Collège Doctoral du Languedoc-Roussillon

# THÈSE

Pour obtenir le grade de

Docteur

Délivré par **Université Montpellier 2**

Préparée au sein de l'école doctorale Science des Procédés-Science des Aliments (SPSA)
Et de l'unité de recherche UMR 95 QUALISUD

Spécialité: **Biotechnologie - Microbiologie - Biologie Moléculaire**

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# IMPACT DES TRAITEMENTS PRÉ-RÉCOLTE (CHIMIQUE ET BIOLOGIQUES) SUR LES ÉCOSYSTÈMES FONGIQUES ET LA CONTAMINATION PAR L'OCHRATOXINE A DE RAISINS

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UNIVERSITÉ MONTPELLIER 2
SCIENCES ET TECHNIQUES

*To EGYPT*

*To the pure spirit of my mother*

*To my dear father and sisters*

*To the beloved husband*

*And to my dear daughters*

# ACKNOWLEDGEMENT

*It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.*

*Above all, I would like to thank my husband* ***Mohamed*** *for his personal support and great patience at all times. My* ***parents, sisters*** *have given me their unequivocal support throughout, as always, for which my* ***mother*** *expression of thanks likewise does not suffice.*

*This thesis would not have been possible without the help, support and patience of my principal supervisor,* ***Prof. Sabine SCHORR-GALINDO****, from finding an appropriate subject in the beginning to the process of writing the thesis, not to mention her advice and knowledge. The good advice, support and friendship of my second supervisor,* ***Dr. Caroline STRUB****, has been invaluable on both an academic and a personal level, for which I am extremely grateful.*

*I would like to acknowledge the financial, support of the* ***Egyptian government*** *particularly all the present (specially,* ***Prof. Aml El-SABBAN****,* ***Dr. Cherif KHATER*** *and* ***Mrs. Hanan El-Sharkawy****) and previous (****Prof, Camelia SOBHY****,) members of the* ***cultural office of Egypt in Paris*** *that provided the necessary financial, moral and other possible supports for this research and my stay in France.*

*I would like to acknowledge the academic and technical support of the University of Montpellier II for the library facilities and computer facilities of the University, particularly Mrs.* ***Cathy HOUEIX***

*I also thank the members of* ***QUALISUD*** *for their support and assistance since the start of my research in 2009, especially the kindness of* ***Dr. Jean-Claude BACCOU****. I remember the generosity and encouragement of the late* ***Dr. Robert RATOMAHENINA*** *who had welcomed this academic study and taught me a lot.*

*I am gratefully appreciate the administration support of* ***Prof. Valérie MICARD*** *and the guidance advice of* ***Mrs. Régine GRASMIK*** *and* ***Mrs. Estelle MONTEIL.***

*I would like to thank* ***Anaïs STEMLEN, Frédérique HILIARE, Gabriel BASTARD****, Master students and* ***Norhan SALAH****, undergraduate student, who were involved in this project at certain stages of this research with a great help, highly responsible and cooperative with solving the problems about running the studies. My special thanks go to* ***Mrs. Veronique MARTINEZ, Dr. Isabelle MARAVAL*** *and* ***Dr. Noël DURAND*** *who helped me with measurements at certain time of this research.*

*I want to thank* ***Dr. Didier MONET*** *and* ***Dr. Jean-Christophe MEILE*** *for their help and laboratory providing for the DGGE technique. Thanks to my supportive friend* ***Dr. Aly EL-SHEIKHA*** *and* ***Miss. Yasmine HAMDOUCHE*** *for teaching me the DGGE technique and the software programs that I used for this part of research.*

*Last, but by no means least, I thank my friends in* ***EGYPT, FRANCE*** *and elsewhere for their support and encouragement throughout.*

*For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.*

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| *Figure N°* | *Figure description* |
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| *Figure N°* | *Figure description* |
|---|---|
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|---|---|
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# LIST OF ABBREVIATIONS

| Abreviation | Description |
|---|---|
| ***AflaA*** | Aflatoxin A |
| ***Afla B*** | Aflatoxin B |
| ***ALR*** | Acquired Local Resistance |
| ***APS*** | Ammonium persulfate |
| ***ASR*** | Acquired Systemic Resistance |
| ***CFU*** | Colony Forming Unit |
| ***CYA*** | Czapek Yeast Extract Agar |
| ***DGGE*** | Denatured Gradient Gel Electrophoresis |
| ***DNA*** | Deoxyribonucleic acid |
| ***dNTP*** | Deoxynucleotide triphosphate |
| ***ds-DNA*** | Double strandes DNA |
| ***EB*** | Ethidium Bromide |
| ***EDTA*** | Ethylenediaminetetraacetic acid |
| ***EEC*** | Council Regulation in the European Union |
| ***e.g. /e.i.*** | example given |
| ***et al.*** | From the latin phrase *et alii* meaning *and others* |
| ***FAO*** | Food and Agriculture Organization of the United Nations |
| ***GC*** | Gas Chromatography |
| ***HPLC*** | High Performance Liquid Chromatography |
| ***IAC*** | Immuno-affinity Columns |
| ***IARC*** | International Agency for Research on Cancer |
| ***IFV*** | French Institute of vine and wine |
| ***ISO*** | International Organization for Standardization |
| ***ITS*** | Internal Transcribed Spacer |
| ***LOD*** | Limit of Detection |
| ***LOQ*** | Limit of Quantification |
| ***MATAB*** | Mixed Alkyl Trimethyl Ammonium Bromide |
| ***MS*** | Mass Spectrometry |
| ***NDS*** | Natural Defense Stimulator |
| ***OTA*** | Ochratoxin A |
| ***OTA-PF*** | OTA-Producing-fungus |
| ***OTB*** | Ochratoxin B |
| ***OTC*** | Ochratoxin C |
| ***pb*** | Base pairs |
| ***PKS*** | Polyketide synthase |
| ***PCR*** | Polymerase Chain Reaction |
| ***PDA*** | Potato Dextrose Agar |
| ***RFLP*** | Restriction Fragment Length Polymorphism |
| ***SCOOP*** | Scientific Cooperation on Questions Relating to Food Projects |
| ***SDS*** | Sodium Dodecyl Sulphate |
| ***sp*** | specie |
| ***spp*** | Species |

*List of Abbreviations*

| | |
|---|---|
| ***SPME*** | Soild Phase Micro-Extraction |
| ***T*** | Tones |
| ***TAE*** | Tris/Ammonium/EDTA (buffer) |
| ***TE*** | Tris/EDTA (biffer) |
| ***TEMED*** | N,N,N',N' Tetramethylethylenediamine |
| ***TLC*** | Thin Layer Chromatography |
| ***UV*** | Ultraviolet radiation |
| ***v/v*** | Volum to volum |
| ***VOC*** | Volatile Organic compound |
| ***YM*** | Yeast extract- malt extract broth |

# General Introduction

# GENERAL INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that possesses a risk to human health due to its nephrotoxic, immunotoxic, mutagenic, teratogenic and carcinogenic effects. This toxin was originally described as a metabolite of *Aspergillus ochraceus* when grown in pure culture. The latest information indicates that *P. verrucosum* is the main species associated with OTA production in foods and feeds in temperate climates, while *Aspergillus* spp. predominate in warmer and tropical countries. These species are included in Circumdati and Nigri sections, such as *A. ochraceus*, *A. melleus*, *Aspergillus carbonarius and Aspergillus niger aggregate.*

OTA has been detected in various foods, including cereal products, coffee, spices, fermentation products such as wine and beer and products of animal origin.

The presence of OTA in grape juices and wines was reported for the first time by Zimmerli and Dick (1995). Later surveys were carried out to evaluate the levels of OTA in grape juices and wines in Europe and South Africa. Data on OTA occurrence in wine showed levels of up to 7.0 ng/mL, and higher levels in red wine produced in Europe (Chulze *et al*., 2006).

The IARC (International Agency for Research on Cancer) have classified OTA as a possible carcinogen (group 2B). OTA toxicity appears to be related to its ability to inhibit protein synthesis by competing with phenylalanine in the reaction catalyzed by phenylalanyl-tRNA synthetase and other systems requiring this amino acid. In the European Union it has only been regulated recently in a few food products, such as cereals and their based products (5 µg /kg) and dry grapes (3 µg/kg). In Italy there is a regulation also for beer (200 ng/Kg). The European Union is considering to impose regulatory limits in more foodstuffs such as wine, grape juice, coffee and cocoa (Turner *et al.,* 2009).

Food safety is a major concern around the world and the driving force of all aspects of mycotoxin research (Schepers and Balázs, 2007). Even though the main objective of the food and feed chain processors and distributors is to avoid the extended contamination of plant-derived foods and animal feeds with mycotoxins, until now, complete OTA removal from foods and feedstuffs is not feasible. Prevention through pre-harvest management is the best method for controlling mycotoxin contamination.

However, in the case that the contamination occurs after this stage, the hazards associated with OTA must be managed through post-harvest strategies. Due to the increasing

number of fungal strains resistant to chemical fungicides and the impact of these pesticides on the environment and human health, maximum levels of chemical residues have been regulated in many products. Alternative methods are necessary to substitute or complement treatments with fungicides to control fungi under field or storage conditions (Ponsone *et al.,* 2012).

The present work was conducted on the context of different global and national projects on OTA prevention and control, such as the European multidisciplinary project "Risk assessment and integrated ochratoxin A management in grape and wine" (2001-2003), which involved several European countries in the Mediterranean basin, relevant for grape growing, the Spanish Project (Evaluación del riesgo de contaminación por ocratoxinas y tricotecenos en alimentos y desarrollo de métodos de control, CICYT, Comisión Interministerial de Ciencia y Tecnología) and the recent Mycored European project "Novel integrated strategies for worldwide mycotoxin reduction in food and feed chains" (2009-2013). Moreover, the new REACH European legislation and the French plan Ecophyto 2018 encourage research on biological treatments against fungal development, as a substitute for chemical treatments whose efficacy is estimated at less than 60% for the vine. France is the first European consumer of pesticides and the third user in the world and 20% of pesticide treatments are for vineyards. Fungicides, used for intensive wine production, contribute to France consumption for about 50% of pesticides. Pesticide residues that can be found in water, soil or food concern overall population and ecosystems. In this context, the subject of this thesis concern the potential use of biological treatments for vine protection against ochratoxigenic fungi in order to reduce both chemical residues and OTA in vine products and preserve environment and producer health.

The first chapter of this thesis is the bibliographic review, which includes a state of art on the global and French viticulture; the most diseases affecting grapevine; a general view on the mycotoxin and its major types; and particularly, on the Ochratoxins (OTA) structure, biosynthesis, producing species, affected food products in general and especially grape & its products; the techniques used for OTA measurement and control strategies (preharvest and post-harvest ones).

The second chapter is the material and methods used during conducting this work.

The third chapter presents the obtained results in a form of publication articles (these articles had already submitted to the mentioned journals. Result chapter consists of two major parts; each part contains two submitted articles, with a brief introduction and conclusion sections. Each article had certain objectives, which will be consequently illustrated:

The **overall objective** was reducing the OTA contamination in grapes and grape juices by alternative safe control treatments to the chemical fungicides. The present thesis was focused in providing tools for preventive action to further reduce the human intake of OTA from grapes and its products while preserving the environment and the health of producers.

**In particular, the objectives were grouped in the following:**

**The first part:** To study the impact of four pre-harvest treatments; chemical fungicide, fungal biocontrol agent, yeast biocontrol agent and plant extract as elicitor regarding to two control modalities.

