatellite-like sequences, some of there are: SED1, AGA1, DAN4 and HSP150. These sequences are highly polymorphic in length and represent a sink of unexplored genetic variability. The primer pairs are designed on the gene open reading frame and yield stable and repeatable amplification profiles, showing a level of resolution that allows the clear discrimination between different strains.

AGA1, DAN4, HSP150 and SED1 were amplified as described below.
AGA1: the reaction mixture contained 1 μl of template, 0,12 mM of each dNTPs, 0.6 U Taq polymerase, 1X Taq reaction buffer, 1,5 mM MgCl2, 3 pmol of each of the primers AGA1f(5^1-PGTGACGATAACCAAGACAAACGATGCAA-3^1) and AGA1r (5^1 CCGTTTCATGCATACTGGTTAATGTGCT-3^1). The PCR reactions were run

for 35 cycles as follows: denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and elongation at 72°C for 2 min.

DAN4: the reaction mixture contained 1 µl of template, 0,12 mM of each dNTPs, 0.6 U Taq polymerase, 1x reaction buffer, 1.5 mM $MgCl_2$, 30 pmol of each primer DAN4f (5^1-AGCGCTTTCAAAGGATGGTATTTACA-3^1) and DAN4r (5P-AAAGTAGACCCGAAGGAAGAAACAGG-3^1). The reactions were run as follows: nine cycles of touch-down PCR with denaturation at 94°C for 45 s, annealing at 70°C for 30 s (with a decrease in the annealing temperature of 0.5°C for each cycle), and elongation at 72°C for 1 min; and 30 cycles with PCR with denaturation at 94°C for 45 s, annealing at 68 C for 30 s, and elongation at 72°C for 1 min.

HSP150: the reaction mixture contained 1 µl of template, 0,12 mM of each dNTPs, 0.6 U Taq polymerase, 1X reaction buffer, 1.5 mM $MgCl_2$, 30 pmol of each of the primers HSP150f (5^1-CACTTTGACTCCAACAGCCACTTACA-3^1) and HSP150r (5^1-TACCGGACAAACATTGGTAGAAGACA-3^1). The reactions were run for 35 cycles as follows: denaturation at 94°C for 45 s, annealing at 68°C for 30 s, and elongation at 72°C for 1 min.

SED1: the reaction mixture contained 1 µl of template DNA, 0.6 U Taq polymerase, 1X reaction buffer, 1.5 mM $MgCl_2$, 0,12 mM each dNTPs, and 2 pmol each of SED1f (5^1-ATGAAATTATCAACTGTCCTATTATCTGCCGG-3^1) and SED1r (5^1-TTATAAGAATAACATAGCAACACCAGCCAAACC-3^1) primers. The reactions were run for 35 cycles as follows: denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and elongation at 72°C for 2 min.

In all four cases an initial denaturation step at 94°C for 7 min and a final 7 min extension step at 72°C were also performed.

The PCR products were analyzed by electrophoresis on a 1.4% agarose gel in 1X Tris-borate-EDTA buffer. The gel images were visualized by means of a Bio-Rad Gel DOC 1000 and acquired with Multi-Analyst software (Bio-Rad).

Inhibition of *A. carbonarius* and *A. ochraceus* by yeast

Yeast and mould spore suspensions were prepared as described above. Inhibition experiments were performed on YPD, CYA and YES agar medium. A top agar was prepared by mixing 6 ml of media with 0.7% agar and 1 ml of yeast suspension containing 10^6 cells. The agar–yeast suspension was poured into Petri dishes that contained 15 ml of the agar media. Once the top agar had set, three 10-µl portions of a mould suspension (10^5 CFU/ml) were separately spotted on each plate. Three replicate experiments for each fungal strain were performed. Moulds were spotted to measure the radial extension rates of the colonies. Plates inoculated only with mould were used as control. Fungal growth inhibition was determined as the percentage of colony diameter decrease compared to control.

Effects of yeast on production of OTA by *A. carbonarius* and *A. ochraceus*

The ability of *A. carbonarius* strain to produce OTA when co-cultured with 10 strains of *S. cerevisiae* and 10 *K. apiculata* on CYA and YES broth was investigated. Yeasts were co-cultured with *A. carbonarius* on CYA and YES broth as described above. *A. carbonarius* was also inoculated in yeast free CYA and YES broth, which were used as controls. After an incubation period of 7 days at 25°C in the dark, production of OTA was estimated by High Performance Liquid Chromatography (HPLC) as follows:

two ml of culture fluid was removed from each flask, filtered through a 0.2-µm syringe filter, and extracted with 2 ml of chloroform. The organic phase was collected, evaporated, and resuspended in 500 µl of methanol. Then 20 µl were injected into an HPLC system (Shimadzu, Milan, Italy).

The ability of *A. carbonarius* and *A. ochraceus* to produce OTA when co-cultured with *S. cerevisiae* and *K. apiculata* on CYA and YES agar plates was also investigated by using a method described by Bragulat et al., (2001). For this HPLC screening method, the strains were three-point inoculated into YES and CYA agar and analyzed after 7, 14 and 21 days growth at 25 °C. At each time, three agar plugs were removed from the central area of the colony, weighed and introduced into a small vial. A volume of 0.5 ml of methanol cloroformic acid 25:1 was added to each vial for 60 min and extracted and quantified as described above.

Inhibition of *A. carbonarius* by yeast isolates on wounded berries

Mature grape berries of different cultivar (Cannonau, Vermentino, Cardinal and Italia), were disinfected with 1% sodium hypochlorite for 10 min and rinsed twice with distilled water. Calibrated wounds (about 2 mm diameter) were made in each berry with a sterile needle to simulate natural damage. Grape berries were dipped in a water suspension of each antagonist yeast (10^8 CFU/ml), followed by inoculation of an aqueous suspension of fungal conidia (10^4 CFU/ml). Each sample, constituted by 5 berries and reproduced with three replicates for each yeast isolate, was incubated for 6 days at 25 °C in a plastic box under high relative humidity (100%). The results obtained are the mean of three independent experiments. A positive control was performed with berries sham treated with sterile water and then with *A. carbonarius* suspension as described.

pks expression and OTA production

Static cultures of fungi were grown in YES at 25°C for 3 days and then were inoculated with 10^6 cells/ml of *Saccharomyces cerevisiae* strain 1182 and grown for a 8 day period. OTA production was daily monitored by HPLC and *pks* gene expression was analysed by RT-PCR

In a further experiment static cultures of fungi were grown in YES:yeast supernatant 1:1 at 25°C for 8 days.

Yeast supernatant (*S. cerevisiae* 1182 and *S. cerevisiae* BY4741) was obtained from overnight YES liquid cultures, centrifuged 20 min at 5000 rpm and filtered (0,22μm). Different experiments were performed using yeast crude supernatant, autoclaved for 1 hour either treated with proteinase K (Sigma) at 37 °C for 60 min.

OTA production were daily monitored by HPLC, *pks* gene expression were analysed by RT-PCR and mould growth were assessed by dry weight mycelium.

Protein were extracted from different treated supernatant and from YES medium and

Effect of ethanol on fungal biomass

Static cultures of fungi were grown in YES added on two different concentration of alcohol 1,8% and 2,4% at 25°C for 8 days. Mould growth were assessed by dry weight mycelium.

Genomic DNA isolation

The fungal DNA was extracted according to Al-Samarrai and Schmid (2000). The initial steps involved suspension of freeze-dried mycelium in buffer containing sodium dodecyl sulphate, detachment of DNA from polysaccharides by mild shearing, NaCl precipitation of polysaccharides and protein, chloroform extraction and ethanol precipitation. The ethanol precipitate was then subjected to a second round of mild shearing, NaCl precipitation, chloroform extraction and ethanol precipitation.

The yeast DNA was extracted from overnight liquid cultures as described by Ushinsky et al. (1997).

Dry weight mycelium

The fungal biomass was filtered, washed with copious amount of deionised water and dried with Whatman paper after which the biomass was placed in a freezer overnight before undergoing the freeze-dry process.

Measurement of OTA production

OTA production was measured by HPLC, using the method described by Sibanda et al. (2002) involving a Beckman system Gold HPLC apparatus and a Beckman Ultrasphere C18 (250 x 4.6mmm, 5µM) reversed-phase column.

The mobile phase was acetonitrile:water:acetic acid 99:99:2. OTA was detected using a Merck –Hitachi fluorescence detector with an excitation wavelength of 333nm and a emission wavelength of 460nm. samples were taken from broth cultures grown statically. To extract OTA the culture media was harvested by filtration through miracloth (Calbiochem) and analysed by HPLC. All samples were diluted 1:1 with HPLC buffer prior to analysis.

RNA preparation and cDNA synthesis

Mycelium samples were taken at day 4 from the YES growing cultures. These were filtered and that mycelium was weighted and stored at -70°C. The stored mycelia was then ground to a fine powder in liquid nitrogen with a mortar and pestle. RNA was extracted using a RNasy plant mini kit (Quiagen). The extracted RNA was treated with DNase I (Roche) to remove contaminating DNA and stored at -70°C until used.
An aliquote of the RNA was separated on an agarose gel, to check the integrity of the RNA. The RNA gel was prepared as described by Sambrook and Russel (2001). Before further experiments the RNA concentration for each sample was determined spectrophotometrically and brought to an identical concentration.

RT-PCR

cDNA was synthesized from mycelia using reverse transcriptase and random hexamer promoter (Roche) as previously described (Soden and Dobson, 2001) this cDNA was used as templates for a PCR amplification with primer specific to the *pks* gene (Table 3).
Primer specific to the *A. carbonarius* housekeeping gene *calmodulin* and to the *A. ochraceus* housekeeping gene *β-tubulin* (table..) were used as a control to monitor expression of those costitutively expressed genes.

Amplification was carried out in 25 µl reaction mixture containing: 2.5 µl of Taq polymerase buffer 10×, 1 µl of 50 mM $MgCl_2$, 1 µl of dNTP 10 mM of each, 1 µM of each primer, 0.5 U of Taq, about 50 ng of genomic DNA, H_2O up to 25 µl. Reaction

conditions were: 94 °C for 3 min (94 °C for 1min, 58 °C for 45 s and 72 °C for 45 s) × 33 cycles followed by an incubation at 72 °C for 10 min. The amplified products were examined by agarose gel electrophoresis.

Table 3. PCR primers used.

Primer name	Sequence (5'⟶3')
B-tub F (*A. ochraceus*)	5'-ggcaaacatctctggcgagcac-3'
B-tub R (*A. ochraceus*)	5'-gaagttgtcggggcggaaaa-3'
PKS F (*A. ochraceus*)	5'-tcacctgtcgtatcagc-3'
PKS R (*A. ochraceus*)	5'-aactcggtcaagcagatc-3'
Camod F (*A. carbonarius*)	5'-ggccagatcaccaccaag-3'
Camod R (*A. carbonarius*)	5'-tcacggatcatcgac-3'
Ac12RL_OTAF (*A. carbonarius*)	5'-aatatatcgactatctggacgagcg-3'
Ac12RL_OTAR (*A. carbonarius*)	5'-ccctctagcgtctcccgaag-3'

Protein extraction from yeast supernatant

For the extraction protocol, about 25ml of yeast supernatant harvested from overnight liquid culture was resuspended in a same volume of acetone, centrifuged for 5 min at 5000 rpm and dissoluted in SDS sample buffer (7M urea, 2M thiurea, 4% CHAPS, 50mM DTT). About 5 ml supernatant was typically loaded per lane of mini-gel.

SDS-PAGE

SDS-PAGE was carried out using 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. After the electrophoresis, the gel was stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol–5% (v/v) acetic acid and de-stained with 10% (v/v) acetic acid–5% (v/v) methanol. Protein Marker, BenchMark (Invitrogen) was used as a molecular mass marker.

Data analysis

Unless otherwise stated, all experiments were performed in triplicate from independent pre-cultures. Statistical analyses of the data were performed using ANOVA followed by Student's t test (two-sided) using JMP version 3.1.5 software (SAS Institute Inc.). Differences were considered significant if p values were less than 0.05.