- **Prevention of OTA contamination:** (Article 1: Crop protection journal, submitted)
  1- Assess a preharvest treatment reducing the OTA accumulation in grapes and its products. Which could substitute the traditional chemical fungicides treatments *(Chromatographical analysis:* Food safety, toxigenicity).
     - To study the presence of OTA in natural grape juice.
     - To test the *in vitro* ability of the ochratoxigenic isolates to produce OTA.
  2- Impact on the fungal ecosystem in general and the black aspergilli strains *(Microbiological studies:* fungal ecosystem, fungal biodiversity, black aspergilli biodiversity)
     - To screen the myco-flora present in grapes.
     - To identify the ochratoxigenic species present in grapes and other non-black aspergilli
  *3-* Assess a fast and accurate monitoring technique evaluating the preharvest treatments and modality; using different molecular techniques; qualitative technique (PCR-DGGE) and quantitative technique (real time PCR)
  *(Molecular approaches:* efficacy, rapidity)
- **The impact of the proposed OTA-control preharvest treatments on the physicochemical characteristics of the grape and its juice:** (Article 2: Journal of Agricultural and Food Chemistry, submitted)
  *(Physiological and physico-chemical study:* Disease resistance, food quality*)*

1- Physical characteristics of the grape berries measurement (berry skin thickness, wax and cuticle masses and berry size) and grape juice (color)
2- Biochemical characteristics of the grape juice (sugar content, acid content, polyphenolic compounds).

**The second part:** Assessment of the mode of action of the plant extract as elicitor

- **Assess the interaction between the non-black-Aspergillus (NBA) strains isolated from the plant extract treatment and *A. carbonarius* growth and OTA production** (Article 3: Food Control, submitted)

  *(Biological control)*

  - To assess *in vitr*o interaction effect on the OTA-PF mycelia growth
  - To assess *in vitr*o interaction effect on the OTA-PF ability of OTA production.

- **Assess the antifungal activity of the volatile compounds identified from the Stifénia treatment** (Article 4: Crop Protection, submitted)

  *(Antifungal activity, volatile compounds)*

  - To determine the direct antifungal effect of the Stifénia powder against the OTA-PF
  - To identify and comparing volatile compound profiles of grapevine leaves from parcels with Stifénia and fungicide treatments.
  - To assess *in vitr*o the effect of identified volatile compound with high concentration in leaves from the plant extract treatment on *A. carbonarius* growth and OTA production.

The fourth chapter included a general conclusion, which resumes the main results obtained from all the different articles and the perspectives for the future work.

# Chapter 1

# Bibliographic Review

# 1. BIBLIOGRAPHIC REVIEW

## 1.1. The grapevine

The grapevine (*Vitis vinifera*) belongs to the family *Vitaceae*, which comprises about 60 inter-fertile wild *Vitis* species distributed in Asia, North America and Europe under subtropical, Mediterranean and continental-temperate climatic conditions. It is the single *Vitis* species that have European origin (Soyer *et al*., 2003; Villa, 2005) and also acquired significant economic interest over time; some other species, for example the North American *V. rupestris*, *V. riparia* or *V. berlandieri*, are used as breeding rootstock due to their resistance against grapevine pathogens, such as Phylloxera, Oidium and mildews (Terral *et al.,* 2010). Indeed, a great majority of cultivars widely cultivated for fruit, juice and mainly for wine, classified as *Vitis vinifera* L. subsp. *vinifera* (or *sativa*), derive from wild forms [*Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi] (Terral *et al.,* 2010).

The fruits of grapes are formed in clusters containing many berries which botanically classified as grapes of small size with light color for the white grapes (greenish, yellowish, walleye) or more darker for the red grapes (pink, black-purple) (Anglade and Puisais, 1987). According to the FAO (Food and Agriculture Organization of the United Nations), for 2011, the global production of grapes was 69 093 293 T which 40% of this production produced in Europe. The French grape production was 6 588 904 T (FAOSTAT/FAO Statistics Division 2013/01 October 2013) (Fig. 1.1.). About half of the European fruit cultivated areas is cultivated by grapevine that produce approximately 40% of total fruit production. That highlights the importance of the grapevine in Europe in general and in France particularly, hence, France occupies the 5$^{th}$ global level regarding the countries grape production) (Fig. 1.1).

The grapes are used primarily in the production of wine from its fermented juice, but are also eaten as a fruit, either fresh (table grapes), either dry raisin which is mainly used in baking or cooking. Other grape products could be found such as grape juice, water spirits of wine, grape sugar, grape seed oil (Kamel *et al.*, 1985; Maier *et al.*, 2009), wine vinegar, and grape jam.

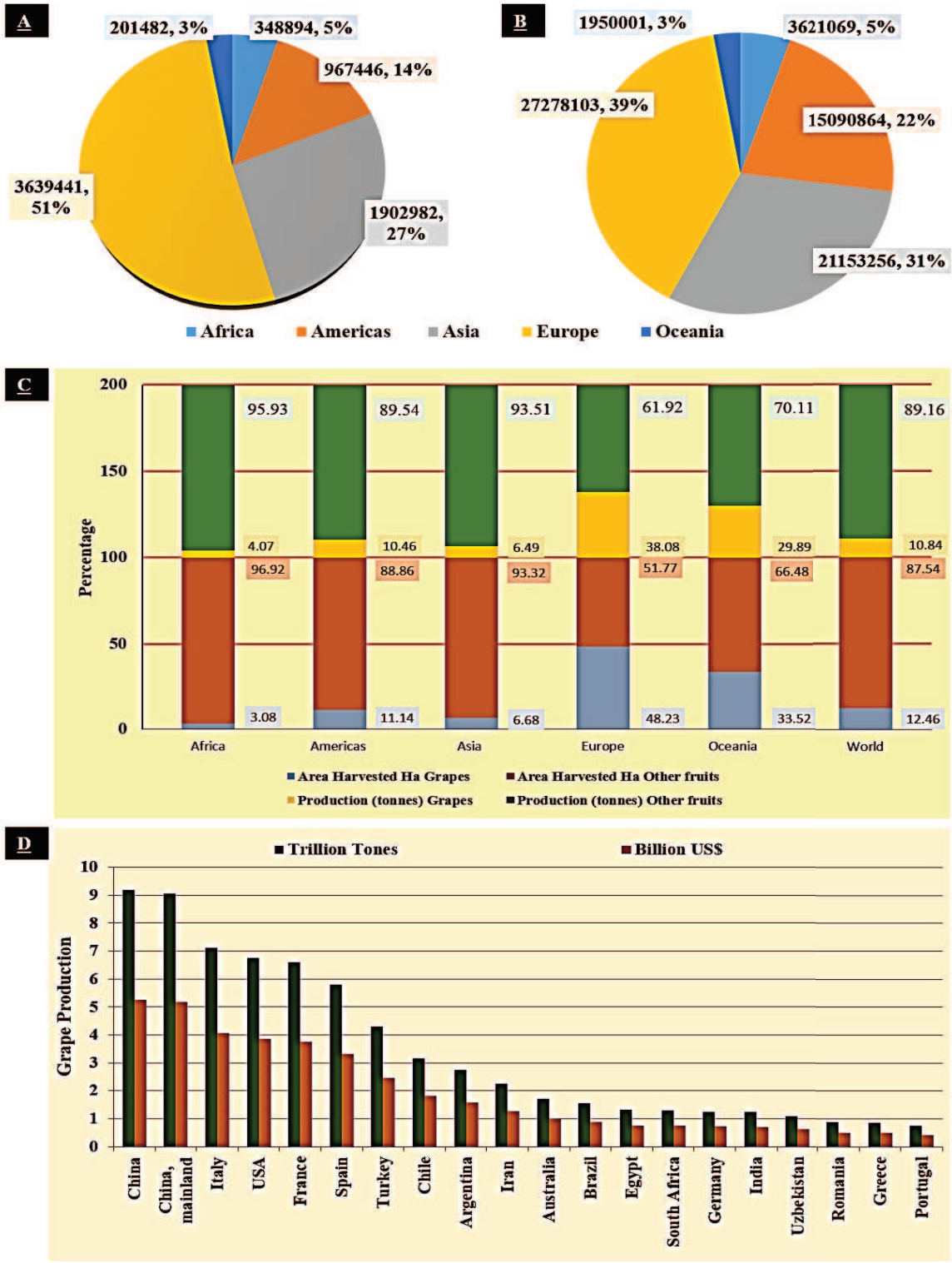



**Figure 1.1.: Grapes; A: Global harvested area (Ha), B: Global Production (T), C: Percentage of harvested area and production of grapes compared to all total fruits, D: Production (quantity and value) in the highest twenty countries all over the world** (FAOSATA, 2011).

## 1.1.1. Grapevine clusters and berries

### 1.1.1.1. Clusters

Grape cluster organized around the rachis (Fig. 1.2.) which is branched. Branching forms the longest principal inflorescence axis or rachis. The shorter branches supporting the grain are called pedicels. These pedicels end with enlargement bead which fit the berries. The relative dimensions of the different branching give a cluster more or less compact. The stalk (rachis) consists essentially of water (78-80%), the lignin, cellulose, tannins and minerals (Navarre, 1994).

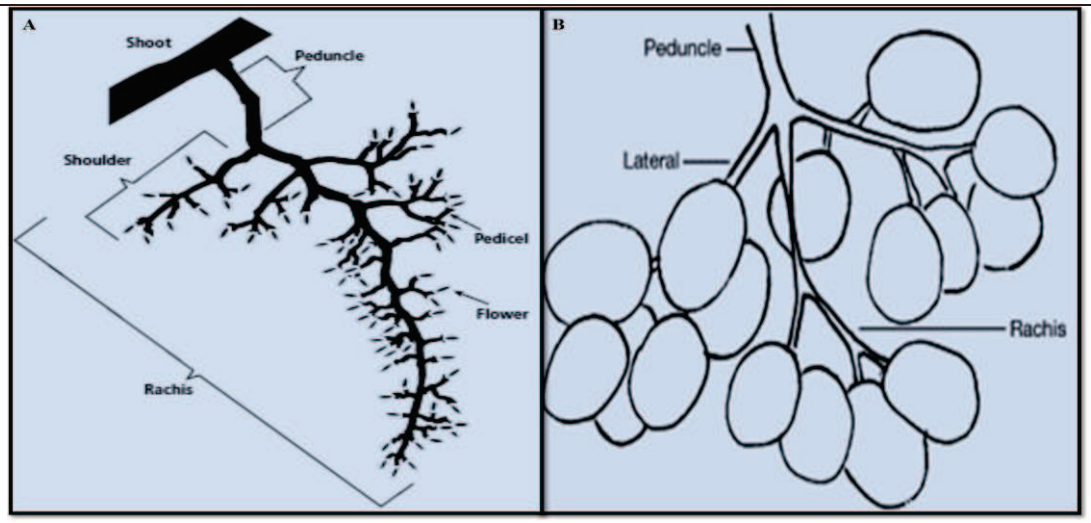



**Figure 1.2: Grape cluster structure, A: Typical 'Thompson Seedless' flower cluster** (Dokoozlian, 2000) **B: Grape berry cluster** ( Margaritis and Jones, 2006)

### 1.1.1.2. Grape Berry

The grape berry has three major types of tissue (Fig. 1.3.): seed, skin and flesh. Grape berries can have a maximum of four seeds, although in practice the seed number is usually one or two (Cawthon *et al.,* 1982; Coombe, 1987). Seeds are made up of the outer and inner integument, which together form the testa (seed coat), the nucellus surrounding the developing endosperm, and the embryo (with two cotyledons, epicotyl, hypocotyl, and radicle). Certain grape varieties are seedless that is an important characteristic for the raisins (dry grape) production.

After fertilization the pistil develops into the fruit, with the ovary wall (pericarp) becoming the skin and flesh of the grape berry. The pericarp consists of three anatomically distinct tissues: the exocarp, mesocarp, and endocarp (Coombe, 1987). The exocarp forms the grape's dermal system, or 'skin', which makes up between 5% and 18% of the fresh weight of mature berries. It is made up of a cuticle-covered (single-layer) epidermis and the

underlying outer hypodermis (Considine and Kriedemann, 1972). Epicuticular wax covers the surface of the cuticle forming a strongly hydrophobic layer that protects the berry from water loss (Rogiers *et al.,* 2004). The structure of the epicuticular wax changes with age of the berry. Although the wax is normally crystalline, the crystals appear to degrade slowly over time (Rogiers *et al.,* 2004). The wax material is rather soft and can be altered or removed by the impact of rain, by abrasion from wind-blown particles, or by contact with other berries and leaves.

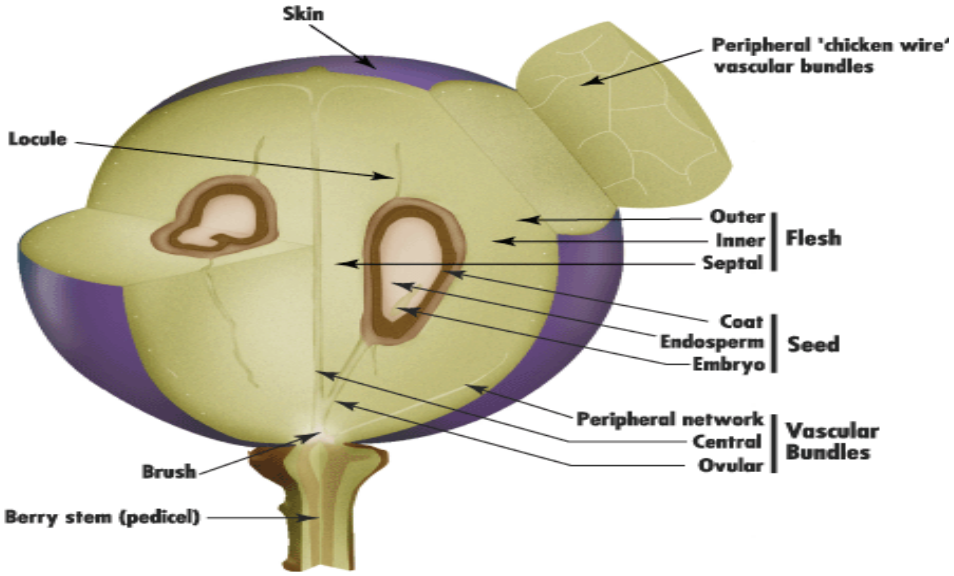



**Figure 1.3.: Structure of a ripe grape berry partially sectioned on the long and central axis** (Coombe, 2001).

The mesocarp, which is commonly called the 'flesh' or 'pulp' of the grape berry, consists of 25 to 30 layers of thin-walled and highly vacuolated parenchyma cells. Vacuoles can make up as much as 99% of the cell volume in ripe grape berries and contain sugars and organic acids. While the mesocarp cell walls remain intact, their polysaccharide components (such as cellulose) are modified at veraison to enable the berry to soften (Coombe, 1987; Ollat *et al.,* 2002). Moreover, the incorporation of soluble proteins (especially glycoproteins, *i.e.* proteins attached to sugar molecules) reinforces the cell walls, so that cellular integrity can be maintained during softening (Davies *et al.,* 1999). The outer mesocarp contains the tissues outside the network of peripheral vascular bundles of the pericarp; the inner mesocarp is inside the network and, at maturity, makes up almost two thirds of the berry volume (Coombe, 1987).

The innermost tissue in the pericarp is the septal tissue, or the endocarp, which surrounds the seeds. Throughout the flesh and under the skin are vascular bundles. The ovular

vascular bundles that previously served the ovary give rise to a complex network of vascular traces (axial and peripheral) that supply the seed and the pericarp (Pratt, 1971). The central vascular bundles (axial) and their associated parenchyma cells are termed the 'brush' and remain attached to the pedicel when a ripe grape is plucked from the cluster. Vascular bundles are composed of xylem with the phloem next to it and are responsible for water and nutrients transport through the plant (Matthews and Shackel, 2005).

### 1.1.2. Chemical cmoposition

Grapevine contains many contents which are generally mostly localized at certain parts of the grape berries as shown in Figure1.4. and Table 1.1.

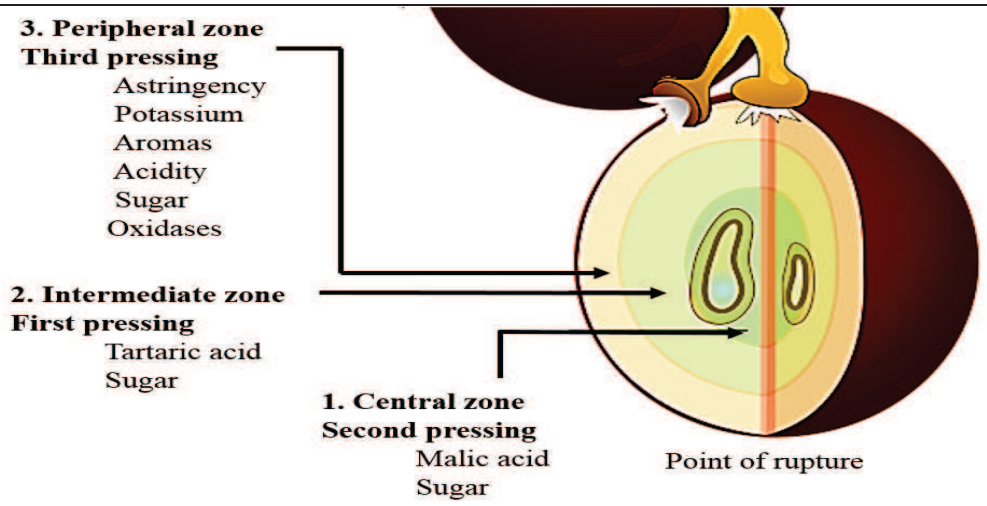



**Figure 1.4.: Schematic structure of a ripe grape berry flavor zones** (Dunsford and Sneyd, 1989)

#### 1.1.2.1. Water

Grape berries require a significant amount of water for growth and development, and water typically contributes 70 to 80 percent of berry fresh weight at harvest. Although they lack functional stomata soon after fruit set, berries transpire significant amounts of water during their development. Prior to veraison, most of the water required by the fruit is supplied by the xylem. However, shortly after veraison the xylem vessels entering the berry are blocked. With water flow via the xylem disrupted, the phloem becomes the primary supplier of water to the berry. Sugar, mineral elements, and other compounds entering the fruit during ripening are also supplied by the phloem (Dokoozlian, 2000).

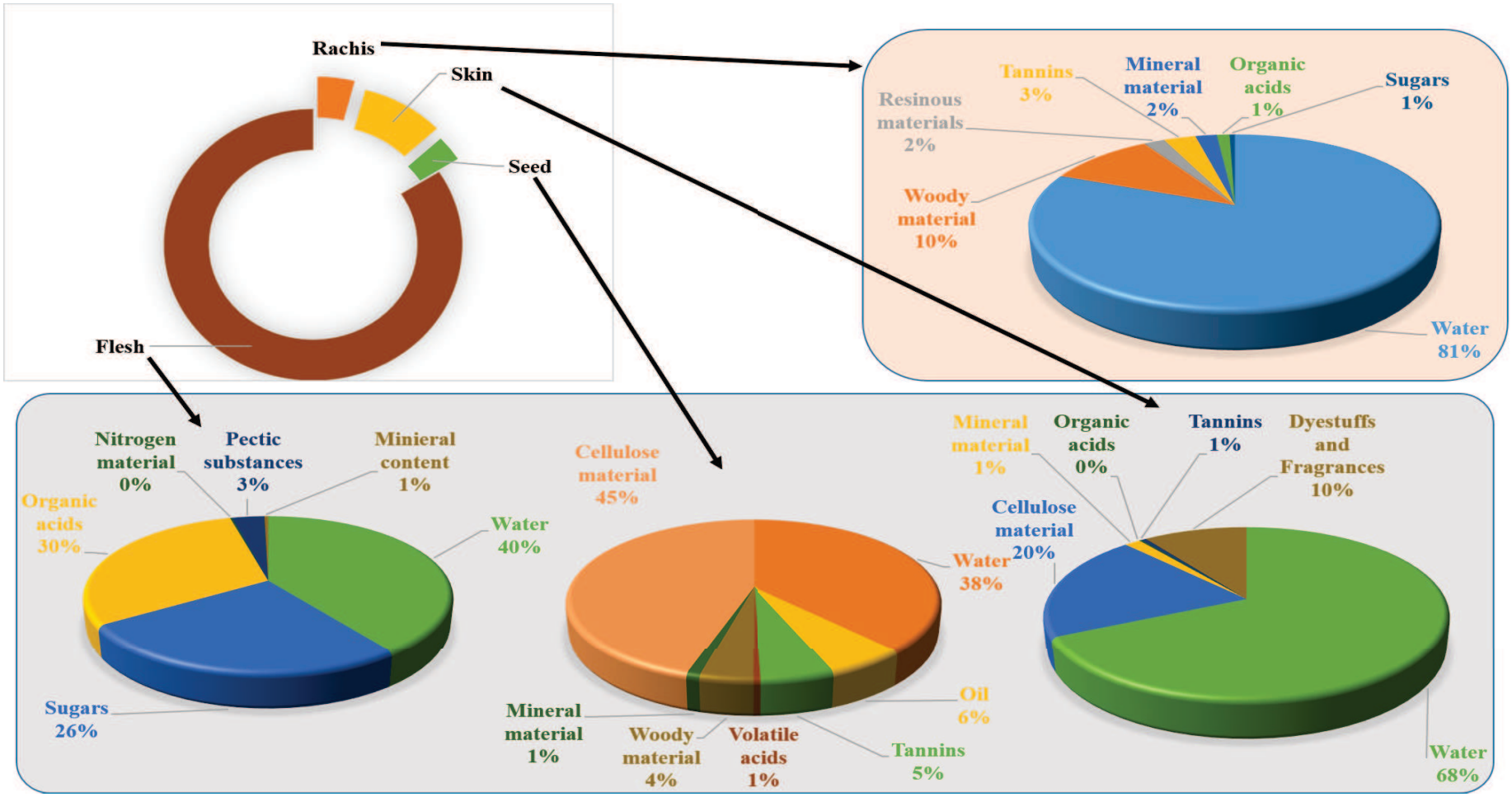



**Figure 1.5.: Chemical composition of the grape cluster (relative to weight)** (Carette, 2005).

### 1.1.2.2. Sugars

Sugar concentration may reach 25 % or more of berry fresh weight by the time of harvest. Sucrose, the predominant sugar transported in the phloem, is made by joining one molecule of glucose with one molecule of fructose. Once sucrose reaches the berry it is converted back to glucose and fructose, and these sugars are found in the fruit (Dokoozlian, 2000). Glucose and fructose are present in approximately equal amounts in grape berries at harvest (Taillandier and Bonnet, 2005), each ranging from 8 to 12 percent of fruit fresh weight. Sucrose and several other sugars are also present in the berry in small amounts. Sugars are found in different plant parts, mainly in the berries flesh (Fig. 1.4 and 1.5).

Glucose and fructose are fermentable sugars by yeasts (*Saccharomyces* spp.). Sucrose (another fermentable sugar) is present in trace concentration in the grape berries (Carette, 2005). Other non-fermentable pentose sugars such as xylose, arabinose and ribose are present in low concentration in grapes (Navarre and Langlade, 2002) Grapes belongs to fruits with high sugar content, for table grapes 120-170 g/L and for wine grapes with 140-240 g/L, and for certain cultivars such as Muscat with the maximum sugar content of 300-400 g/L (Blouin and Cruège, 2003). The sugar content varies according to several factors: the temperature, sun light, soil humidity, grape cultivar and the level of maturation (Blouin and Cruège, 2003).

### 1.1.2.3. Organic Acids

Next to sugars, organic acids are the most abundant solids present in grape juice. They are a very important component of juice and wine. They are responsible for the tart taste and have a marked influence on wine stability, color, and pH. The principal organic acids found in grapes are tartaric, malic, and to a small extent, citric acids (Saccani *et al*., 1995 Mato *et al*., 2006). Organic acids are well distributed in the grape flesh (Fig. 1.4). Many other organic acids, including amino acids, are also found in juice and wines, but tartaric and malic acid account for over 90% of the total acids present. During the early period of berry growth, concentration of both acids increases in the fruit. At the onset of ripening, the sugar accumulates in the fruit and the acid concentration decreases. Generally the reduction in malic acid is greater, and consequently, at maturity, the fruit contains more tartaric than malic acid (Kliewer *et al.,* 1967; Saito and Kasai, 1968; Ong and Nagel, 1978; Kupina *et al.,* 1991). Grapes are one of the rare fruits that contain tartaric acid. It is present as free acid and a salt, such as potassium bitartrate. Bitartrate is an important constituent since it affects pH and the cold stability of the wine (Boulton, 1980). The acid composition of grapes is influenced by many factors such as variety, climatic region, and cultural practices. Generally in ripe grapes,

the acid levels are lower in a warmer climatic region than in a cooler region (Jackson, 1986). The acidity is expressed as titratable acidity (TA). It is an important parameter used in quality evaluation of juice and wine. Acid content of the juice has an important bearing on juice and wine pH. Acids upon dissociation liberate H+ ions, which are measured and expressed in terms of pH. Thus acidity and pH are related. However, the relationship is neither direct nor predictable. Due to the presence of various kinds of acids and their salts, the relationship between acidity and pH is a complex one. Understanding the role of pH in winemaking is crucial to making good wines (Conde *et al.,* 2007).