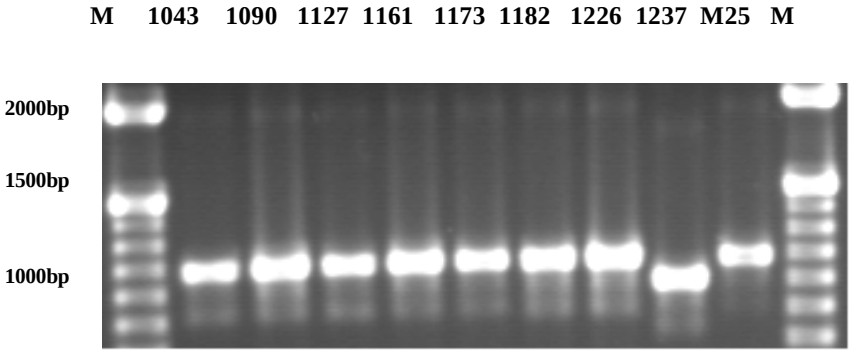
RESULTS

Results

Effect of yeasts on ochratoxigenic fungi: *A. carbonarius* and *A. ochraceus* strains

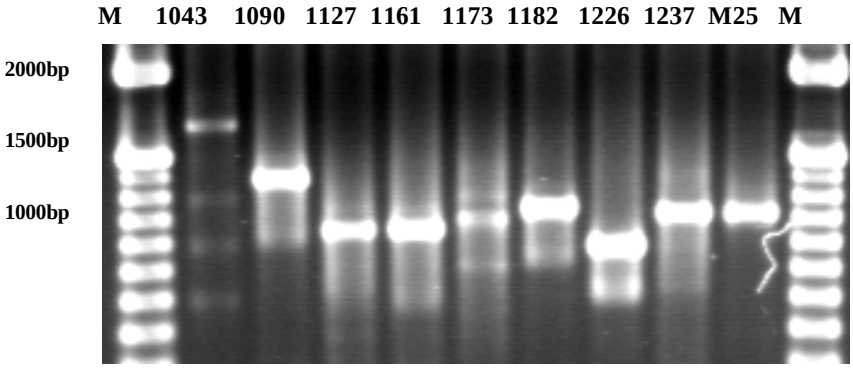
Wine yeasts used in this study were identified by PCR amplification of the 5.8-ITS region, which had proven to be highly discriminative for yeast identification (White et al., 1990). Coupled digestion analyses for *Hinf*I/*Hae*III/CfoI revealed two different profiles which were identified by comparison, with those previously described for several yeast species (Esteve-Zarzoso et al., 1999) belonging to *Saccharomyces cerevisiae* and *Kloeckera apiculata* species. The molecular characterisation of *S. cerevisiae* isolates was carried out according to Marinangeli et al (2004).

The primer pairs specific for AGA1, DAN4, HSP150 and SED1 were used under highly stringent PCR conditions to amplify the total DNA from a population of wild isolates of *S. cerevisiae* selected for their ability to reduce OTA. As expected, the four sets of primer pairs highlighted the existence of extensive length polymorphism in each of the cell wall genes analysed (Fig. 5).



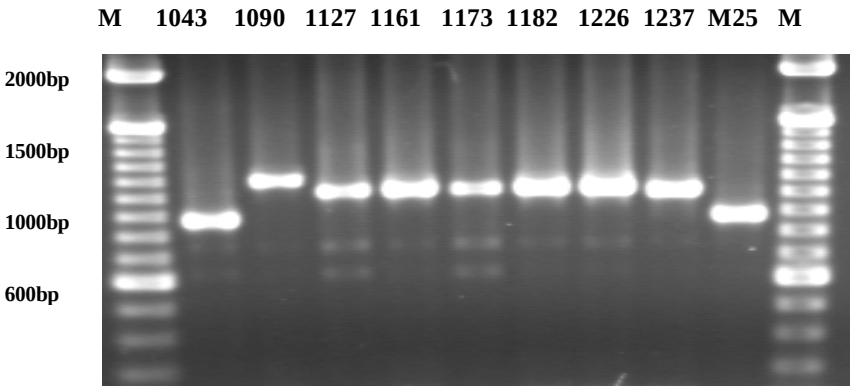
A

AGA1



B

DAN4



C

HSP150

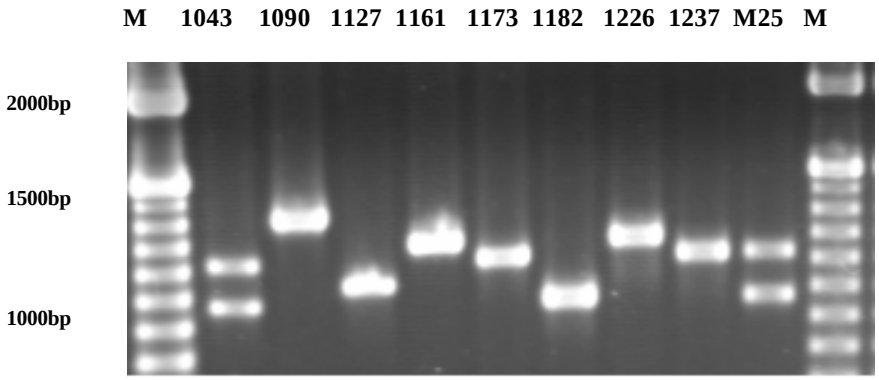


Fig. 5. Cell wall gene polymorphisms in *S. cerevisiae*. PCR primers designed on the AGA1 (A), DAN4 (B), HSP150 (C) and SED1 (D) sequences were used to amplify the corresponding genes of 9 isolates of *S. cerevisiae*. Lanes 1-9: PCR profiles observed within the population analysed with each of the primer pairs; M: 100 bp ladder .

Inhibition of *A. carbonarius* and *A. ochraceus* by yeast strains

Yeast strains were further characterised by using a nutritional competition assay in YES and CYA agar media. This test was proposed in order to select yeast strains able to overwhelm the co-inoculated fungi when colonising a common ecological niche. All yeast strains were able to inhibit fungal growth when co-cultured in CYA and YES media. Some of the strains analysed caused severe growth reduction in *A. carbonarius* (MPVP A 0566) and *A. ochraceus* (MPVP A 0703). Growth inhibition was significantly higher on YES than on CYA (Fig. 6-7). This inhibitory effect was induced at a yeast concentration of 10^6 CFU/ml.

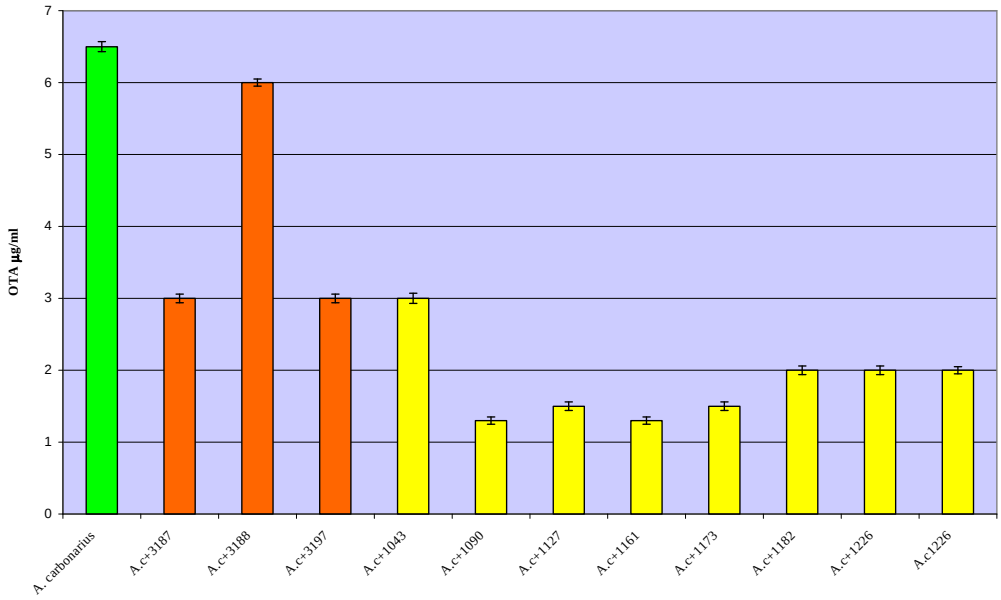
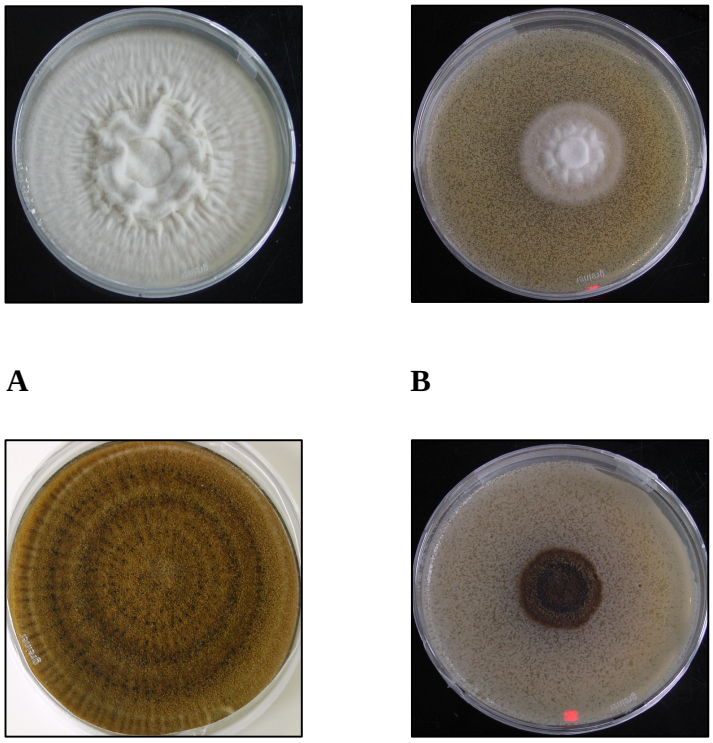


Fig. 6. Growth inhibition (cm) of *Aspergillus carbonarius* MPV A703 in YES medium in Petri dish when co-cultured with yeast strains.



A **B**

C **D**

Fig. 7. *Aspergillus ochraceus* MPV A703 on YES agar plate A); *Aspergillus ochraceus* and yeast strain *K. apiculata* 3187 on YES B); *Aspergillus carbonarius* MPV A566 on YES medium C) and co-cultured with yeast strain *S. cerevisiae* 1182 D).

Effects of yeast on production of OTA by *A. carbonarius* and *A. ochraceus*

Some yeasts were able to significantly reduce OTA content in the culture filtrates when co-cultivated with the ochratoxigenic strains of *A. carbonarius* and *A.ochraceus* (Fig. 8-9). Only the strain *K.apiculata* 3199 did not reduce OTA content.

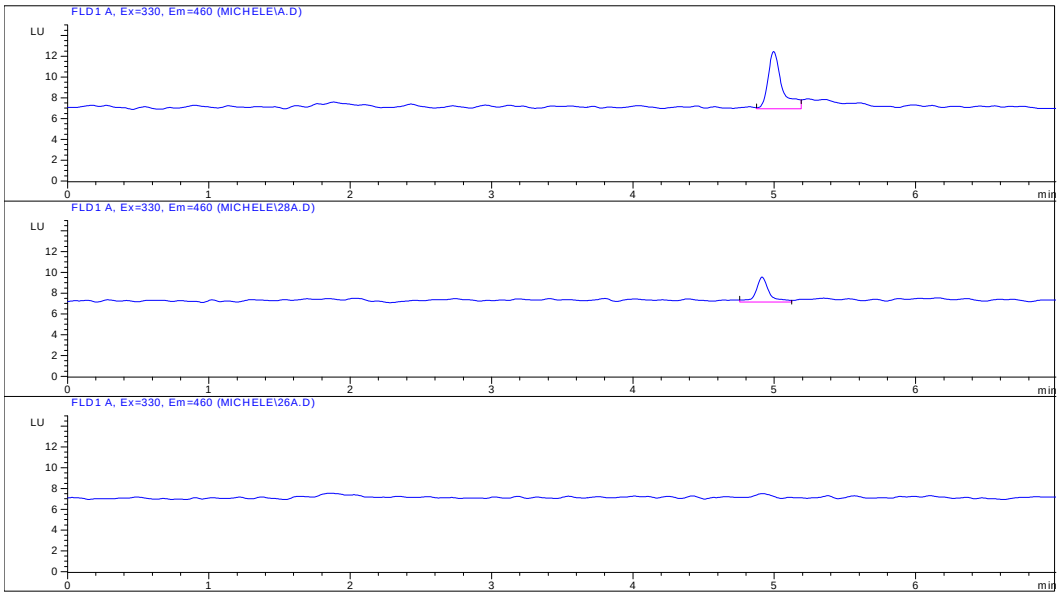


Fig. 8. Chromatograms of Ochratoxin A from the culture filtrates of *A. carbonarius* (A), *A. carbonarius* + *S. cerevisiae* 1226 (B) and *S. cerevisiae* 1161(C)