#### 1.1.2.4. Minerals materials

Potassium is the most abundant element with higher rates of accumulation after veraison compared to the other elements in various *V. vinifera* cultivars (Schaller *et al*., 1992; Creasy *et al*., 1993; Ollat and Gaudillère, 1996; Rogiers *et al*., 2006). Grapes have the same elements of minerals that found in plant (Pereira *et al., 2006)*. They are very rich in potassium (K) (2.2 g/L) (Carette, 2005) and that plays important role in the pH and sugar accumulation. Rogiers *et al*. (2006) support the linkage between potassium and sugar accumulation into the berry. The high concentration of potassium contributes significantly to uptake of water, especially after veraison and combines with sugar and fresh weight increment.

The pulp and skin were the strongest sinks for potassium and boron, seeds were the strongest sinks for calcium, phosphorus, sulphur, manganese and zinc. With the exception of calcium and manganese, seeds ceased to accumulate most elements during late ripening (Rogiers *et al*., 2006)

#### 1.1.2.5. Nitrogen content

Nitrogen is found in all parts of grape berry from the skins to the seeds at the nitrogen mineral form ($NO_3$, $NH_4$) (Rapp and Versini, 1996; Keller *et al.,* 1998; Dokoozlian, 2000). Other forms of nitrogen maily found as free amino acids, peptides and proteins in the berries (juice, skins, and seeds). A comparative study was conducted by Yokotsuka and Fukui (2002) evaluating the nitrogen compounds changes of six grape cultivars during ripening. The amount of protein in juice and skins increased over time during ripening, whereas the amount of protein in seeds changed only slightly. Approximately 63% of total soluble and/or extractable nitrogen compounds in berries were proteins. Free amino acids and peptides comprised approximately 20 and 17% of all nitrogen compounds, respectively. Seeds contained on average approximately 50% of all the nitrogen compounds found in berries,

primarily in the form of proteins. Juice and skins both contained approximately 25% of nitrogen compounds, and in juices these were primarily in the form of free amino acids. Proteins from these three tissues were rich in aspartic acid and glutamic acid.

#### 1.1.2.6. Polyphenol content

Phenolic compounds are important constituents of grapes and wine. Following sugars and acids, they are the most abundant constituents present in grapes. Phenolic compounds are a group of substances that are structurally diverse and are present in various amounts. They are involved in browning reactions in grapes and wines and also play a key role in the aging and maturation of wines (Jensen *et al*., 2008). The majority of different grape phenolic compounds are found in the skin and seeds (Adams, 2006; Conde *et al*., 2007; Keller, 2010). Grape phenols contribute to color, flavor, texture and astringency of wine and to its antioxidant properties (Teixeira *et al*., 2013). Two distinguishable tissues compose the grape skin, representing the hydrophobic barrier of the pericarp. The outermost (the epidermis) is strongly cutinized, while the inner thick-walled layers of hypodermis (assumed to consist of several layers, depending on the variety), contain most of the skin flavonoids. In this fraction, the major class of flavonoids is represented by anthocyanins, proanthocyanidins and, to a minor extent, simple flavan-3-ols and flavonols (Braidot *et al*., 2008) of a ripe grape berry with the distribution pattern of secondary metabolites between tissues is shown in Figure 1.6.

The grape phenols belong to three families: the phenolic acids (benzoic acid, hydroxycinnamic acid), the flavonoids (the flavonols, the catechins, the anthocyanins), and the tannins (Kennedy *et al*., 2006; Pinelo *et al*., 2006).

Anthocyanins are synthesized in the cytosol of the epidermal cells, are co-localized with proanthocyanidins in the skin hypodermal layers and then stored in the vacuole (Braidot *et al*., 2008; Guerreroa *et al.*, 2009; Fontes *et al.*, 2011). The biosynthesis of soluble phenolics begins with the aromatic amino acid phenylalanine, a product of the shikimate pathway. The early precursors of the shikimate pathway are erythrose-4-phosphate and phosphoenol pyruvate. This pathway is responsible for producing phenylalanine and the other amino acids tyrosine and tryptophan (Conde *et al*., 2007; Castellarin *et al.,* 2012; Baiano and Terracone, 2011).

The sugars in the must were directly related to the total anthocyanins in the must and closely related to the derivatives of peonidin, while the sugars in the skin were closely related to each of the anthocyanins and to the other phenolic compounds present in the skin (Gonzalezsanjose and Diez (1992).

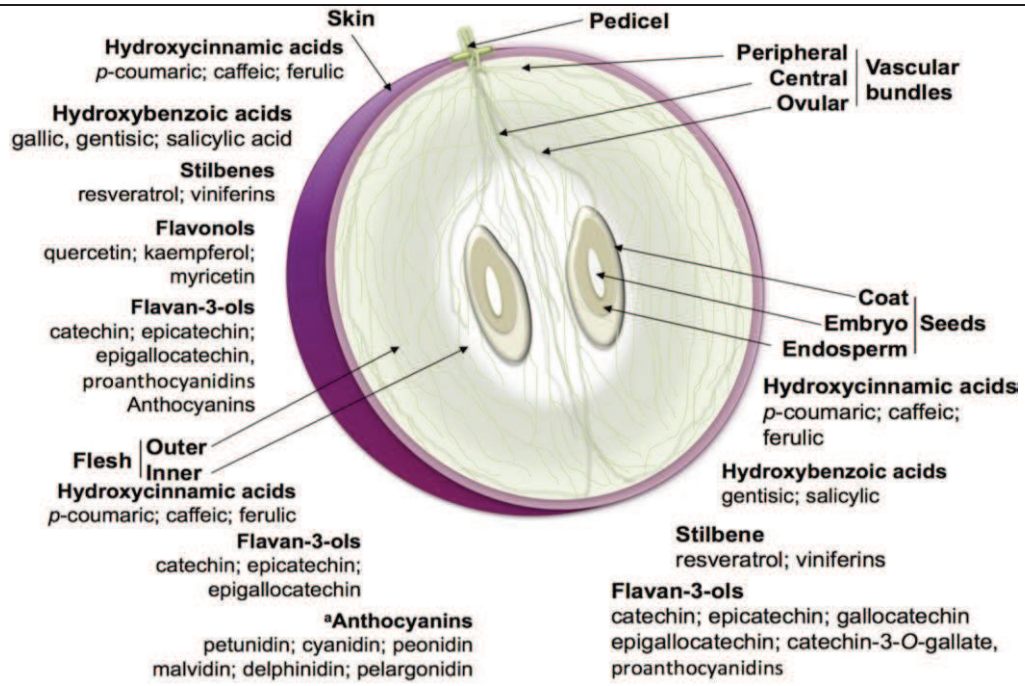



**Figure 1.6.: Schematic structure of a ripe grape berry and pattern phenolic biosynthesis distribution between several organs and tissues** (Teixeira *et al*., 2013)

### 1.1.2.7. Aromatic composition

The odorous compounds in grapes are largely present in the skin and the layers of cells immediately beneath it. Their concentration (flavor compounds) tends to increase during ripening. It is important that the grapes be harvested when the flavor is at its peak. Many factors affect the concentration of aroma compounds in grapes. Manipulation and control of these factors is necessary for attaining the desired flavor level at harvest because flavor analysis of grape is a key step in quality evaluation (Gomez *et al*., 1994; Ferrandino *et al*., 2012). Iyer *et al.* (2012) assessed that pH, titratable acidity (TA), and total soluble solids (TSS)/TA ratio were all significantly correlated with methyl anthranilate and trans-2-hexenal concentrations) in samples equal to or exceeding 15 Brix, the industry minimum for grape maturity. These results indicate that parameters related to acidity are better predictors of aroma composition than TSS, which may aid in efforts to minimize herbaceous off-aromas and optimize the aroma composition of the finished juice.

Recently, certain studies were conducted for determining the flavor and volatile compound profile of different varieties, such as Genovese *et al*. (2013) found 26 volatile compounds, of total of 37 quantified free and bound grape aroma compounds, resulted validated in grape skin and pulp juice. Pedneault *et al.* (2013) determined the aroma profiles of two grape cultivars 'Frontenac and Marquette' were dominated by C6 compounds (hexanal, trans-2-hexenal, 1-hexanol, cis-3-hexenol, and hexanoic acid), acetic acid, β-

damascenone, and 2-phenylethanol, with Marquette additionally showing significant levels of monoterpenes (linalool, geraniol, and α-citral) and 1-octen-3-ol.

### 1.1.3. Cultivars

There are thousands of cultivars all over the world, which are cultivated for table or wine production. The majority of wine cultivars belong to *Vitis vinifera* L. (Anglade and Puisais, 1987; Villa, 2005) which distributed in middle Europe, Mediterranean countries and western Asia (Anglade and Puisais, 1987; Soyer *et al.*, 2003). The different grape cultivars are distinguished by: cluster dimensions, morphological characteristics [color of buds or berries, shape of leaves and branches, sexual type of flowers (males, females, bisexual flowers) etc.], grape size, juice importance, grape skin thickness and the chemical constitution (sugars, organic acids, polyphenols, aroma, etc.) that usually adapted to certain regions (Huglin, 1986; Villa, 2005; Carbonneau *et al*., 2007).

### 1.1.4. Grapevine growth cycle

#### 1.1.4.1. Vegetative development of grapevines

Several distinct developmental stages such as dormancy, budbreak (budburst), bloom (anthesis, flowering), fruit set (berry set, setting), veraison (berry softening, color change, onset of ripening), harvest (ripeness, maturity), and leaf fall (abscission) occur in the annual vine growth cycle (*Appendix 1*).

The vegetative cycle starts with bleeding in March, which refers to the exudation of xylem sap from pruning surfaces and is a transition from dormancy to active growth. Bleeding is caused by root pressure, which is generated by remobilization of nutrient reserves (especially carbohydrates) and pumping of sugar into the xylem (Sperry *et al.*, 1987). Root pressure serves to dissolve and push out air bubbles that have formed in the xylem vessels during the winter. In temperate climates budbreak and shoot growth are induced by mean daily temperatures above 10°C (Galet, 2000). Budbreak is affected also by the grape variety, the time of pruning, the vigor of the vine, water stress and cold hardiness (Mills *et al.,* 2006). Budbreak is followed by a period of exponential shoot growth (Williams and Matthews, 1990). This phase is characterized by strong apical dominance, whereby release of auxin by the growing main-shoot tip inhibits lateral shoot growth. However, lateral shoots can supply up to 40% the total leaf area (Candolfi-Vasconcelos and Koblet, 1990) and lateral leaves are important contributors to sugar accumulation in the fruit and starch accumulation in the wood (Candolfi-Vasconcelos and Koblet, 1990; Mabrouk *et al.,* 1997; Schultz *et al.,* 1996).

Unlike many fruit trees which produce specialized fruiting buds and vegetative buds, grapevines produce both fruit and foliage from the same buds. Buds are particularly numerous and complex. Latent buds can remain dormant for several years, before some event activates them. In the axil of each leaf on the main shoot there are lateral buds which can development and enrich leaf area (Pratt, 1971). The petiole is the leaf stem that connects the leaf to the shoot and contains multiple vascular bundles from a separate shoot vascular bundle (Pratt *et al.*, 1974). As perennial plants, grapevines annually store and remobilize carbohydrates and mineral nutrients in response to changing conditions (Galet, 2000). After budbreak when the cells of the young shoot are dividing, growth is slow. Then, as mean temperatures rise, growth and shoot elongation accelerates from day to day (Williams*.,* 1987). Unless growth conditions become unfavorable (e.g. with water stress), the shoots will continue to grow (Winkler *et al.*, 1974). Shoots and leaves position themselves on the outside of the vine's canopy to capture sunlight. The plant leaf area depends on the training system, plant vigor, pruning intensity and genotype; (Williams, 1987; Mabrouk *et al.,* 1997; Palliotti *et al.*, 2000). The basic function of the leaf is to capture sunlight for energy (ATP) production and carbon dioxide (CO2) for carbohydrate production to support the vine's metabolism.