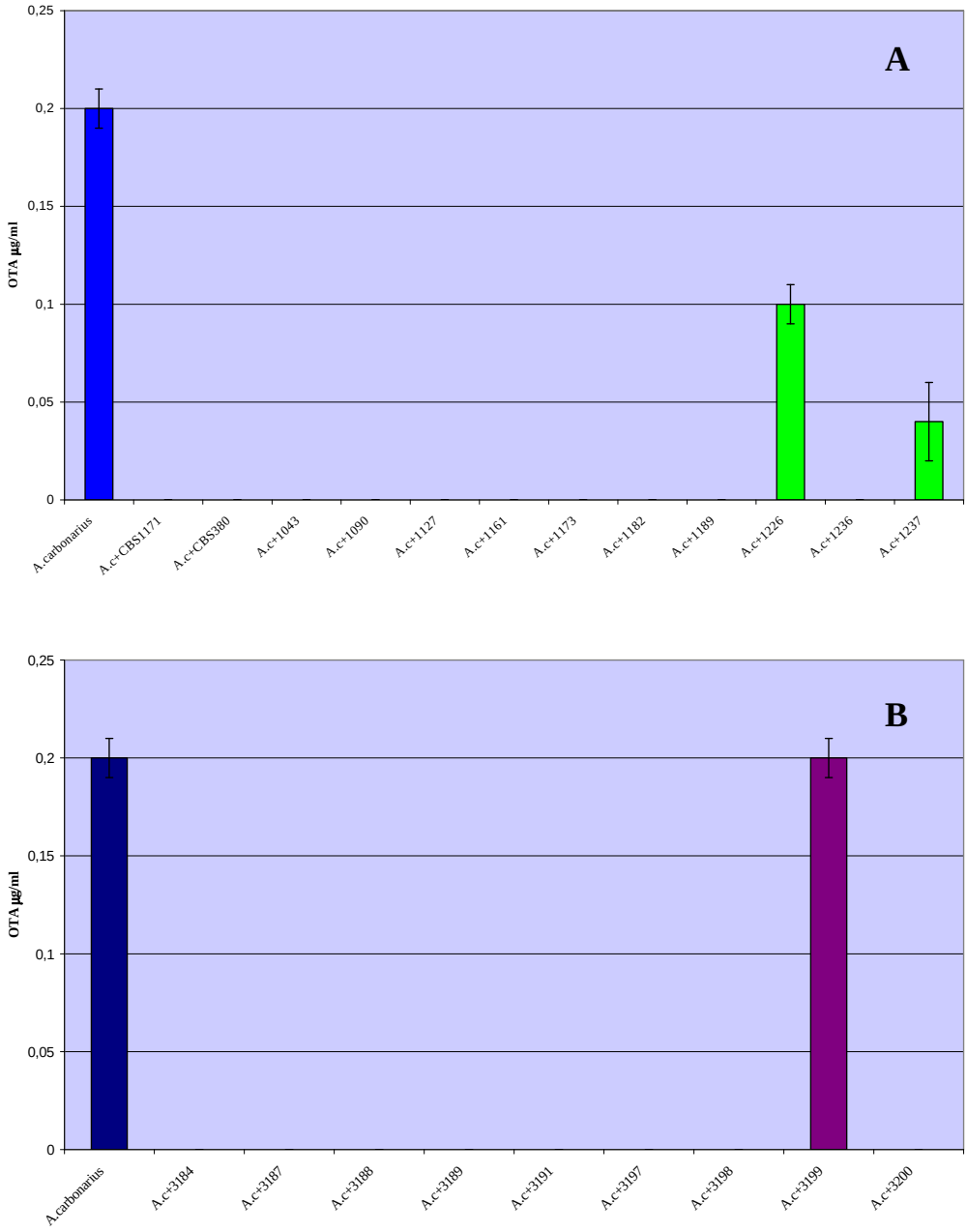


Fig. 9. Ochratoxin A production by *Aspergillus carbonarius* MPVP A 0566 and *Aspergillus carbonarius* MPVP A 0566 co-cultured in CYB medium with some *S. cerevisiae* strains(A) and with some *K. apiculata* strains (B). after 7 days of growth in

static cultures at 25°C. OTA was determined by HPLC and each value is the mean of three replicates from independent cultures.

Inhibition of *A. carbonarius* by yeast isolates on wounded berries

The inhibitory effect of the different yeast strains was further tested on wounded grape berries by co-inoculation of each yeast strain with *A. carbonarius* MPV A703. When applied at a concentration of 10^8 CFU/wound, all yeasts strains reduced significantly fungal colonisation on artificially inoculated grape berries (Fig.10-11).

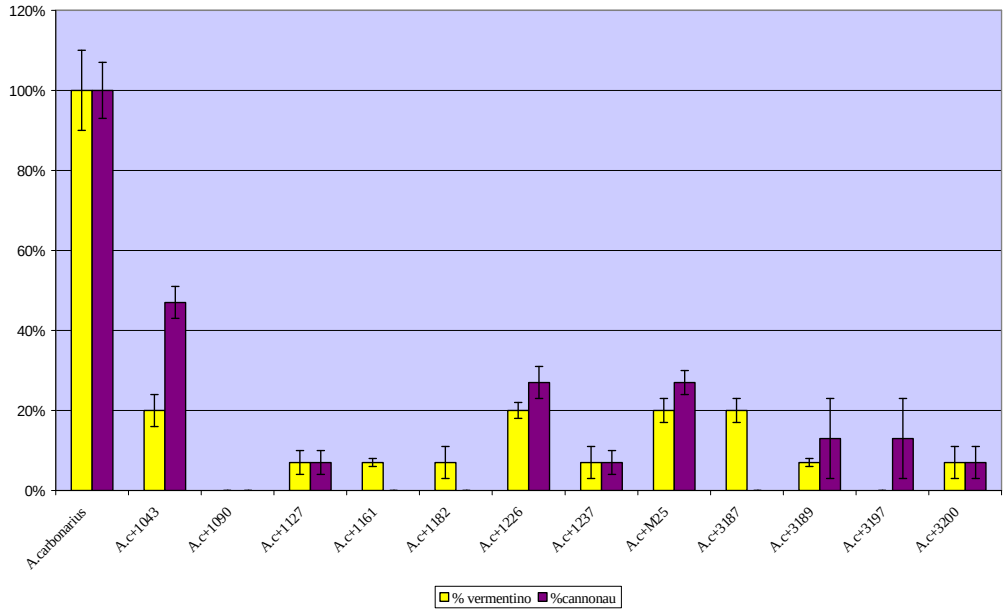


Fig. 10. Percent of inhibition of *A. carbonarius* infection by yeast strains on grape berries (cultivars Vermentino and Cannonau) after 7 days.

A

B

Fig. 11. Infection of *A.carbonarius* on grape berries of cultivar Cardinal and Italia (A), complete inhibition of *A.carbonarius* growth on grape berries by yeast strain *S. cerevisiae* 1182 (B).

Effects of *S. cerevisiae* 1182 supernatants on OTA production and *A. ochraceus* growth

In order to provide insight on the mechanism of OTA reduction caused by yeast strains, *A .ochraceus* MPV A703 and *A. carbonarius* MPV A566 were grown in YES amended with *S. cerevisiae* 1182 supernatant in static cultures for 6 days at 25°C.

Figure 12. *A. ochraceus* MPV A703 and *A. ochraceus* grown in *S. cerevisiae* 1182 supernatant after 4 days of growth in static cultures at 25°C.

In figure 13 the OTA production by *A. ochraceus* and *A. ochraceus* in *S. cerevisiae* 1182 supernatant during 6 days is reported. When *A. ochraceus* was grown in *S. cerevisiae* 1182 supernatant a decrease in OTA values and a reduction of fungal biomass up to 96% were observed.

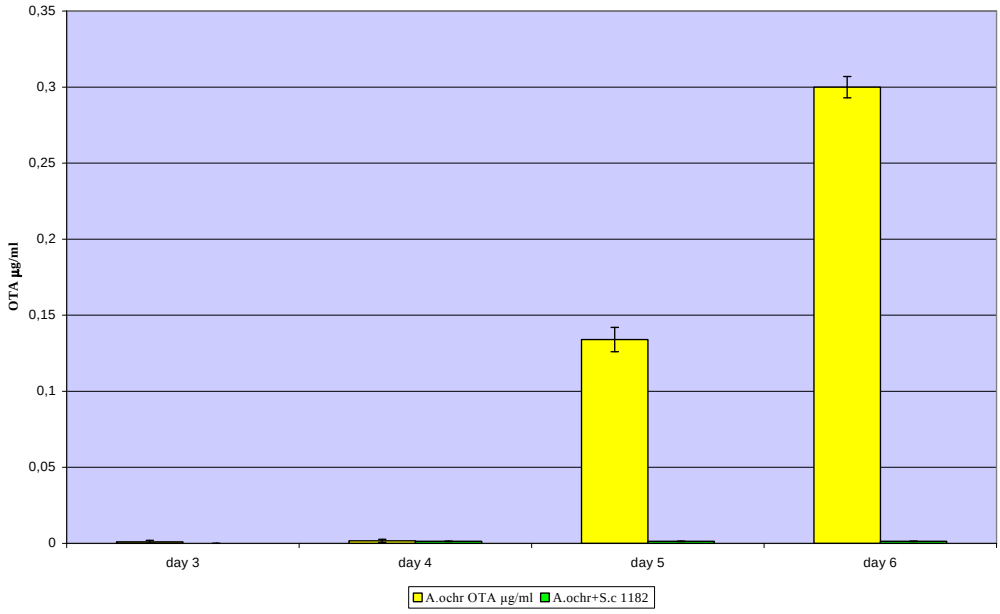


Figure 13. Ochratoxin A production by *A. ochraceus* MPV A703 on YES medium and in *S.cerevisiae* 1182 supernatant during 6 days of growth. HPLC values of OTA expressed as µg/ml. Each experiment is the mean of three different replicates.

A different experiment was performed by growing *A. ochraceus* in *S. cerevisiae* 1182 yeast supernatant either treated with proteinase K at 37 °C for 60 min or boiled for 1 hour in order to investigate the nature of the yeast's antifungal activity (Figure 14, 15).

In figure 14 the decrease of OTA production in *A.ochraceus* when grown in different *S. cerevisiae* supernatants is reported. There was no significative difference in OTA production among the supernatants in spite of a reduction in biomass yields were observed in supernatants (Figure 15).

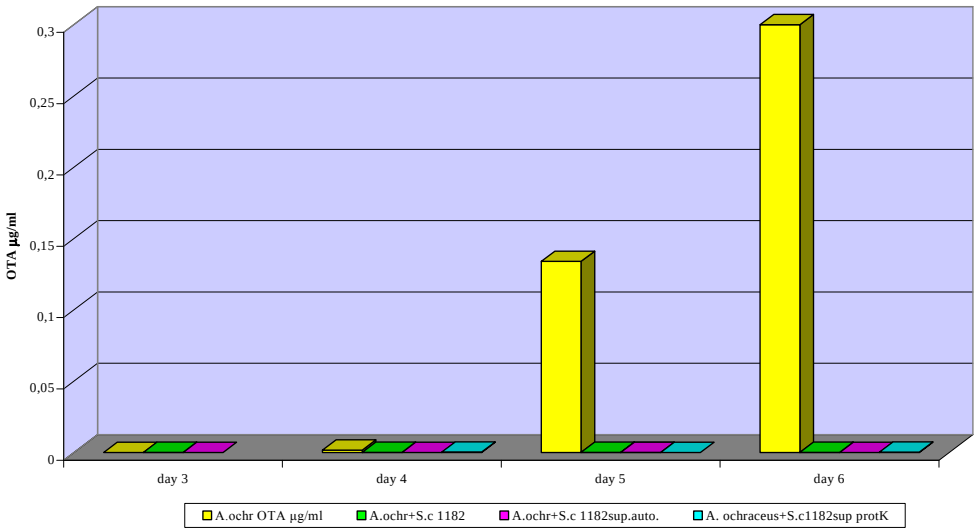


Figure 14. Ochratoxin A production by *A. ochraceus* on YES medium and *A. ochraceus* in *S.cerevisiae* 1182 crude supernatant, autoclaved and amended with proteinase K during 6 days. OTA accumulation was determined by HCPL and expressed in µg/ml.

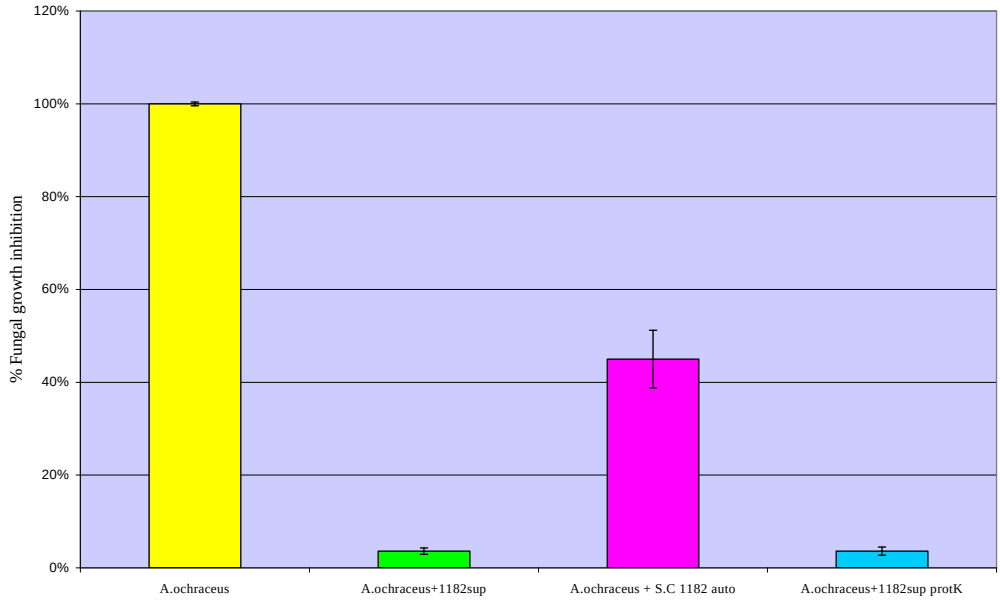


Figure 15. Percent of fungal growth inhibition of *A.ochraceus* grown in YES amended with *S. cerevisiae* 1182 crude supernatant, autoclaved and either treated with proteinase K after 6 days compared to *A. ochraceus* grown in YES medium (100% of growth). Results were the mean of three independent experiments.

Effects of *S. cerevisiae* BY4741 supernatant on OTA production and *A. ochraceus* growth

The potential influence of a widely used laboratory strain on OTA production and fungal growth was studied. *S.cerevisiae* BY4741 *(MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)* was selected as a model strain in order to establish if the biocontrol activity was a characteristic quality of *S. cerevisiae* 1182.

When *A. ochraceus* was grown in *S. cervisiae* BY4741 crude supernatant a reduction on OTA production was observed (Figure 16). No significative difference were observed in fungal biomass when *A. ochraceus* was grown in *S.cerevisiae* BY4741 supernatant.

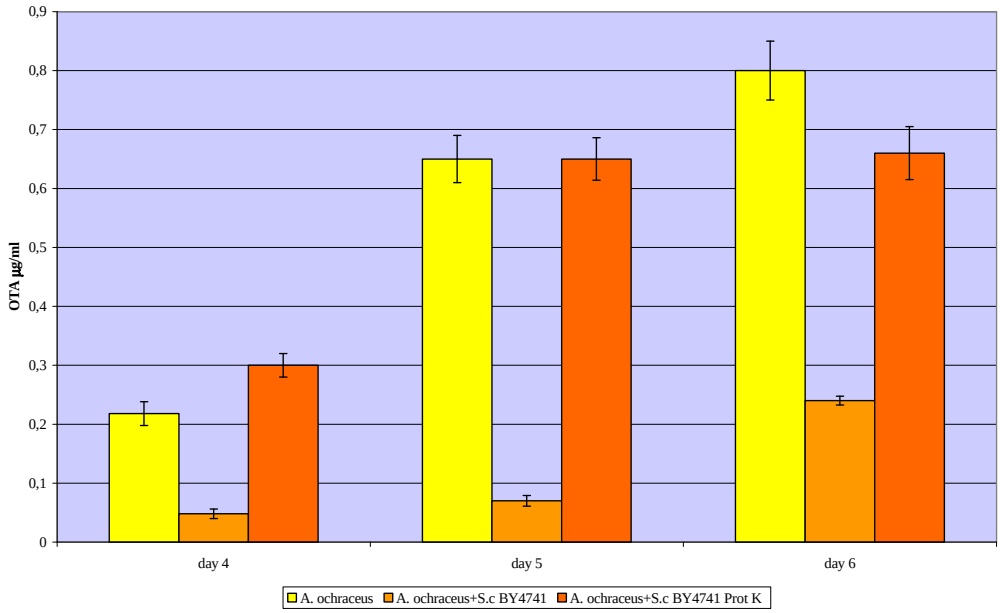


Figure 16 OTA production by *A. ochraceus* on YES medium and *A. ochraceus* in *S.cerevisiae* BY4741 crude supernatant and amended with proteinase K during 6 days in static cultures at 25°C. OTA values were expressed in μg/ml.

In figure 17 the activity of *S. cerevisae* 1182 and *S. cerevisiae* BY4741 supernatant on OTA production and fungal growth of *A.ochraceus* is compared. *S. cerevisae* 1182 has an effect on both OTA production and fungal growth.

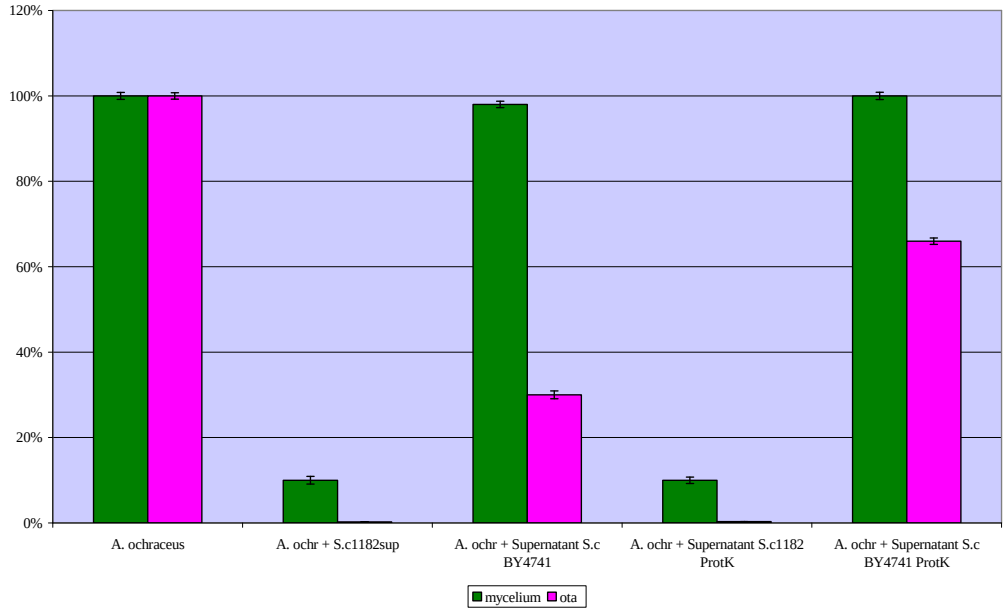


Figure 17. Percent of OTA production and fungal biomass in *A. ochraceus*, *A.ochraceus* in *S. cerevisiae* 1182 and in *S.cerevisiae* BY4741 supernatant (crude and amended with proteinase K) after 6 days grown in static cultures at 25°C.

Effects of *S. cerevisiae* 1182 supernatant on OTA production and *A. carbonarius* growth

When *A .carbonarius* MPV A566 was grown in YES amended with *S.cerevisiae* 1182 crude supernatant a reduction on OTA production followed by a decrease of 99% in fungal biomass was observed (Figure 19, 21).

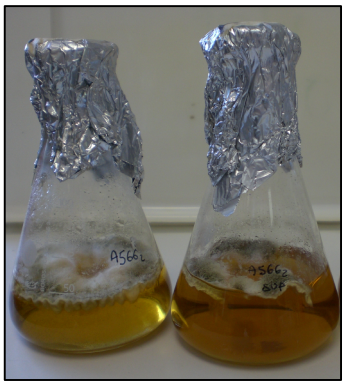


Figure 18. *A. carbonarius* MPV A566 in YES medium and *A. carbonarius* in *S. cerevisiae* 1182 supernatant after 4 days of growth at 25°C.

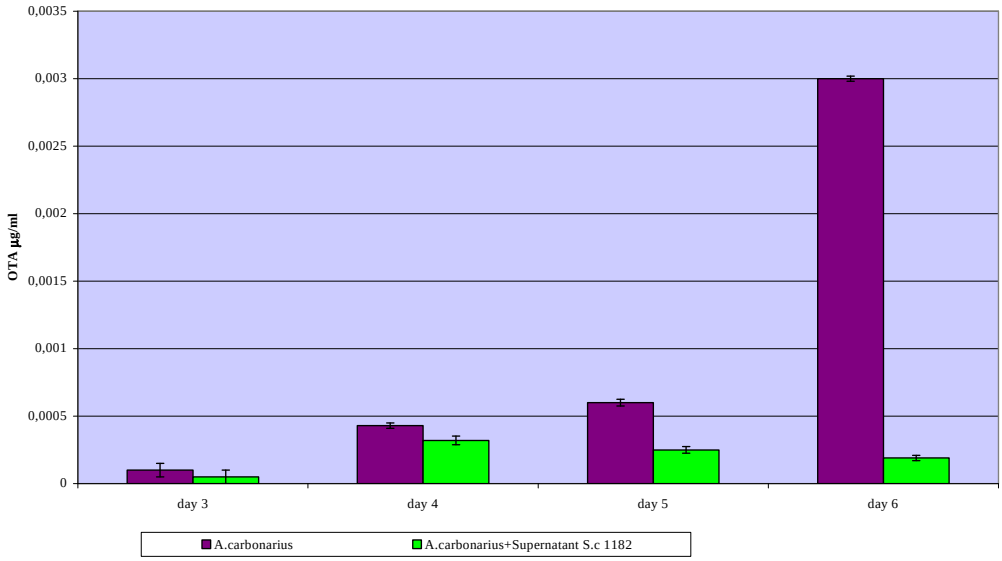


Figure 19. Ochratoxin A production by *A. carbonarius* MPV A566 on YES medium and in *S.cerevisiae* 1182 crude supernatant during 6 days of growth in static cultures at 25°C. HPLC values of OTA expressed as µg/ml. Each experiment is the mean of three different replicates.

In figure 20 is shown the decrease in OTA production of *A. carbonarius* MPV A566 when grown in *S. cerevisiae* 1182 supernatant (crude, autoclaved and treated with proteinase K), the OTA values decrease is supported also by a fungal biomass reduction (Figure 21)

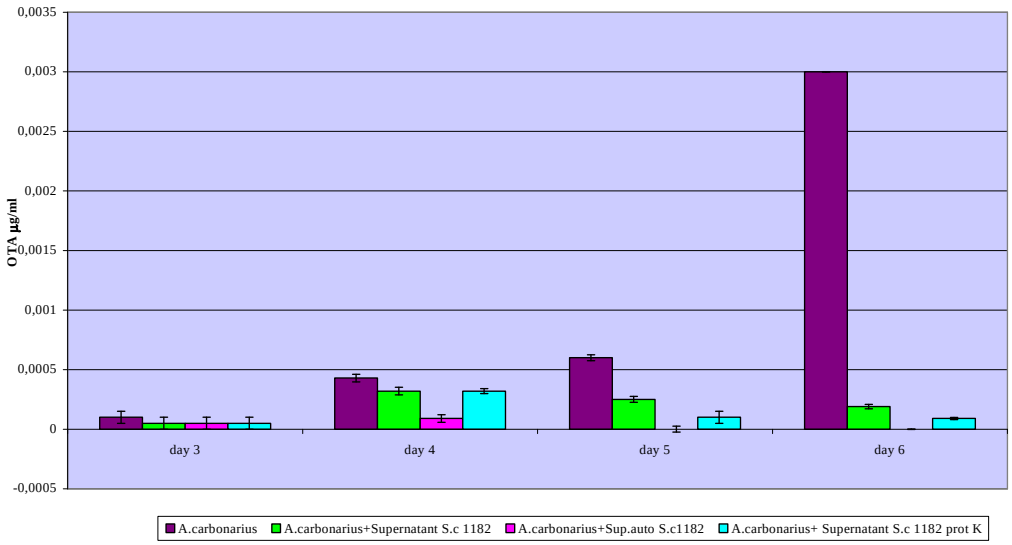


Figure 20. Ochratoxin A production by *A. carbonarius* MPV A566 on YES medium and in *S.cerevisiae* 1182 supernatant (crude, autoclaved and trated with proteinase K) during 6 days of growth in static cultures at 25°C. HPLC values of OTA expressed as µg/ml. Each experiment is the mean of three different replicates.