Grapevine leaves reach light saturation at photosynthetic photon fluxes between 700 and 1200 μmol/$m^2$s, which is well below the photon flux of full sunlight (up to ≥2000 μmol/$m^2$s) (Mullins *et al.,* 2003). However, the conditions under which vines are grown may cause some change in the value for light saturation (Kriedemann, 1968). Temperature is another important environmental factor that can influence the rate of photosynthesis in grape leaves. The optimum leaf temperature for photosynthesis of field grown vines is generally between 25 and 35°C (Kriedemann, 1968). Vine leaves typically have between 100 and 400 stomata per $mm^2$ (Mullins *et al.,* 2003). Stomata are responsible for regulating the gas exchange (mainly $CO_2$ for photosynthesis, oxygen ($O_2$) for respiration, and water ($H_2O$) vapor from transpiration) between the leaf and the atmosphere.

Vines, like other plants, are stationary so they cannot move from place to place to find better food sources. Because uptake of water and nutrients by the roots from the surrounding soil quickly depletes available resources, the roots must keep growing throughout the vine's life and the season in order to maintain the supply of these raw materials (Williams and Biscay, 1991; Araujo *et al.,* 1988).

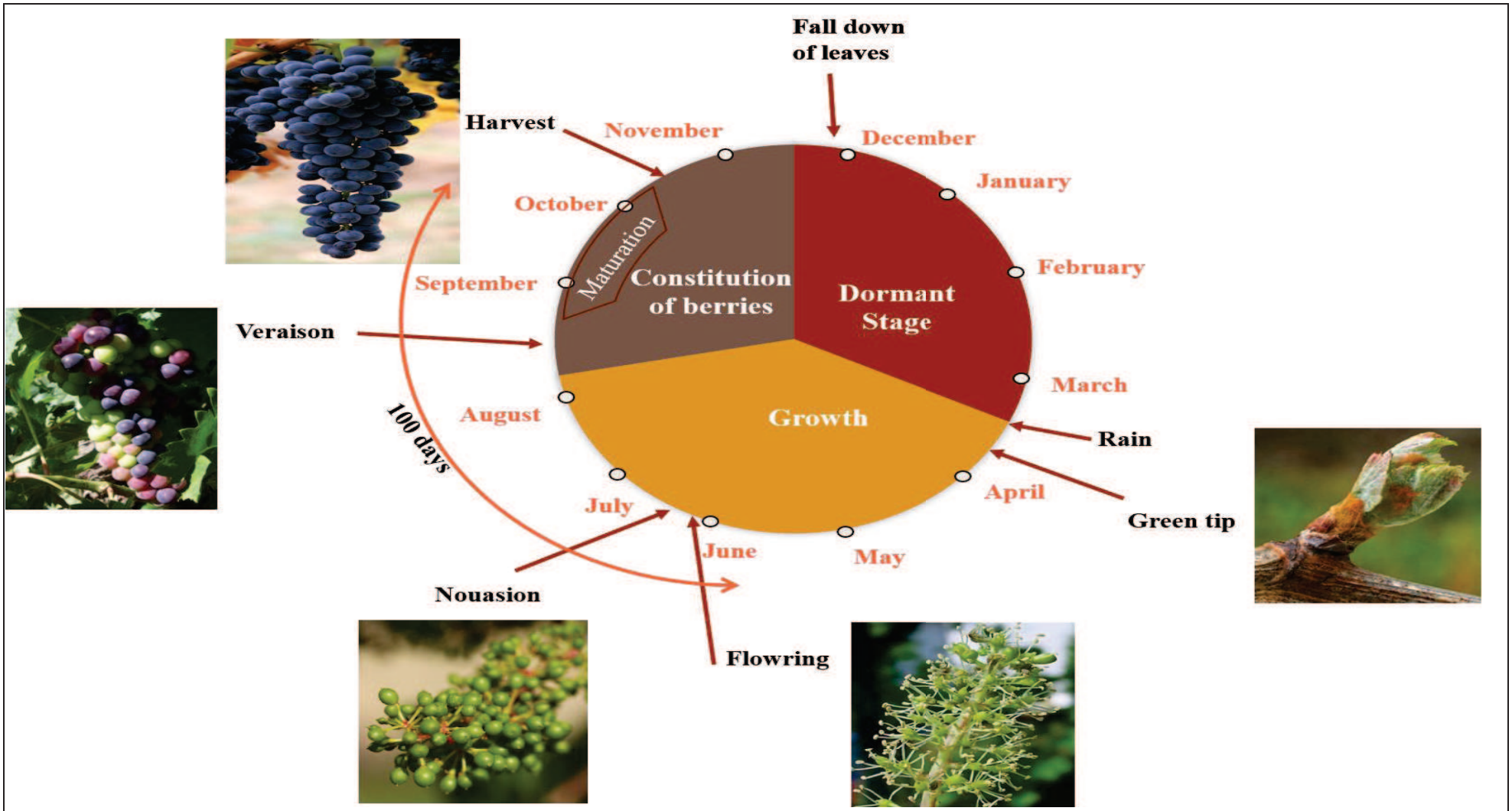



**Figure 1.7.: Scheme of Grapevine life cycle** (Puisais, 1984).

### 1.1.4.2. Reproductive development of grapevines

Grapevines require two consecutive growing seasons for flower and fruit production. Inflorescences are initiated the year before bloom. Environmental conditions required for the formation of the maximum number of inflorescence include high light intensity, optimal temperature (25-30°C), and adequate water and nutrient availability (Mullins *et al.,* 2003). Shoots and inflorescence develop between inflorescence initiation and anthesis. Depending on the species and cultivar, flowers might be female, male, or hermaphroditic (also known as "perfect", which means that each flower contains both male and female structures). By far, the majority of cultivated varieties are hermaphroditic (Pratt, 1971). Anthesis is the period during which a flower is fully open and functional. After bloom, fertilized flowers set fruit, although about 2/3 of the individual florets in the cluster drop off. Grape fruit set and growth is triggered by pollination and correlates with elevated endogenous auxin indole-3-acetic acid (IAA) levels (Cawthon and Morris, 1982). As berries develop, the fruit starts to be a greater 'sink' for photosynthetic products (Coombe, 1989; Ho 1988; Conradie, 1980), and the growth rate of shoots declines. Unfavorable environmental conditions (e.g. cloudy, cool, wet bloom period, water or nutrient stress), insufficient or inefficient leaf area (e.g. due to hail, insect or disease attack) or excessively vigorous shoot growth (competing with inflorescences for assimilates) often result in poor fruit set and loose clusters.

Growth of some fleshy fruit such as grape exhibits a double sigmoid pattern in which there are two periods of growth separated by a lag phase (Coombe, 2001; Harris *et al.,* 1968; Ollat *et al.,* 2002) (Fig. 1.7). The first stage is the cell division phase (phase I) throughout mid-July. Cell division and cell enlargement both contribute to pericarp growth in the early post-anthesis stage. Cell division in the grape pericarp begins 5-10 days before anthesis and continues for approximately 25 days (Harris *et al.,* 1968). Following the cell division phase, is a pause in berry growth called the “lag phase” or phase II, during which seed formation progresses. Berry turgor pressure also declines about tenfold during this period (Matthews and Shackel, 2005). The length of the lag phase (one to six weeks) depends largely on the cultivar and is important in determining the time of fruit maturity (early vs. late ripening varieties). After the lag phase, the number of cells in each berry is set. Further berry growth is due to cell expansion only (Hardie *et al.,* 1996; Ollat *et al*., 2002; Coombe 1976; Pratt, 1971).

## 1.1.5. Grapevine diseases

Grapes are subject to attack by many different pests, including nematodes, fungal, bacterial, and viral pathogens and insects (Belhadj *et al*., 2006). Weeds, which compete with the vines for soil moisture and nutrients, may also be included in this list. The fungal and viral diseases are the most dangerous disease affecting the viticulture

### 1.1.5.1. Fungal diseases

The main fungal diseases infecting the grapevines are listed in Tab. 1.1.

Downy mildew, powdery mildew and gray mold are the most frequent diseases in the vineyards (Fig. 1.8.).

#### 1.1.5.1.1. Downy mildew

The downy mildew (caused by *Plasmopara viticola*, obligate parasite of grapevine) causes an economically very important disease (Dai *et al.,* 1995). *Plasmopara viticola* is endemic on wild *Vitis* species of North America. It was first observed in Europe in 1878. It was probably introduced into Europe with American grape cuttings used to replant the French vineyards destroyed by phylloxera (Reynier, 2003; Gessler *et al.*, 2011). Downy mildew injures grapes by causing deformed shoots, tendrils and clusters. The fungus also causes premature defoliation which impacts fruit ripening and increases susceptibility to winter damage. A major outbreak of this disease can cause severe losses in yield and quality (Villa, 2005; Pérez Marín, 2007). Downy mildew epidemics involve primary (sexual) and secondary (asexual) infection cycles. Oospores, which represent the sexual stage of the pathogen (Berlese, 1898; Caffi *et al*., 2009), are the sole relevant source of inoculum for primary infections in the following season (Galbiati and Longhin, 1984; Rumbou and Gessler, 2004; Gobbin *et al.,* 2005; Kennely *et al.,* 2007). Oospores overwinter in leaf debris above ground or buried in soil; in spring they germinate to form a macrosporangium that releases zoospores which are responsible for primary infections on grape leaves and clusters. After 5 to 18 days, depending on the temperature, the pathogen produces sporangia containing asexually produced zoospores (Agrios, 1988). First infections are therefore followed by successive asexual cycles (Blaeser and Weltzien, 1979). Increased knowledge of the biological cycle of *P. viticola* opened up the possibility of applying the most effective chemical dosage at the most suitable time. An important set of studies were devoted to optimizing the application of copper based products (*i.e.* Bordeaux mixture is based on copper sulfate) (Pérez Marín, 2007; Gessler *et al.*, 2011).

**Table 1.1.: Main fungal diseases of grapevines**

| Disease | Fungal pathogen (s) | Infected parts | Reference |
|---|---|---|---|
| **Anthracnose, Bird's-Eye Rot** | *Elsinoe ampelina.* | Young shoots, Grape bunches | Brooka (1973); Magarey *et al*. (1993) |
| **Bitter Rot** | *Greeneria uvicola* (syn. *Melanconium fuligineum*) | Leaves, Grape bunches | Navarrete *et al*. (2009); Samuelian *et al*. (2011) |
| **Black Rot** | *Guignardia bidwellii* | leaves, shoots, tendrils, grape berries | Ullrich *et al.* (2009) |
| **Botrytis Bunch Rot, Gray Mold** | *Botrytis cinerea* | Grape bunches, Leaves | Aziz *et al.* (2003); Trotel-Aziz (2006) |
| **Downy Mildew** | *Plasmopara viticola* | Leaves, grape bunches, young shoots | Oliva et al. (1999); Aziz *et al.* (2003) |
| **Macrophoma Rot** | *Botryosphaeria* spp. | Grape berries | Amponsah *et al*. (2009) |
| **Phomopsis Cane and Leaf Spot and Fruit Rot** | *Phomopsis viticola* | Shoots, grape bunches, leaves, grape berries | Mostert *et al*. (2001) |
| **Powdery Mildew** | *Uncinula necator* | Leaves, grape bunches, young shoots | Staudt *et* al. (1997);Oliva *et al.* (1999) |
| **Ripe Rot** | *Colletotrichum gloeosporioides, C. acutatum, and Glomerella cingulata.* | Grape berries | Jeffries *et al*. (1990) |
| **Sour Rot** | *Aspergillus, Alternaria, Penicillium, and Rhizopus* | Grape berries | Oliva *et al.* (1999) |

1.1.5.1.2. Powdery mildew

Grape powdery mildew, caused by *Uncinula necator* (Schw.) Burr., is the most widespread and destructive disease of grapevine (Pearson & Gadoury, 1992; Halleen and Holz, 2011). Powdery mildew may be observed on all the green parts of the vine: leaves, branches, inflorescences, rachises, pedicels and berries (Bulit and Lafon, 1978; Villa, 2005; Pérez de Obanos, 2007). The mass of canes pruned from infected vines is lower than that from healthy vines, indicating lower yield (Reuveni and Reuveni, 1995). Infection reduces the winter hardiness of canes, expressed as a reduction in bud survival (Pool *et al*., 1984). Fruit infection lowers wine quality, both as a result of increased acid concentration and as a direct result of the fungus itself producing off-flavours (Pool *et al.,* 1984). Wines made from infected grapes have a lower color intensity associated with a higher tonality index and a

lower concentration of total anthocyanins. Infection of immature fruit causes tissue scarring and berry splitting (Chellemi & Marois, 1991). Infected berries may also crack and provide entry sites for pathogens like *Botrytis cinerea* and organisms associated with sour-rot (Pearson & Gadoury, 1992). Rainfall is a critical event in the release of asco-spores and the initiation of powdery mildew epidemics in areas where cleistothecia are sources of primary inoculum (Halleen and Holz, 2011).