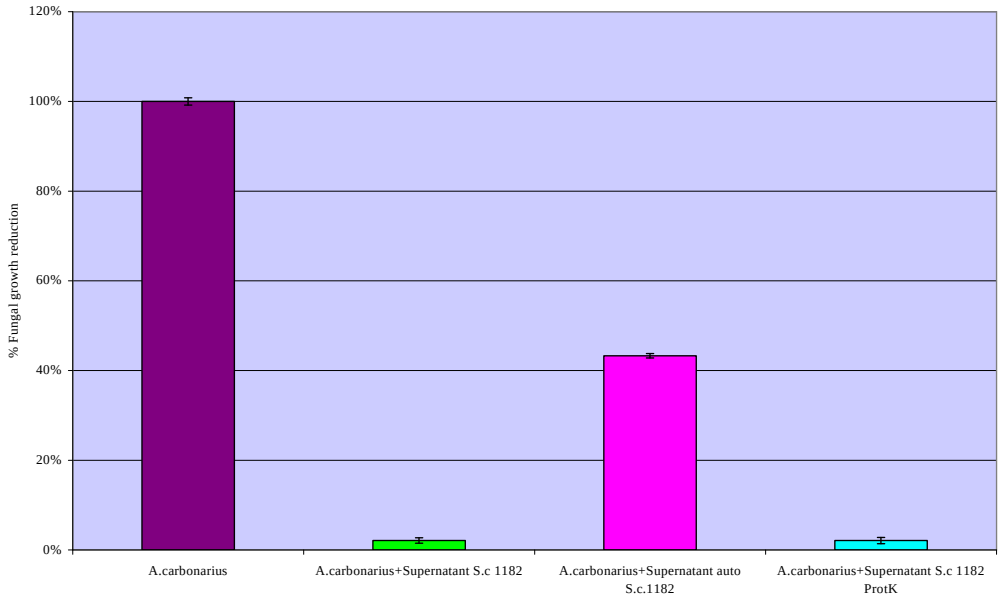


Figure 21. % of Fungal biomass reduction in *A. carbonarius* grown in YES medium and in *S. cerevisiae* 1182 supernatant (crude, autoclaved and amended with proteinase K after 6 days in static cultures at 25°C .

Effects of *S. cerevisiae* BY4741 supernatants on OTA production and *A. carbonarius* growth

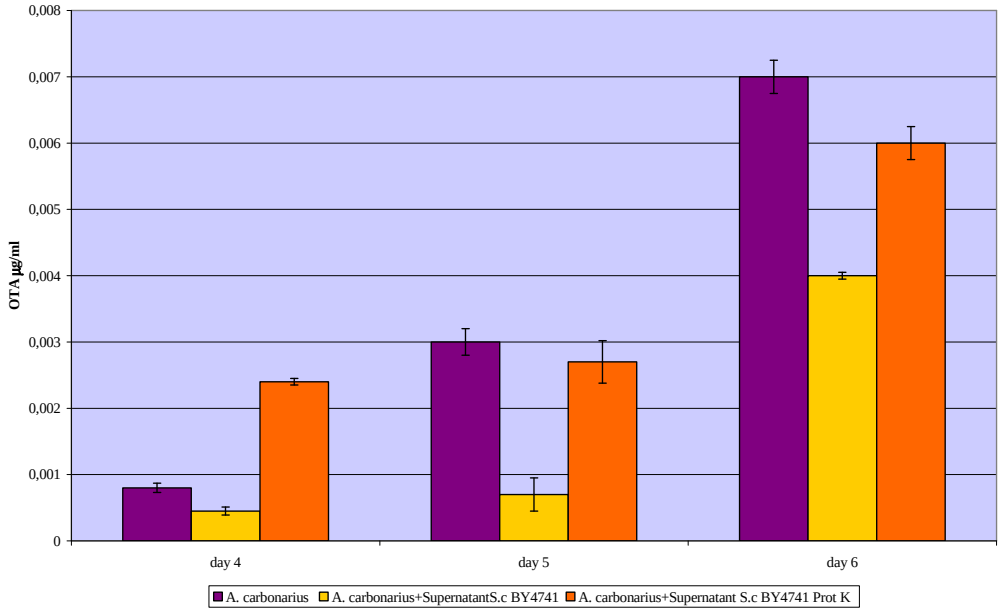


Figure 22. OTA production by *A. carbonarius* on YES medium and *A. carbonarius* in *S.cerevisiae* BY4741 supernatant (crude and amended with proteinase K) during 6 days of static growth at 25°C. OTA values were the means of three different replicates.

The activity of *S. cerevisae* 1182 supernatant was higher than *S.cerevisiae* BY4741 either on OTA production than in fungal growth of *A. carbonarius* .

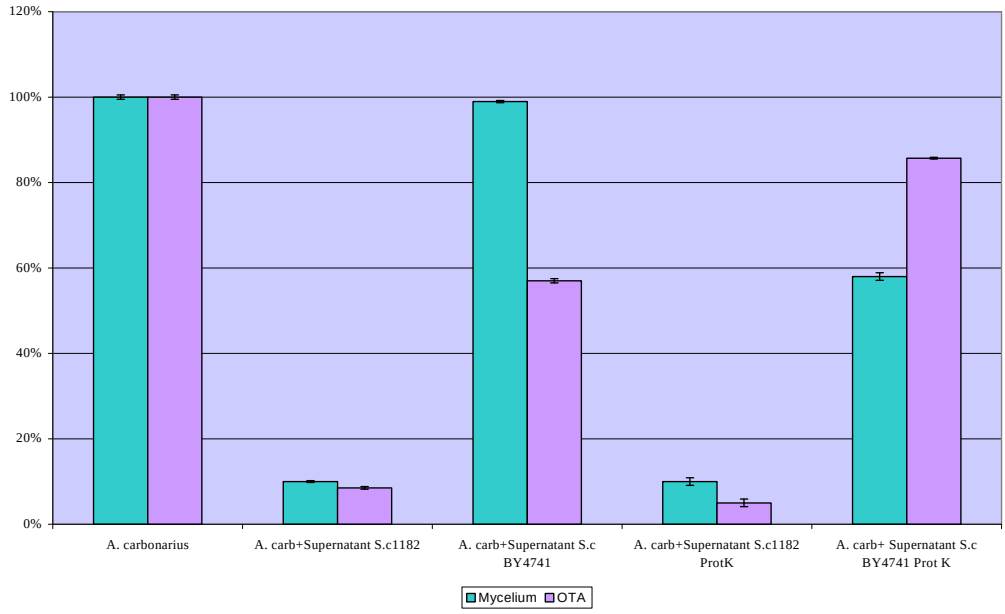


Figure 23. Percent of OTA production and fungal biomass in *A. carbonarius*, *grown* in *S. cerevisiae* 1182 and *S.cerevisiae* BY4741 supernatant (crude and amended with proteinase K) after 6 days in static cultures at 25°C.

SDS PAGE of yeasts supernatants

In order to attempt elucidating the inhibitory mechanism of yeast, *S. cerevisiae*1182 and *S. cerevisiae* BY4741 supernatants (crude, autoclaved and amended with proteinase K) were analysed by SDS PAGE.

Figure 24 shows the absence of polysaccharides in the autoclaved supernatants (A) and the presence of a single protein band corresponding to proteinase K (B).

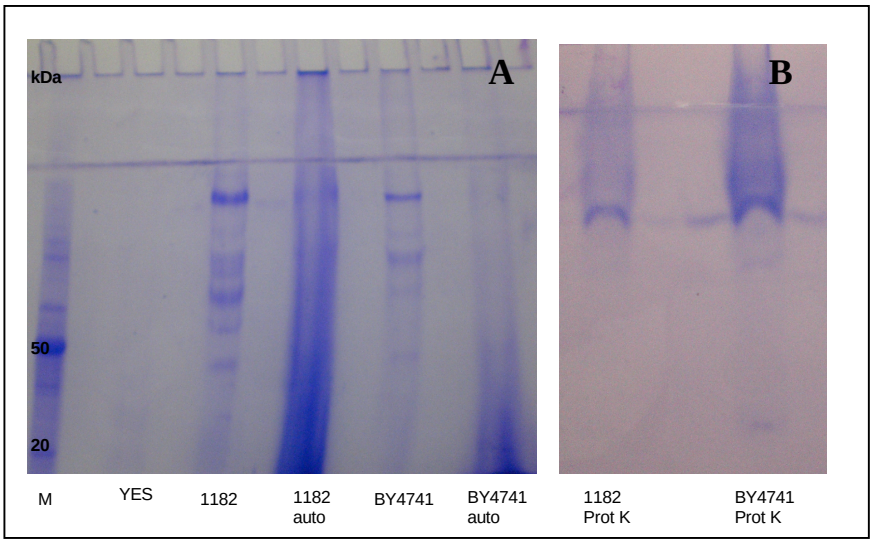


Figure 24. SDS PAGE of YES, *S.cerevisiae* 1182 crude and autoclaved supernatant, *S .cerevisiae* BY4741 crude and autoclaved supernatant (A); SDS PAGE of *S. cerevisae* 1182 supernatant amended with proteinase K and *S. cerevisiae* BY4741 supernatant amended with proteinase K

Killer activity strain screenings

The killer activity of the two *S. cerevisiae* strains used in the present work was tested against the classical killer toxins K1. Tests revealed that *S. cerevisiae* 1182 and *S. cerevisiae* BY4741 are killer neutral.

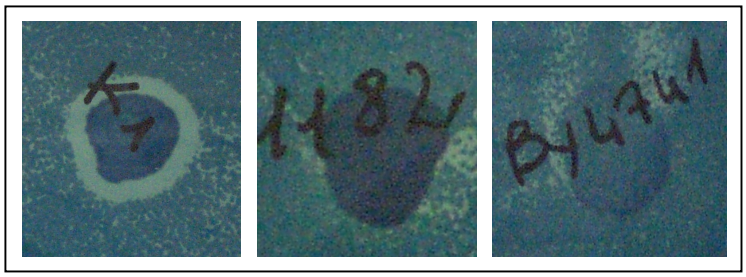


Figure 25. Pictures of the killer activity plate tests performed for *S. cerevisiae* 1182 and *S. cerevisiae* BY4741 with the reference killer strain (K1).

Effect of ethanol on fungal biomass

There are several compounds produced by yeasts during alcoholic fermentations that may become inhibitory to other species and ethanol is one of the main responsible. In order to establish if *S. cerevisiae* 1182 activity on *A. carbonarius* and *A. ochraceus* could be related to the presence of ethanol in the culture medium, moulds were grown in YES medium added with the same amount of ethanol present in supernatants.

In *S. cerevisiae* 1182 crude supernatant an amount of ethanol of 1,8% and of 2,4% in the autoclaved supernatant was found.

When *A. ochraceus* was grown in YES amended with 1,8% and of 2,4% of ethanol a reduction of fungal biomass was observed, while smaller biomass yields were observed when *A. ochraceus* was grown in *S. cerevisiae* 1182 supernatants (Figure 26).

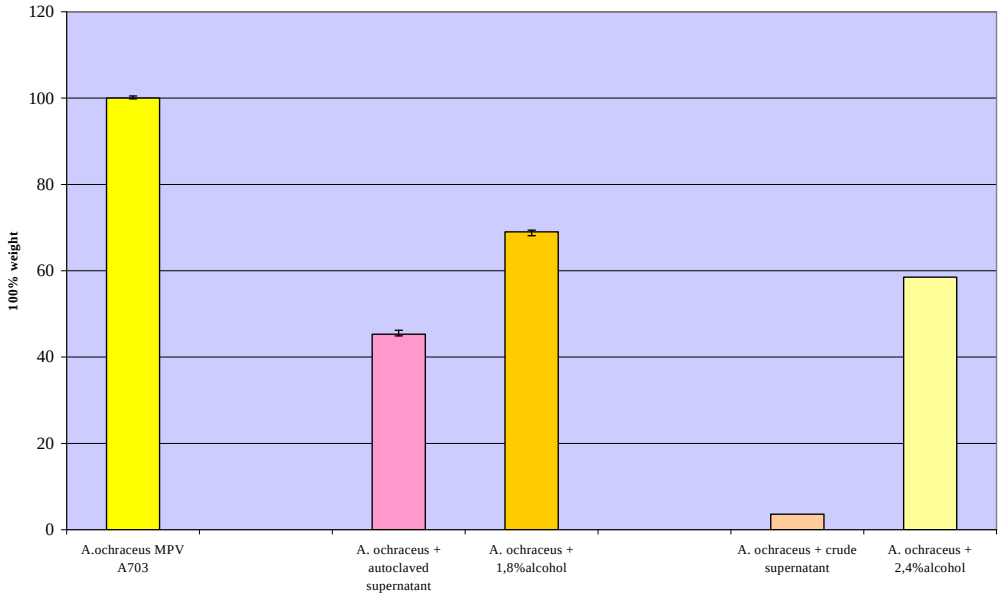


Figure 26. Percent of *A. ochraceus* MPV A 703 growth inhibition after 6 days in static cultures at 25°C. *A. ochraceus* was grown in *S. cerevisiae* 1182 crude and autoclaved supernatant and in YES amended with 1,8% and 2,4% of ethanol

When the same experiment was performed with *A. carbonarius* smaller biomass yields were observed when the mould was grown in *S.cerevisiae* 1182 supernatants (Figure 27).

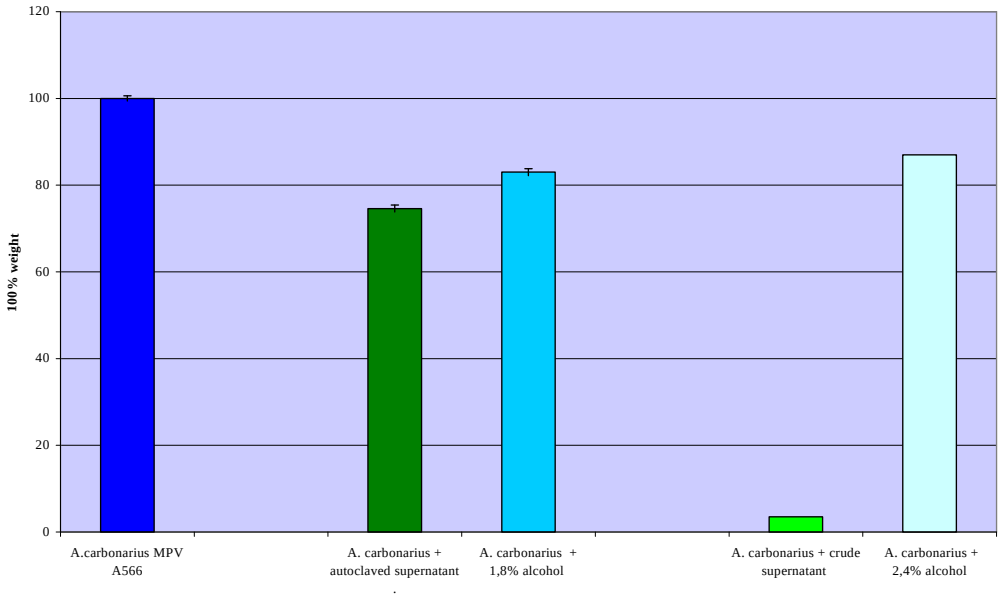


Figure 27. Percent of *A. carbonarius* MPV A566 growth inhibition after 6 days in static cultures at 25°C. *A. carbonarius* was grown in *S. cerevisiae* 1182 crude and autoclaved supernatant and in YES amended with 1,8% and 2,4% of ethanol.

Regulation of *pks* genes involved in OTA production by yeast strains

In order to examine a possible activity of yeasts on the expression of the *pks* gene and OTA production, *A.carbonarius* and *A.ochraceus* were grown in YES liquid medium with *S. cerevisiae* 1182 (selected as the best antagonist strain).

The transcription of each gene was monitored using a reverse transcription (RT)-PCR based approach, and OTA production was monitored in parallel by HPLC.

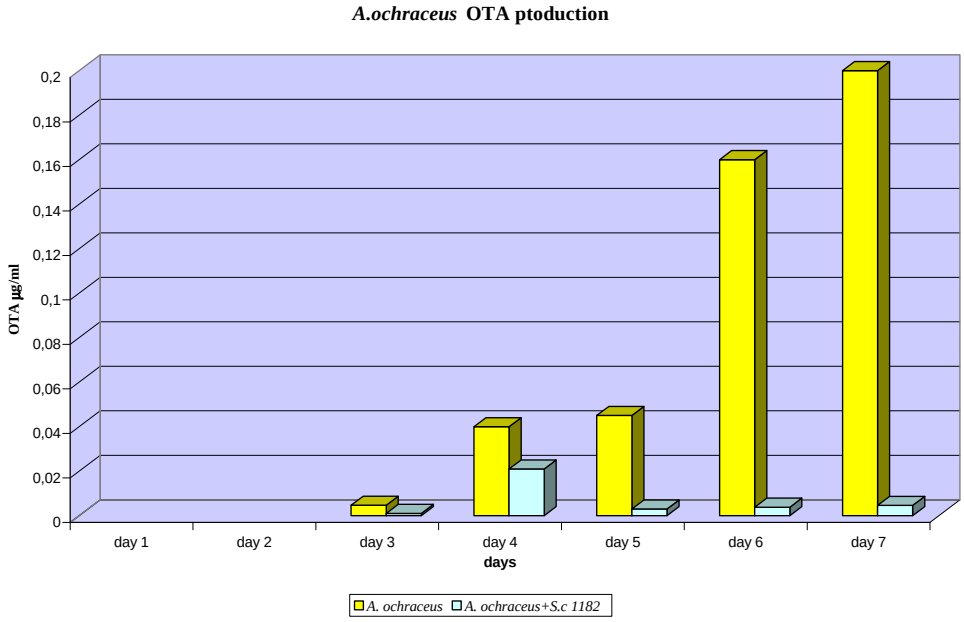


Fig. 28. OTA production by *A.ochraceus* and *A. ochraceus* co-cultured with *S. cerevisiae* 1182 on YES medium during 7 days of growth. OTA accumulation at different incubation days was determined by HPLC. Each OTA value are expressed as µg/ml and is the mean of three replicates from independent cultures.

When *A. ochraceus* was grown on YES in static at 25°C,OTA production was initially observed on day 3 with levels increasing to reach a maximum level on day 7; when *A. ochraceus* was grown in co-culture with *S. cerevisiae* 1182 a decrease in OTA values was observed (Figure 28). Analysis of *A. ochraceus* polyketide synthase gene transcript levels clearly indicate that the *pks* gene is underexpressed in the co-culture (Figure 29). The similar expression levels of β-tubulin gene showed that the changes observed with the *pks* gene were specific and not simply a result of changes in overall gene transcription levels in the fungus.

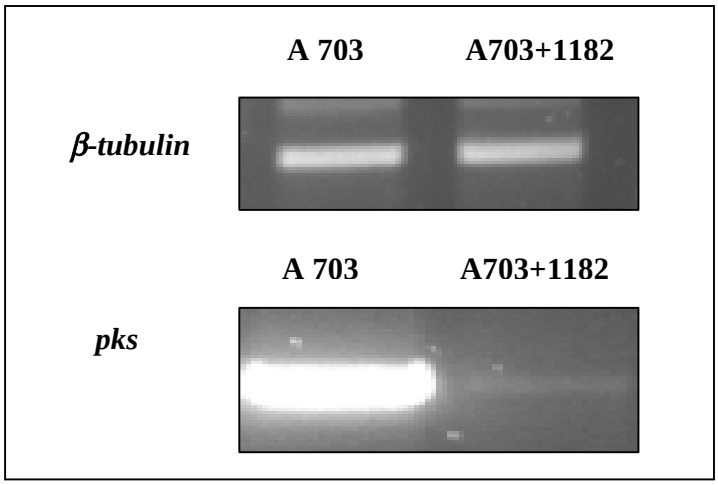


Fig. 29. RT-PCR analysis of *pks* gene and *β-tubulin* gene in *A ochraceus* MPV A703 alone or in co-culture with *S. cerevisiae* 1182, performed on RNA of 4 day-old mycelium grown in YES medium in static cultures at 25°C.

Figure 30 and 31 represent the effects observed on OTA production and polyketide gene expression when *A. carbonarius* was grown in YES medium in co-culture with *S. cerevisiae* 1182.

OTA production in *A. carbonarius* was initially observed on day 3 with levels increasing to reach a maximum level on day 6 followed by a decrease on day 7; OTA decreased in the co-culture and *carbonarius pks* gene is undexpressed in presence of *S. cerevisiae* 1182.

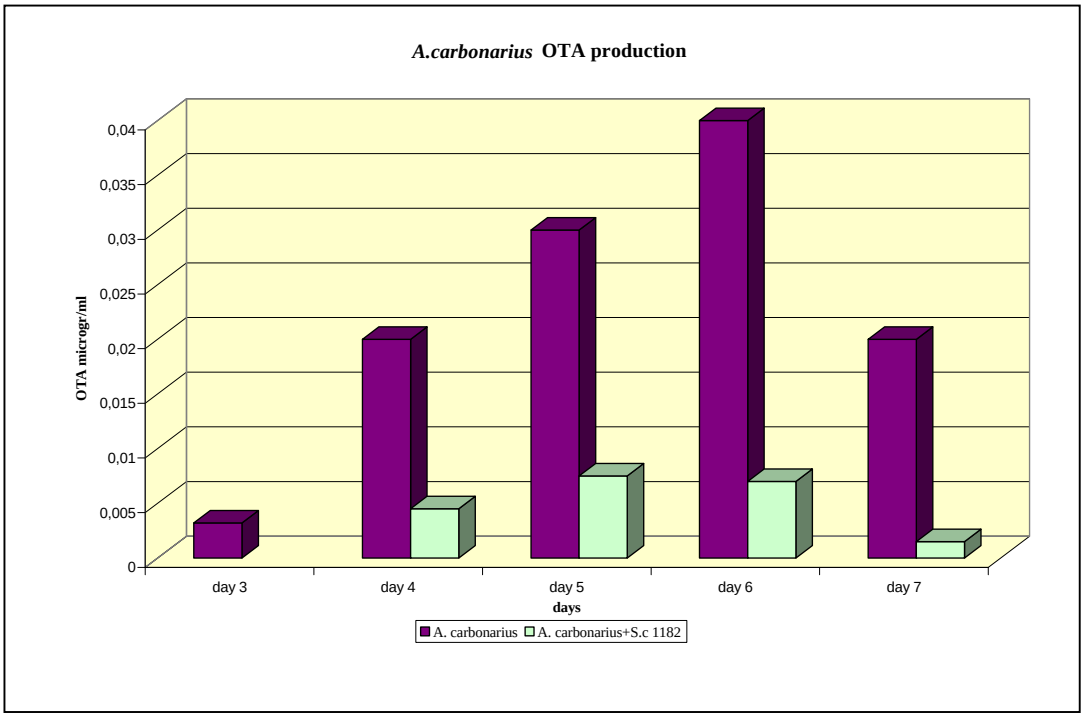


Fig. 30. OTA production by *A.carbonarius* and *A. carbonarius* co-cultured with *S. cerevisiae* 1182 on YES medium during 7 days of growth. OTA accumulation at

different incubation days was determined by HPLC. Each OTA value are expressed as µg/ml and is the mean of three replicates from independent cultures.

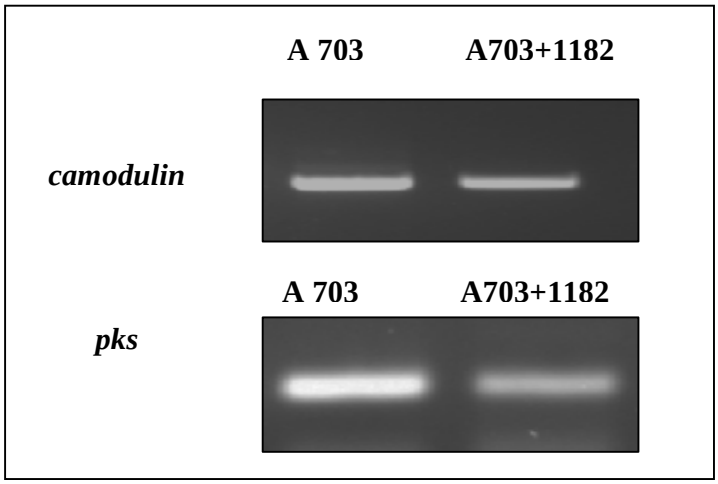


Fig. 31. RT-PCR analysis of *pks* gene and *calmodulin* gene in *A. carbonarius* MPV A566 alone or in co-culture with *S. cerevisiae* 1182, performed on RNA of 4 day-old mycelium grown in YES medium in static at 25°C.