1.1.5.1.3. Gray mold

*Botrytis cinerea*, the causal agent of gray mold, is a highly variable fungus with strains displaying very different degrees of virulence toward one given host plant species (Derckel *et al*., 1999) *Botrytis* is regarded as the most important post-harvest fungal pathogen that causes significant losses in fresh fruits, vegetables and ornamentals. Its ability to attack a wide range of crops in a variety of modes of infection and its ability to develop under conditions prevailing during storage, shipment and marketing make its control a challenge. Harvested crops are particularly vulnerable to Botrytis infection because unlike vegetative tissue harvested commodities are senescing rather than developing (Droby and Lichter, 2007).

Fungicide-based management, once an accepted practice, is becoming increasingly restricted, a trend likely to continue in the future. Greater emphasis on alternative, non-chemical control will require improved knowledge of *B. cinerea* ecology and epidemiology in affected crops. Epidemics are often initiated in the spring from conidial inoculum produced on over-wintering structures on a very wide range of plant species (Derckel *et al*., 1999). The growth of organic production in the last decade has high-lighted the need for a greater understanding of the complexities of epidemic development in order to develop durable and sustainable disease control strategies (Elmer and Michailides, 2007).

Other saprophytic fungal contaminants can also affecting the grape quality. Indeed, the majority of these fungi are airborne fungi (*Aspergillus* or *Penicillium*) which are easily contaminating the grapes. Climate conditions play major role in the development of these fungi. Temperatures from 15 to 30°C favorite the proliferation and certain *Aspergillus* conidia resist beyond 35°C (Chapeland-Lecler *et al*., 2005). 80-90% humidity and the nutrition availability via grapes permit the fast fungal development. In addition, certain strains those are able to produce mycotoxins could contaminate the grapes and wine. These fungi are not infectious ones as the previously described diseases (downy or powdery mildew) (Benette and Klich, 2003).

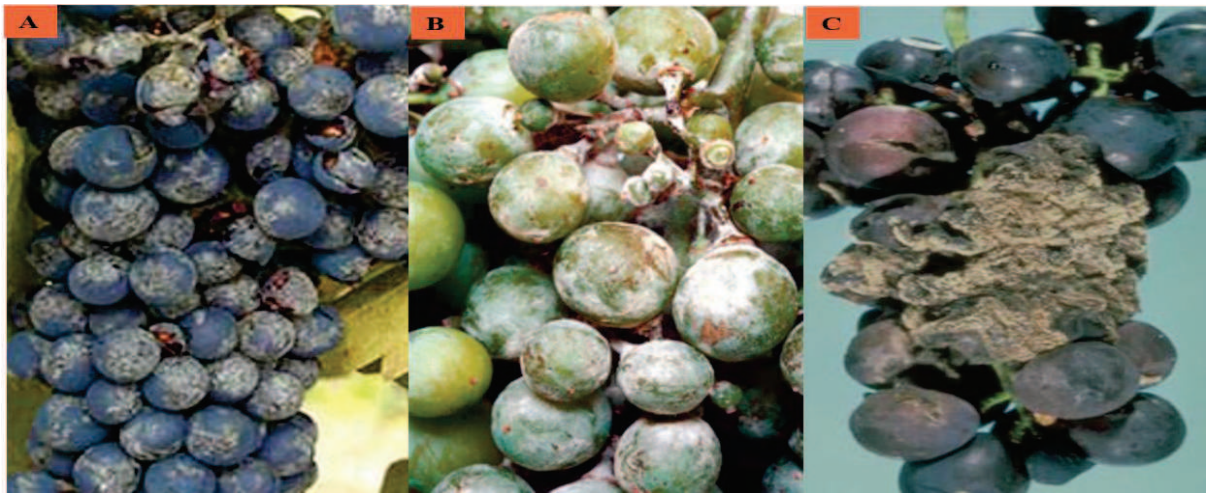



**Figure 1.8.: Main fungal diseases affecting grape clusters; A: downy mildew, B: powdery mildew, C: gray rot.**
**http://www.agf.gov.bc.ca/cropprot/grapeipm/mildew.htm**
**http://www.hortscience.com.au/pest-disease/powdery-mildew/**
**http://www.oardc.ohio-state.edu/fruitpathology/organic/Grape/botrytis.html**

#### 1.1.5.2. Viral diseases

There are many viral diseases, and other graft transmissible diseases believed to be caused by viruses, that affect grapes. The presence of one or more virus diseases in a mother vine block or in a commercial vineyard can have quite variable consequences. Viruses affect wood production, graft take, rooting capacity, longevity of vines, quantity and quality of yield, and composition of must (Walter and Martelli, 1998 Ahmed-Hoda *et al.,* 2004). The major viral diseases affecting grapevine are fanleaf (grapevine degeneration), Leafroll and rugose wood complex, diseases (Martelli, 1993; Ahmed-Hoda *et al.,* 2004).

In 2007, the *V. vinifera* genome was sequenced, which probably used for the creation of new grape cultivars resistant for certain diseases (Jaillon *et al*., 2007).

## 1.2. Mycotoxins

The term mycotoxin is derived from the Greek word ‘mycos’ meaning mould, and the Latin word ‘toxicum’, which means poison. While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins. The target and concentration of the metabolite are both important. Fungal products that are mainly toxic to bacteria (such as penicillin) are usually called antibiotics. Fungal products that are toxic to plants are called phytotoxins by plant pathologists (the term of phytotoxin can also refer to toxins made by plants) (Pestska *et al*., 1990; Bennett and Klich, 2003). These metabolites are restricted to a certain number of fungal species (Smith and Moss, 1985). Mycotoxins are considered

secondary metabolic products because they are not necessary for fungal growth and are simply a product of the primary metabolic processes.

Secondary metabolism usually occurs after a phase of balanced growth and it is often associated with developmental processes. Thus, sometimes mycotoxins are secreted by growing colonies at the approximate time of sporulation (Calvo *et al.*, 2002), but the functions of mycotoxins are still an enigma. They are believed to protect the mould and act as a defense mechanism by excluding or poisoning animals, plants or other competing fungal species in the same environment.

The majority of mycotoxicoses (toxic effect of the mycotoxins) result from eating contaminated foods. Skin contact with mold infested substrates and inhalation of spore-borne toxins are also important sources of exposure. Except for supportive therapy (e.g., diet, hydration), there are almost no treatments for mycotoxin exposure (Fischer *et al.*, 2000; Nielsen; 2003; Bennett and Kich, 2003). Mycotoxins are made by fungi and are toxic to vertebrates and other animal groups in low concentrations. Other low-molecular-weight fungal metabolites such as ethanol that are toxic only in high concentrations are not considered as mycotoxins (Bennett, 1987).

Mycotoxin contamination of the food chain (Tab. 1.2.) has a major economic impact. However, the insidious nature of many mycotoxicoses makes it difficult to estimate incidence and cost (Cast, 1989; Bryden, 2007). In addition to crop losses and reduced animal productivity, costs are derived from the efforts made by producers and distributors to counteract their initial loss, the cost of improved technologies for production, storage and transport, the cost of analytical testing, especially as detection or regulations become more stringent and the development of sampling plans (Bryden, 2007). There is also a considerable cost to society as a whole, in terms of monitoring; extra handling and distribution costs increased processing costs and loss of consumer confidence in the safety of food products. It is estimated that in developing countries the greatest economic impact is associated with human health (Miller, 1998; Bryden, 2007)

The amount of mycotoxins needed to produce adverse health effects varies widely among toxins, as well as for each animal or person's immune system. Two concepts are needed to understand the negative effects of mycotoxins on human health. These concepts are acute toxicity (which defined as the rapid onset of an adverse effect from a single exposure) and chronic toxicity (the slow or delayed onset of an adverse effect, usually from multiple, long term exposures).

**Table 1.2: Probable routes for mycotoxin contamination of foods and feeds** (www.ifst.org)

| | ***Agricultural products*** | | |
|---|---|---|---|
| **Mould damaged foodstuffs** | *Major source* | *Minor source* | *Secondary infection* |
| | Cereals | Fruits | Consumer foods |
| | Herbs, spices | Vegetables | Compounded animal feeds |
| | Oil seeds | | |
| | | | |
| **Residues in animal (tissues & products)** | Milk (animal and human) | Dairy produce | Meats (liver, kidney) |
| | | | |
| **Mould fermented foods** | Cheeses | Fermented Meat products | Oriental and other fermented products |
| | | | |
| **Fermenter-derived products** | Microbial proteins | | Food additives |

Mycotoxins can be acutely or chronically toxic, or both, depending on the kind of toxin and the dose. In terms of acute toxicity, the mycotoxins most commonly encountered in food are about a factor of a million times less toxic than the most virulent of the botulism toxins (Moss, 1996). It is the long term toxicity which is of special concern because certain mycotoxins ingested in minor quantities with the daily diet for an extended period are known to be carcinogenic and to influence the immune response of a number of animal species, being also a risk to human health (Table 1.3.).

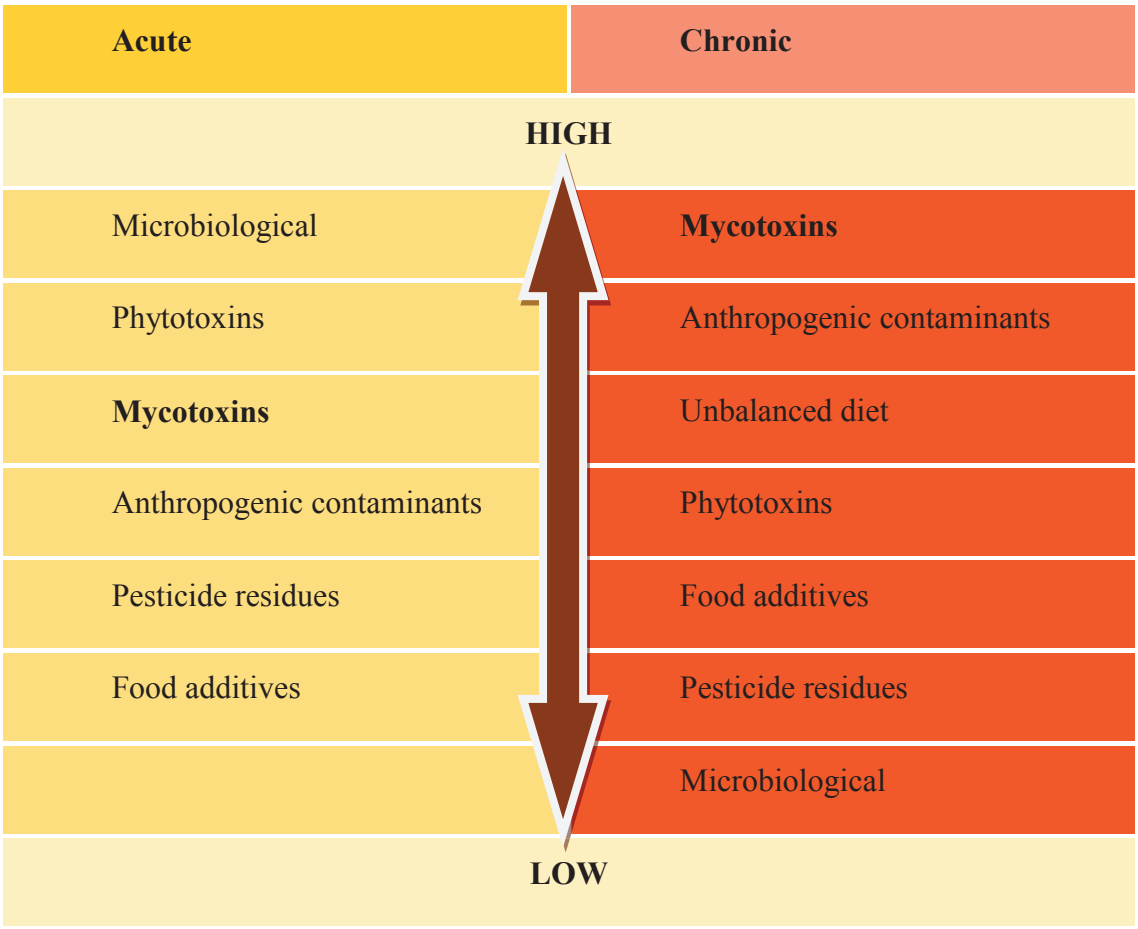
### 1.2.1. Major mycotoxins

Numbers of mycotoxins produced have been identified with several researches such as Betina (1989) had identified approximately 300 mycotoxins produced by 350 species of fungi. This number has increased to 400 mycotoxins (Bennet and Klich, 2003), but the exact number has never been accurately determined. However, not all fungi produce mycotoxins and among the toxigenic species, some only produce one type of mycotoxin, while others are able to produce several. Also, a specific type of mycotoxin can be produced by different

fungal species (Boutrif and Bessy, 2001). Some of the fungi producing mycotoxins are listed in Tab. 1.4. Certain species are capable to produce multiple kinds of toxins (Tab. 1.5.).

The main mycotoxins that have been related to human intoxication include aflatoxins, cyclopiazonic acid, citreoviridin, fumonisins, 3-nitropropionic acid, ochratoxins, certain trichothecenes and zearalenone (Peraica and Dominjan, 2001; Reverberi *et al.*, 2010).

**Table 1. 3. Rating health risks from foods** (Kuiper-Goodman, 1998).




The food can contain concomitantly different mycotoxins (Table 1.4.). As these combined mycotoxins occur simultaneously in the food item, consumption of the food will lead to a combined intake depending on the absorption rates of the different mycotoxins. A combined intake of mycotoxins would lead to a possible higher risk for adverse health effects than the intake of one of these mycotoxins alone. When the mycotoxins are of similar structure and of the same species, or of the same families, it is likely to expect that the mode of action of the mycotoxins and or the toxicity profiles will be quite similar. This indicates that such related mycotoxins are likely to exert only additive effects (Speijers and Speijers, 2004).

**Table 1.4.: Fungal species producing mycotoxins** (Bennett and Klich, 2003; Ehrlich *et al.*, 2011)

| Mycotoxin | Fungal species |
|---|---|
| **Aflatoxins** | *Aspergillus flavus*, *A. parasiticus*; *A. bombycis*, *A. ochraceoroseus*, *A. nomius*, and *A. pseudotamari* |
| **Citrinin** | *Penicillium citrinum, P. camemberti, A. terreus, A. niveus*, *A. oryzae, Monascus ruber* and *M. purpureus* |
| **Ergot Alkaloids** | *Claviceps* spp. |
| **Fumonisins** | *Fusarium verticillioides* (*F. moniliforme* = *Gibberella fujikuroi*), *F. proliferatum, F. nygamai, Alternaria alternata* f.sp. *lycopersici* |
| **Ochratoxin** | *A. ochraceus, A. alliaceus*, *A. auricomus*, *A. carbonarius*, *A. glaucus*, *A. melleus*, and *A. niger, P. verrucosum, P. nordicum* |
| **Patulin** | *P. patulum (later called P. urticae, now P. griseofulvum),* |
| **Trichothecenes**<br>- **Deoxynivalenol (DON)**<br>- **T-2 toxin** | *F. tricinctum, F. sporotrichoides, F. graminearum, F. culmorum, F. poae, F. roseum, F. acuminatum, Myrothecium* spp., *Phomopsis* spp., *Stachybotrys* spp., *Trichoderma* spp., *Trichothecium* spp. |
| **Zearalenone** | *F. graminearum (teleomorph Gibberella zeae), F. culmorum,*<br>*F. equiseti, F. crookwellense, F. semitectum* |
| **Rubratoxins** | *P. purpurogenum* |

**Table1.5: Some toxigenic fungal species producing multiple mycotoxins** (www.ifst.org).

| Fungal species | Toxin |
|---|---|
| ***A. flavus*** | Aflatoxins B1, B2, cyclopiazonic acid |
| ***A. parasiticus*** | Aflatoxins B1, B2, G1, G2 |
| ***A. ochraceus*** | Ochratoxin A; Penicillic acid |
| ***A. versicolor*** | Sterigmatocystin, cyclopiazonic acid |
| ***P. verrucosum*** | Ochratoxin A, citrinin |
| ***P. expansum*** | Patulin, citrinin |
| ***F. graminearum*** | Deoxynivalenol, nivalenol, zearalenone |

Some of these mycotoxins will be described briefly below; aflatoxins, fumonisins, and patuline. Ochratoxins will be described more fully.

#### 1.2.1.1. Aflatoxins

The aflatoxins were isolated and characterized after the death of more than 100,000 turkey poults (turkey X disease) was traced to the consumption of a mold-contaminated peanut meal (Klich, 2007). The four major aflatoxins are called B1, B2, G1, and G2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography. Aflatoxin B1 (Fig. 1.9) is the most potent natural carcinogen known (Squire, 1981; Murphy *et al*., 2006) and is usually the major aflatoxin produced by toxigenic strains. It is also the best studied: in a large percentage of the papers published, the term Aflatoxin can be construed to mean aflatoxin B1. Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus flavus* and *A. parasiticus*; in particular, *A. flavus* is a common contaminant in agriculture (Davis *et al.*, 1966). *A. bombycis*, *A. ochraceoroseus*, *A. nomius*, and *A. pseudotamari* are also aflatoxin-producing species, but they are encountered less frequently (Kurtzman *et al*., 1987; Goto *et al*., 1996; Klich *et al*., 2000; Peterson *et al*., 2001). From the mycological perspective, there are great qualitative and quantitative differences in the toxigenic abilities displayed by different strains within each aflatoxigenic species. For example, only about half of *A. flavus* strains produce aflatoxins (Klich and Pitt, 1988), while those that do may produce more than 106 µg/kg (Cotty *et al.*, 1994).

#### 1.2.1.2. Fumonisins

Fumonisins were first described and characterized in 1988 (Bezuidenhout *et al*., 1988; Gelderblom *et al*., 1988). The most abundantly produced member of the family is fumonisin B1 (Fig. 1.9.). They are thought to be synthesized by condensation of the amino acid alanine into an acetate-derived precursor (Sweeney *et al.,* 2000; Bennett and Klich, 2003). Fumonisins are produced by a number of *Fusarium* species, notably *F. verticillioides* (formerly *F. moniliforme* = *Gibberella fujikuroi*), *F. proliferatum,* and *F. nygamai,* as well as *Alternaria alternata* f. sp. *lycopersici* (Marasas *et al.,* 2001; Rheeder *et al.,* 2002). The major species of economic importance is *Fusarium verticillioides*, which grows as a corn endophyte in both vegetative and reproductive tissues, often without causing disease symptoms in the plant. However, when weather conditions, insect damage, and the appropriate fungal and plant genotype are present, it can cause seedling blight, stalk rot, and ear rot (Nelson *et al.,* 1993; Bennett and Klich, 2003).

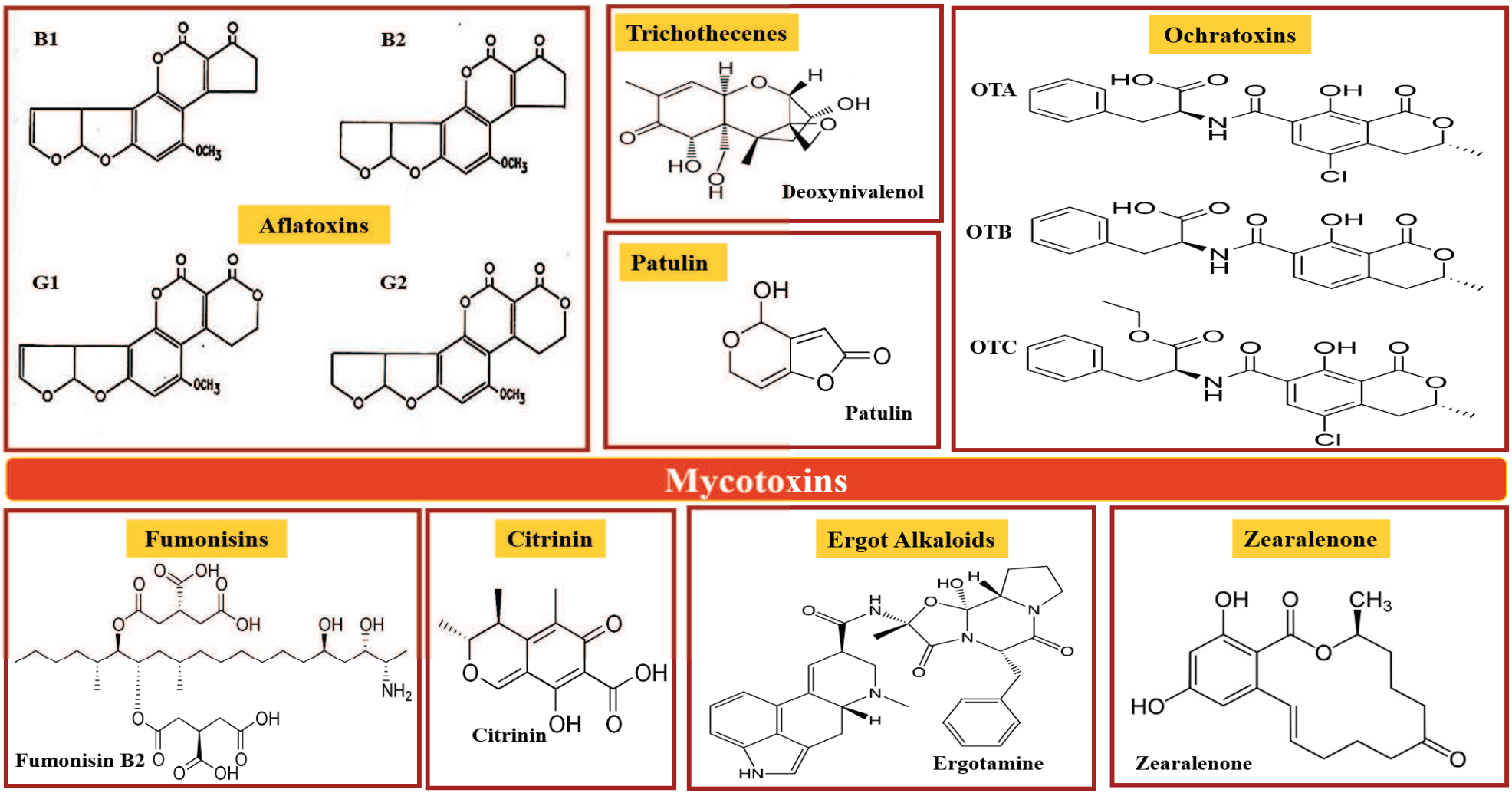



**Figure 1.9.: The chemical structure of the main mycotoxins belonging to different groups**

1.2.1.3. Patulin

Patulin, 4-hydroxy-4H-furo [3, 2c] pyran-2(6H)-one (Fig. 1.9.), is produced by many different molds but was first isolated as an antimicrobial active principle during the 1940s from *Penicillium patulum* (later called *P. urticae,* now *P. griseofulvum*). The same metabolite was also isolated from other species and given the names clavacin, claviformin, expansin and penicidin (Ciegler *et al*., 1971). *P. expansum*, the blue mold that causes soft rot of apples, pears, cherries, and other fruits, is recognized as one of the most common offenders in patulin contamination. Patulin is regularly found in unfermented apple juice, although it does not survive the fermentation into cider products (Trucksess and Tang, 2001). Patulin is toxic at high concentration in laboratory settings, but evidence for natural poisoning is indirect and inconclusive. Nevertheless, the Joint Food and Agriculture Organization–World Health Organization Expert Committee on Food Additives has established a provisional maximum tolerable daily intake for patulin of 0.4 mg/kg of body weight per day (Trucksess and Tang, 2001; Bennett and Klich, 2003).