Regulation of *pks* genes involved in OTA production by yeast supernatants

Growing *A. ochraceus* in S.*cerevisiae* 1182 supernatant a decrease in OTA levels was observed starting from day 3. A smaller reduction was observed when *A. ochraceus* where grown in *S. cerevisiae* BY4741 supernatant (Figure 31). *A. ochraceus pks* gene is underexpressed in the supernatant culture and the gene expression is correlated with the OTA production (Figure 32)

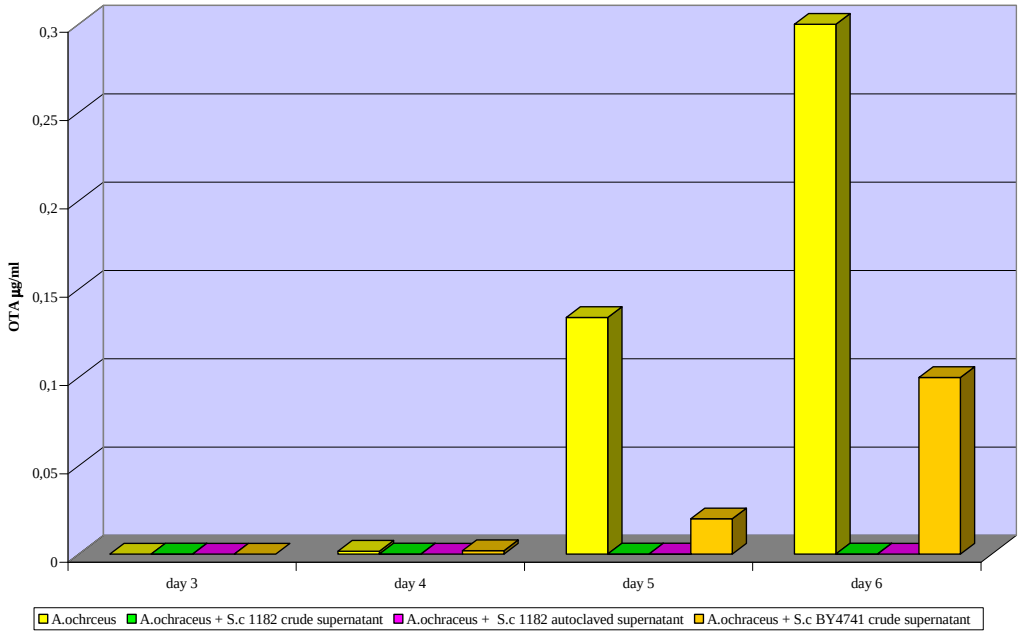


Figure 32. OTA production by *A.ochraceus* and *A. ochraceus* co-cultured with *S. cerevisiae* 1182 supernatant (crude and autoclaved) and *S. cerevisiae* BY4741 crude supernatant during 6 days of growth. OTA accumulation at different incubation days was determined by HPLC. Each OTA value are expressed in µg/ml and is the mean of three replicates from independent cultures.

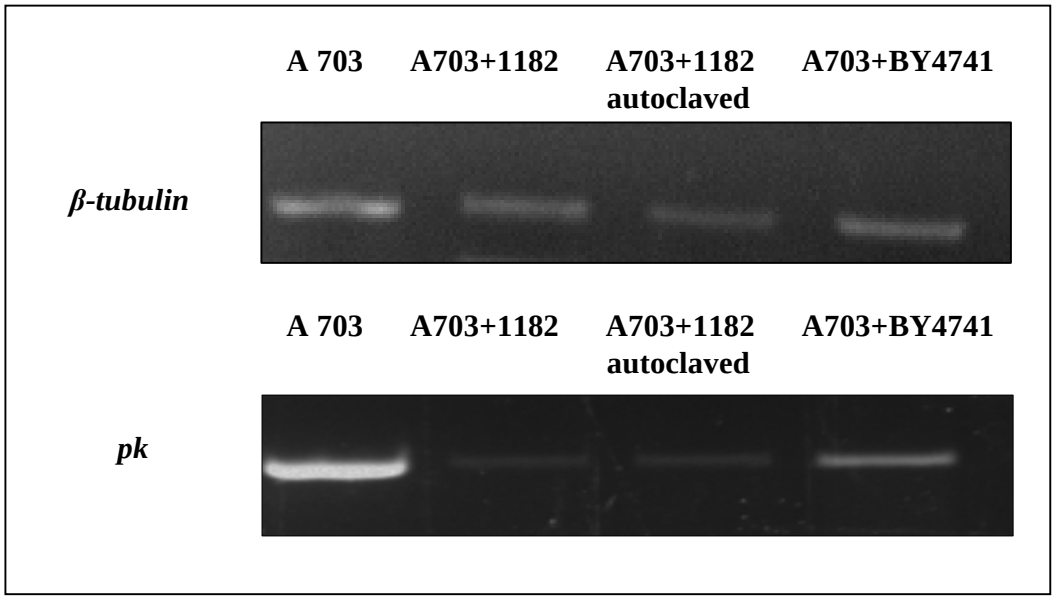


Figure 33. RT-PCR analysis of *β-tubulin* gene and *pks* gene in *A ochraceus* MPV A703 alone or cultured in *S. cerevisiae* 1182 supernatant (crude and autoclaved) and *S. cerevisiae* BY4741 crude supernatant, performed on RNA of 6 day-old mycelium grown in static at 25°C.

When the same experiment was performed in *A. carbonarius* OTA production decreased in the S.*cerevisiae* 1182 supernatant co-culture either crude or autoclaved. (Figure 34).

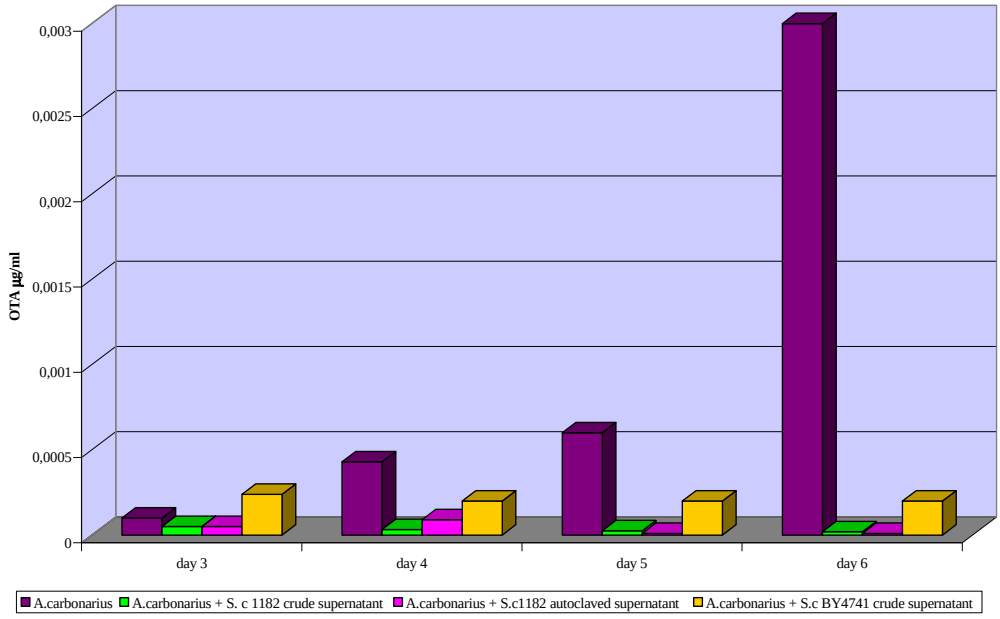


Figure 34. OTA production by *A.carbonarius* MPV A566 and *A. carbonarius* co-cultured with *S. cerevisiae* 1182 supernatant (crude and autoclaved) and *S. cerevisiae* BY4741 crude supernatant during 6 days of growth. OTA accumulation at different incubation days was determined by HPLC. Each OTA value are expressed in µg/ml and is the mean of three replicates from independent cultures.

The underexpression of the *pks* gene in the supernatant co-cultures, suggested a link between *pks* gene expression and OTA production, no difference in the expression levels of the costitutively expressed calmodulin gene was observed (Figure 35).

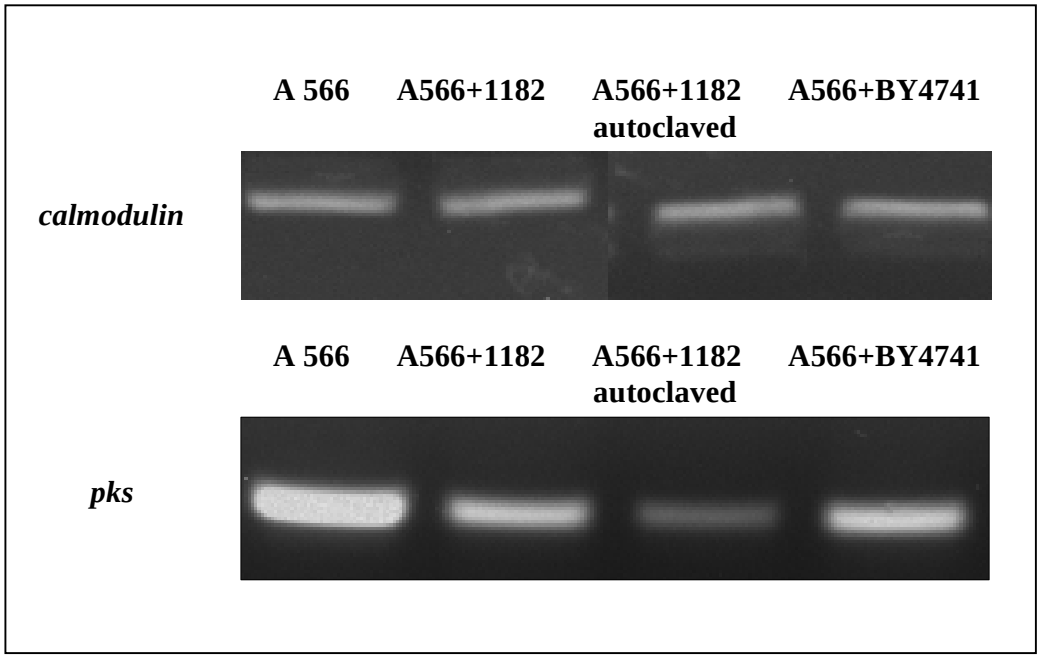


Figure 35. RT-PCR analysis of *calmodulin* gene and *pks* gene in *A carbonarius* MPV A566 and *A. carbonarius* co-cultured with *S. cerevisiae* 1182 supernatant (crude and autoclaved) and *S. cerevisiae* BY4741 crude supernatant, performed on RNA of 6 day-old mycelium grown in static at 25°C.

DISCUSSION

Discussion

One of the aim of this thesis was to identify yeast species which show antagonist activity against *A. carbonarius* and *A. ochraceus* and which may have the potential to prevent the proliferation of these ochratoxigenic fungi in grapes and derived products. The OTA-producing isolates belonging to *A. carbonarius* and *A. ochraceus* species were selected because they play a significant role as the source of OTA contamination in wine (Cabanes et al., 2002).

Yeasts have a long history of proven safe use as fermentative starters in food and beverages (Jakobsen and Narvhus, 1996). Moreover, the need to develop natural alternatives to chemical preservation, has led to the use of yeasts as biocontrol agents toward other yeasts, moulds and even bacteria. Numerous reports showed that yeasts appear to be promising for the control of spoilage microorganisms. This property has been well studied and exploited in the biological control of postharvest diseases of fruits (Fleet, 2003).

During wine making, OTA levels were found to decrease during fermentation, and this decrease was suggested to be due to the action of lactic acid bacteria and yeasts. The mycotoxin level reduction, is supposed to be due to its adsorption on the surface of *Saccharomyces* cells, to its interaction with metabolites produced by yeasts or to its degradation by the lactic bacteria present in wine (Bejaoui et al., 2004, and ICV, 2002).

The yeast isolates used in this study belong to Di.S.A.A.B.A Culture Collection of the University of Sassari, Italy. 32 yeast strains, isolated in Sardinia from grape, must and wine samples were ascribed to *Saccharomyces cerevisiae* (23 isolates) and *Kloeckera apiculata* (9 strains) species following identification by PCR amplification of the 5.8-ITS region. *S. cerevisiae* strains were further characterised according to Marinangeli et al., (2004) which showed that *S. cerevisiae* genes encoding cell wall proteins, such as *AGA1*, *DAN4*, *HSP150* and *SED1*, are highly polymorphic in length and are preferential targets for PCR-based typing of *S. cerevisiae* wine strains. Within the *S. cerevisiae* population analysed, the four sets of primer pairs highlighted the existence of extensive length polymorphisms in each of the cell wall genes analysed.

A preliminary bio-typing of the identified yeast isolates was obtained by evaluating their antagonistic effects against *A. carbonarius* and *A. ochraceus* in a co-inoculation (fungus–yeast) assay performed *in vitro* on agar plates using different culture media. This effect was considered indicative of the fungal sensitivity to the action of a yeast isolate present in the same biological niche.

Culture media played an important role in fungal growth, yeast isolates representing 35% of the analyzed population, showed a mean of reduction up to 65% in *A. carbonarius* growth on YES medium When *A. carbonarius* and *A. ochraceus* were co-cultured with yeast strains in liquid media a higher recovery of OTA was obtained from CYB than from YES, although no statistically significant differences were found. Abramson and Clear 1996 reported that differences in recoveries from both

culture media could be related to sucrose content: compared to YES (15% sucrose), CYB (3% sucrose) probably presents a less hydrophilic layer more permeable to lipophilic solvents used for OTA recovery.

The percentage of OTA reduction by yeast was between 50% and 100% depending on the yeast strain used. The significant decrease of OTA contamination in the co-culture with yeast could be due to an inhibition of *A. carbonarius* growth. Nevertheless, the production of the mycotoxin is not necessarily proportional to the biomass of the mycotoxigenic fungi, as was shown in studies on other mycotoxins (Xu et al., 2007). An increase of OTA production per pathogen biomass could take place as a consequence of competition among microorganisms for essential environmental factors. Inter-microbial competition is a stressful condition and is expected to have a dramatic effect on the secondary metabolism of spoilage fungi. Nutrient availability strongly affects mycotoxin production. It was observed that the amounts of OTA decreased when increasing incubation time. Some authors suggested that microorganism such as other fungi could remove and assimilate the phenylalanine moiety from the OTA molecule, as other nitrogen sources of the culture medium become exhausted (Téren et al., 1996).

In vivo trials where carried out to verify the degree of infection of *A. carbonarius* co-cultured with yeast incubated at 25°C for 7days

Different grape varieties could also affect fungal invasion, as skin hardness and thickness may become a hurdle for the penetration of the mould. On this assumption

in this study two Sardinian grapes's cultivars (Vermentino and Cannonau) were infected with *A. carbonarius* at 25°C.

A. carbonarius is a very invasive fungus, able to colonise and penetrate berries even without skin damage and to grow at 25-35 ºC and 0.95-0.99 aw respectively (Leong et al., 2004). .Differences of more than 80 % in the percentage of intact and damaged wine berries colonised both internally and superficially by *A. carbonarius* were observed In figure …is show the percent of fungal growth reduction by yeast on Vermentino and Cannonnau berries after 7 days at 25°C. The reduction was between 80% and 99% for Vermentino and between 45% and 99% for Cannonau suggesting that white grapes can be affected easily by mould infection, and this characteristic could be correlated probably with skin thickness.

The experimental conditions used to assess the biocontrol activity of the yeast strains against *A. carbonarius* in laboratory were strongly favorable to the pathogen and to its production of OTA. In fact, OTA accumulation occurs mainly at ripening, when the fungus preferentially infects berries by entering skin wounds made by insects and/or injuries by meteorological phenomena. High levels of fungal infections and of the consequent wine contaminations with OTA take place when high humidity and temperature as well as damage to berries co-occur. Furthermore, the levels of infection by *A. carbonarius* and the synthesis of OTA are the highest on wounded berries that are detached and that are subjected to the temperatures that were used in our laboratory experiments. Although we had provided environmental conditions that were highly conducive to infection, almost all yeast strains provided an efficient

protection of wine grape berries from *A. carbonarius* for up to 4 days after the beginning of these experiments.

All the yeast strains selected in this study for the biocontrol of the two ochratoxigenic strains of *A. ochraceus* and *A.carbonarius* on grape showed a comparable growth ratewhen inoculated in the absence of fungal spores as control,, into grape wounds. Because growth to similar biomass levels would require consumption of approximately equal amounts of nutrient, general nutrient competition in the grape berry is not by itself sufficient to explain yeast biocontrol activity. Nevertheless, this finding does not exclude the hypothesis that the antagonistic behaviour shown by the yeast isolates could be the effect of competition for a specific growth limiting factor, e.g., a vitamin or another particular metabolite.

Another hypothesis that may explain the reduction of moulds growth is that yeasts produce secondary metabolite(s) with antifungal properties. On the basis of this hypothesis either the combination of extracellular hydrolytic enzymes and secondary antifungal metabolite(s) or the secondary antifungal metabolite(s) alone can be assumed to play a major role in the inhibition of OTA production and fungal growth. To investigate over this assumption, yeast supernatants were treated by heating at 100 °C for 1 hour or with proteinase K at 37 °C for 60 min before the antifungal bioassay. *S. cerevisiae* 1182, which showed the best antagonist activity ,and *S. cerevisiae* BY4741, a widely used laboratory strain, were selected for this experiment. A difference in the percentage of growth inhibition for both experimental fungi was observed by using the heat-treated or the proteinase K-treated culture

filtrates. Heat treated supernatant reduced *A. ochraceus* and *A. carbonarius* growth up to 55% while a reduction of about 95% was observed in in both untreated and heat treated supernatant. The antifungal potential of *S. cerevisiae* BY4741 supernatant against *A. ochraceus* and *A. carbonarius* was lower than *S. cerevisiae* 1182. No significative reduction in fungal biomass was observed in *A. ochraceus*, instead of a reduction of 55% by *S. cerevisiae* BY4741 proteinase K-treated supernatant in *A. carbonarius* growth.

Considering OTA production, the results showed that *A. ochraceus* and *A. carbonarius* OTA production was reduced about 10 fold by *S. cerevisiae* 1182 culture filtrate either untreated, heat-treated and proteinase K-treated culture filtrate as opposed to *S. cerevisiae* BY4741 culture filtrates. *A. ochraceus* and *A. carbonarius* OTA production was inhibited about 4 fold by untreated supernatant and about 1 fold by proteinase K-treated culture filtrate. *S. cerevisiae* BY4741 proteinase K-treated supernatant seemed to have a higher effect on both OTA and fungal biomass reduction compared to untreated supernatant.

SDS page analysis of yeast supernatant showed that no protein was involved in the antagonistic activity. Fungal growth inhibition by yeast culture filtrate was probably related to the presence of unknown thermostable antifungal compound(s) rather than to the presence of a proteic compound. And the origin of a possible antifungal compound should be further elucidated.

It is well known that during wine fermentations yeasts can produce, besides ethanol, other toxic compounds, namely, killer toxins, short- and medium-chain fatty acids

and sulphite, able to induce death of other yeasts. (Fleet, 2003). Several killer strains of *S. cerevisiae*, isolated from wine musts, have been found to kill other sensitive yeasts within the firsts days of fermentation, although the majority of these toxins are only effective against other *S. cerevisiae* strains (Pérez et al., 2001). To assure that classical killer toxins produced by *S. cerevisiae* were not involved in this biocontrol phenomenon, killer activity of the two yeast strains used in the present work was tested against the classical killer toxins K1. Tests revealed that *S. cerevisiae* 1182 and *S. cerevisiae* BY4741 are killer neutral. Although significant, these results do not exclude the production by *S. cerevisiae* of any other unknown killer-like toxins (e.g. peptides, proteins or glicoproteins) or even some fermentative metabolites potentially toxic. To determine the nature of the possible toxic compounds involved in the activity of these *Saccharomyces* strains, further investigation will be carried out.

Among the physicochemical hurdles cited in the literature, ethanol is recognized as a mould inhibitor (Legan, 1993) and ethanol is also well known for its antomycotic effect in foods (Daifas, 2000). The major target of ethanol is the lipid membrane, but it has many other effects on fungal cells. Survival of fungi following ethanol stress is strain dependent and some ethanol producing yeasts can survive in up to 20% ethanol. Thus, it is to be expected that high concentrations (5%) of ethanol will be required to to affect survival of fungal spores upon short exposure times. Fungal growth involves germination and hyphal extension, eventually forming visible mycelium. A food matrix will be spoiled shortly after spores are germinated.

Therefore, prevention of germination will prevent fungal growth, the subsequent spoilage of food and the possible production of mycotoxins.

Yeasts during alcoholic fermentation can produce different amount of ethanol and it is species and strain specific. *S. cerevisiae* 1182 is a wine strain able to produce 16,5% of ethanol at 25 °C after 9 days in rich medium. In order to establish if the presence of ethanol was responsible of *S. cerevisiae* 1182 activity on *A. carbonarius* and *A. ochraceus*, moulds were grown in YES medium added with the same amount of ethanol present in supernatants. The amount of ethanol founded in *S. cerevisiae* 1182 supernatants was 1,8% in the autoclaved supernatant and 2,4% in the crude supernatant. The influence of ethanol on mycelium development of *A. ochraceus* and *A. carbonarius* was assessed by growing the two moulds in YES amended with 1,8% and 2,4% of ethanol and in *S. cerevisiae* 1182 supernatant. Comparing the fungal biomass yields of *A. ochraceus* grown in *S. cerevisiae* 1182 supernatant and *A. ochraceus* grown in YES amended with 1,8% and 2,4% of ethanol, a higher reduction on mould growth was observed in yeast supernatant. Ethanol had an evident impact on fungal growth, as fungi exposed to ethanol correspondingly modified their intracellular metabolisms. In presence of 1,8% of ethanol *A. ochraceus* biomass reduction was up to 31% and to 41,5% in presence of 2,4% of ethanol.

S. cerevisiae 1182 crude supernatant reduced both *A. ochraceus* and *A. carbonarius* growth up to 96,5%. A reduction of 55% in *A. ochraceus* and of 25% in *A carbonarius* biomass were observed in autoclaved supernatant, suggesting that the anti-fugal activity could be ascribed to a termolable compound. The higher activity of

yeast supernatant could be done by the action of an antimicrobial molecule eventually present in the yeast culture filtrate instead of the ethanol inhibition.

In order to examine a possible correlation between OTA production and the expression of *pks* genes regulated by the yeast antagonistic activity, *A. carbonarius* and *A. ochraceus* were exposed to *S. cerevisiae* 1182 (co-cultured), to *S. cerevisiae* 1182 supernatant and to *S. cerevisiae* BY4741 supernatant which have previously shown to affect OTA production.

Polyketide synthase (PKS) proteins are commonly found in many fungi and are primarily involved in the synthesis of a wide variety of different secondary metabolites. PKSs are multifunctional enzymes encoded by a single gene and typically possess up to eight types of functional domains (Bingle et al. 1999). The cloning and molecular characterisation of many of the genes encoding fungal PKSs has been greatly facilitated by the very conserved nature of these functional domains, which has allowed the design of gene probes and degenerate primers which have been used to isolate the gene fragments encoding the PKS domains (Cox et al., 2004; Nicholson et al., 2001). The diversity of PKS genes in *A. carbonarius* (Atoui et al., 2006) and in *A. ocharceus* (O'Challagan et al 2003) have been already investigated.

The transcriptional profiling carried out in Botton et al., 2008 allowed the identification of some differentially expressed genes putatively involved in the biosynthesis of OTA and its regulation.

In this work the transcription of the genes was monitored using a reverse transcription (RT)-PCR based approach, and OTA production was monitored in parallel by HPLC. Analysis of *A. carbonarius* and *A.ochraceus* polyketide synthase gene transcript levels indicate that *pks* gene transcripts seemed to correlate closely with OTA production. Moreover the *pks* gene is under-expressed in presence of *S. cerevisiae* 1182 and . *S. cerevisiae* 1182 supernatant.

In Botton et al. 2008, a likely connection was pointed out between OTA biosynthesis and sexual/asexual sporulation, along with common signalling pathways. Indeed, the involvement of G protein and Ca^{2+}/calmodulin signalling was proposed, important transcription factors were also identified, such as the Cys_2His_2 and the $Zn(II)_2Cys_6$ zinc fingers, possibly acting antagonistically. On this assumption, a post-transcriptional regulatory mechanisms due by the presence of *S. cerevisae* 1182 could act on protein stability and activity. One more plausible explanation for the *pks* under expression, observed here, is that the genes involved in the polyketide biosynthesis, may be transcriptionally regulated in presence of *S. cerevisiae* 1182 and that the observed reductions in OTA levels may result from the regulation of gene involved in OTA biosynthesis.

Specific studies should be addressed to the functional characterization of the genes involved in the OTA production and their regulation by *S. cerevisiae* 1182, either by silencing or knock-out approaches. Such experiments may have important

implications in the full comprehension of the biosynthesis of OTA, a toxin that may become a serious problem for the worldwide wine industry.

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