## 1.3. Ochratoxins

Ochratoxin A is, a mycotoxin produced by two main genera of fungi, *Aspergillus* and *Penicillium,* a potent nephrotoxin that also displays hepatotoxic, teratogenic, immunosuppressive, and carcinogenic effects (Petzinger and Ziegler, 2000; Abouzied *et al.*, 2002; Pfohl-Leszkowicz and Manderville, 2007); it has been classified as a class 2B human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1993). At present, wine is considered, after cereals, the second major source of OTA, and strict European Union regulation has been established, setting maximum levels for OTA of 2 µg/kg in wine, musts, and grape juice and 10 µg/kg for dried vine fruits (Commission regulation no. 1881/2006).

### 1.3.1. Chemical structure

Chemically, ochratoxins are described as weak organic acids consisting of a dihydroisocumarin moiety joined by a peptide bond to 1-phenylalanine (O'Brien and Dietrich, 2005). There are three generally recognized ochratoxins, designated A, B and C (Fig. 1.9). Structurally, these three toxins differ only very slightly from each other; however, ochratoxin A (OTA) is chlorinated and is the most toxic, followed by OTB (substitution of chloride for a hydrogen atom in the isocumarin moiety), which is at least an order of

magnitude less toxic, and OTC, or ethyl OTA, with little or no toxic potential (van der Merwe *et al.,* 1965; Li *et al.,* 1997).

OTA chemical name is L-phenylalanine-*N*-[(5-chloro-3, 4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyrane-7-yl) carbonyl]-(*R*)-isocoumarin and its chemical structure is presented in Figure 1.10.

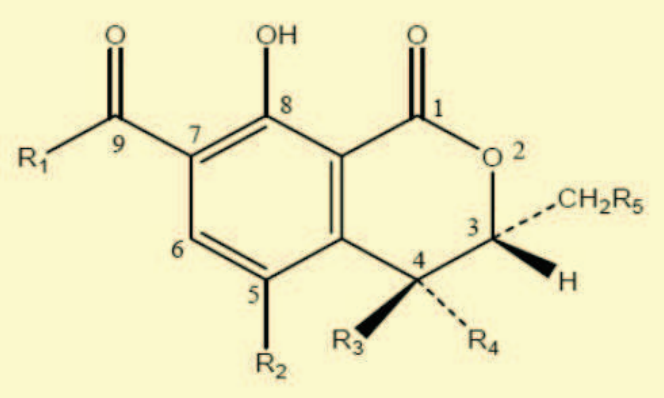
### 1.3.2. Ochratoxin A derived metabolites

OTA, and occasionally OTB, occur naturally in mouldy products. However, a wide range of related compounds like ochratoxin α (Otα) -the isocoumarin nucleus of OTA-, its dechlorinated analogue known as OTβ, methyl and ethyl esters, and several amino acid analogues, are synthesized in laboratory cultures (Moss, 1996; Xiao *et al.*, 1995). Ochratoxin α and β, are hydrolysis products of OTA and OTB respectively, and as consequence of the lack of the phenylalanine molecule, they are not toxics.

Several metabolites related to OTA have been also identified, particularly, OTB the dechloro analog of OTA, OTC its ethyl ester, the isocoumaric derivative of OTA, Otα, and its dechloro analog, OTβ. Figure 2 presents the general structure common to these different metabolites and Table 1 shows the characteristic composition of each one. Recently, new OTA derived metabolites have been characterized, which include a dechlorinated ochratoxin A derivative identified by nono-ESI-IT-MS (Faucet-Marquis *et al*., 2006) and a quinone/hydroquinone metabolite showing toxicological properties (Tozlovanu *et al.,* 2006). In addition, it is predicted that OTA will form a benzoquinone electrophile following activation by cytochrome P450 enzymes, and radical species following activation by enzymes with peroxidase activities (Pfohl-Leszkowicz and Manderville, 2007; Manderville and Pfohl-Leszkowicz, 2008). These electrophiles react preferentially with deoxyguanosine (dG) to form benzetheno adducts and C8-dG adducts, respectively (Manderville and Pfohl-Leszkowicz, 2008; El-Khoury and Atoui, 2010).

### 1.3.3. Physico-chemicals properties of ochratoxin A

OTA is a weak organic acid with a pKa value of 7.1 (Bredenkamp *et al*., 1989; El-Khoury and Atoui, 2010) and a molar mass of 403.8 g.mol-1. With crystalline structure varying from colorless to white, this molecule posses an intense green fluorescence under UV light in acid medium and blue fluorescence in alkaline conditions (El-Khoury and Atoui, 2010).




| | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|
| Natural ochratoxins | | | | | |
| **Ochratoxin A** | Phenylalanine | Cl | H | H | H |
| **Ochratoxin B** | Phenylalanine | H | H | H | H |
| **Ochratoxin C** | Ethyl-ester, phenylalanine | Cl | H | H | H |
| **Ochratoxin A Methyl-ester** | Methyl-ester, phenylalanine | Cl | H | H | H |
| **Ochratoxin B Methyl-ester** | Methyl-ester, phenylalanine | H | H | H | H |
| **Ochratoxin B Ethyl-ester** | Ethyl-ester, phenylalanine | H | H | H | H |
| **Ochratoxin α** | OH | Cl | H | H | H |
| **Ochratoxin β** | OH | H | H | H | H |
| **4-R-Hydroxyochratixn A** | Phenylalanine | Cl | H | OH | H |
| **4-s-Hydroxyochratoxin A** | Phenylalanine | Cl | OH | H | H |
| **10-Hydroxyochratoxin A** | Phenylalanine | Cl | H | H | OH |
| **Tyrosine analog of OTA** | Tyrosine | Cl | H | H | H |
| **Serine analog of OTA** | Serine | Cl | H | H | H |
| **Hydroxyproline analog of OTA** | Hydroxyproline | Cl | H | H | H |
| **Lysine analog of OTA** | Lysine | Cl | H | H | H |
| Synthetic ochratoxins | | | | | |
| **d-Ochratoxin A** | d-phenylalanine | Cl | H | H | H |
| **Ochratoxin A Ethyl amid** | Ethyl amid, phenylalanine | Cl | H | H | H |
| **O-methyl Ochratoxin A** | Phenylalanine, $OHCH_3$ on C-8 | Cl | H | H | H |

**Figure 1.10: Characteristic composition of ochratoxin A derived metabolites** (El-Khoury and Atoui, 2010, Wu *et al*., 2011).

In acid and neutral pH, OTA is soluble in polar organic solvents (alcohols, ketones, chloroform), slightly soluble in water and insoluble in petroleum ethers and saturated hydrocarbons. While in alkaline conditions, this molecule is soluble in aqueous sodium bicarbonate solution and in all alkaline solutions in general. It has a melting point of about 90 °C when crystallized from benzene as a solvate (El-Khoury and Atoui, 2010).

However, non-solvated crystals of melting point 169 °C have been obtained from xylene, which are suitable for X-ray structural analysis. OTA is optically active and its spectral characteristics are shown in Tab. 1.6.

**Table 1.6.: Spectral characteristics of ochratoxin A** (El-Khoury and Atoui, 2010).

| Spectral | Solvents | Characteristics |
|---|---|---|
| **UV-VIS** | ETOH | λmax = 213nm (ε36.800)<br>λmax = 332nm (ε 6.400) |
| **Fluorescence** | ETOH 96%<br>ETOH /ABS | λmax = 467nm<br>λmax = 428nm |
| **IR** | $CHCl_3$ | 3380; 2988; 1723; 1674; 1528; 1425; 1381; 1304; 1260; 1170; 1140; 1107; 827 cm-1 |
| **NMR 1H250- MHZ** | $CDCl_3$ | δ 12,70; δ 10,80; δ 8,55 (3H); δ 7,23; δ 7,15 (H Aromatic); δ 4,71; δ 5,07 (CH); δ 2,78; δ 3,2 (CH2); δ1,55 (CH3) |
| **MS** | — | m/z 239/241<br>m/z 255/257<br>molecular ion m/z 403 |

The particularity of OTA is due to its high stability. It has been shown that it possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule.

Müller (1982) showed that the OTA is only partially degraded at normal conditions of cooking. Moreover, this molecule can resist three hours of high pressure steam sterilization of 121 °C (Trivedi *et al.*, 1992), and even at 250 °C its destruction is not complete (Boudra *et al.*, 1995).

### 1.3.4. OTA biosynthesis

Structurally, OTA comprises adihydrocoumarin moiety linked to a molecule of L-β-phenylalanine (Phe), derived from the shikimic acid pathway, by an amide bond. Several related compounds were also reported to occur in OTA-producing organism cultures, such as the dechlorinated analog ochratoxin B (OTB), the isocoumarin nucleus of OTA ochratoxin α (OTα) and its dechlorinated analogue ochratoxin β (OTβ), which are not linked to phenylalanine, methyl and ethyl esters, including ochratoxin C (OTC), which is an ethyl ester derivative of OTA and several amino acid analogues (Moss, 1996; 1998; Xiao *et al.*, 1995; Gallo *et al.*, 2012).

Unlike other important mycotoxins, the biosynthesis pathway of OTA has not yet been completely elucidated in detail. However, it is clear that the pathway involves some

crucial steps, such as the biosynthesis of the isocoumarin group through the catalyzing action of a polyketide synthase (PKS) (Bacha *et al.,* 2009), its ligation with the amino acid phenylalanine through the carboxyl group in a reaction catalyzed by a peptide synthetase, and the chlorination step, but the order of the reactions is not yet well defined (Gallo *et al.*, 2012). Several schemes have been proposed that have resulted from different studies. Huff and Hamilton (1979) suggested a role in the polyketide synthesis for mellein, which would be carboxylated to OTβ and then transformed through a chloroperoxidase to OTα. According to their hypothesis, in the subsequent step, OTα would be phosphorylated and linked to the ethyl ester of phenylalanine to generate OTC, and then a deesterification would lead to the final product OTA (Fig. 1.11.). Precursor feeding experiments with *A. ochraceus*, carried out by Harris and Mantle (2001), suggested that one, possibly dominant, biosynthetic pathway involves the passage from OTβ to OTα and then to OTA, with a chlorinating step prior to the ligation of the polyketide (OTα) to phenylalanine, without OTC and phenylalanine ethyl ester intermediates. Since they could not rule out the role of OTB, they also maintain a possible alternative pathway in which the formation of OTA goes through the synthesis from OTβ to OTB, but in this case, a biosynthetic role for OTα, which occurs naturally, could not be explained.

However, several reports have now been published about the presence of OTα as a product of OTA biodegradation by different microorganisms, among which are various *Aspergillus* species (Varga *et al.,* 2000; Abrunhosa *et al.,* 2002; Bejaoui *et al*., 2006). The amide bond between OTα and phenylalanine in OTA is hydrolyzed by several different enzymes, including a carboxypeptidase A (Deberghes *et al*., 1995), lipase (Stander *et al.,* 2002), commercial proteases, and as recently determined, a metalloprotease isolated in *A. niger* (Abrunhosa and Venancio, 2007). Gallo *et al.* (2012) proposed a hypothesis regarding the OTA biosynthetic pathway (Fig. 1.12), the presence of OTα in the OTA-producing *A. carbonarius* strain culture is most likely due to the biodegradation activity of the fungus and is independent of OTA biosynthesis. Whereas, they did not indicate of whether the hydrolysis activity occurred in the medium due to an enzyme excreted out of the cell or if externally applied OTA was absorbed into the cell where the degradation process would have taken place.

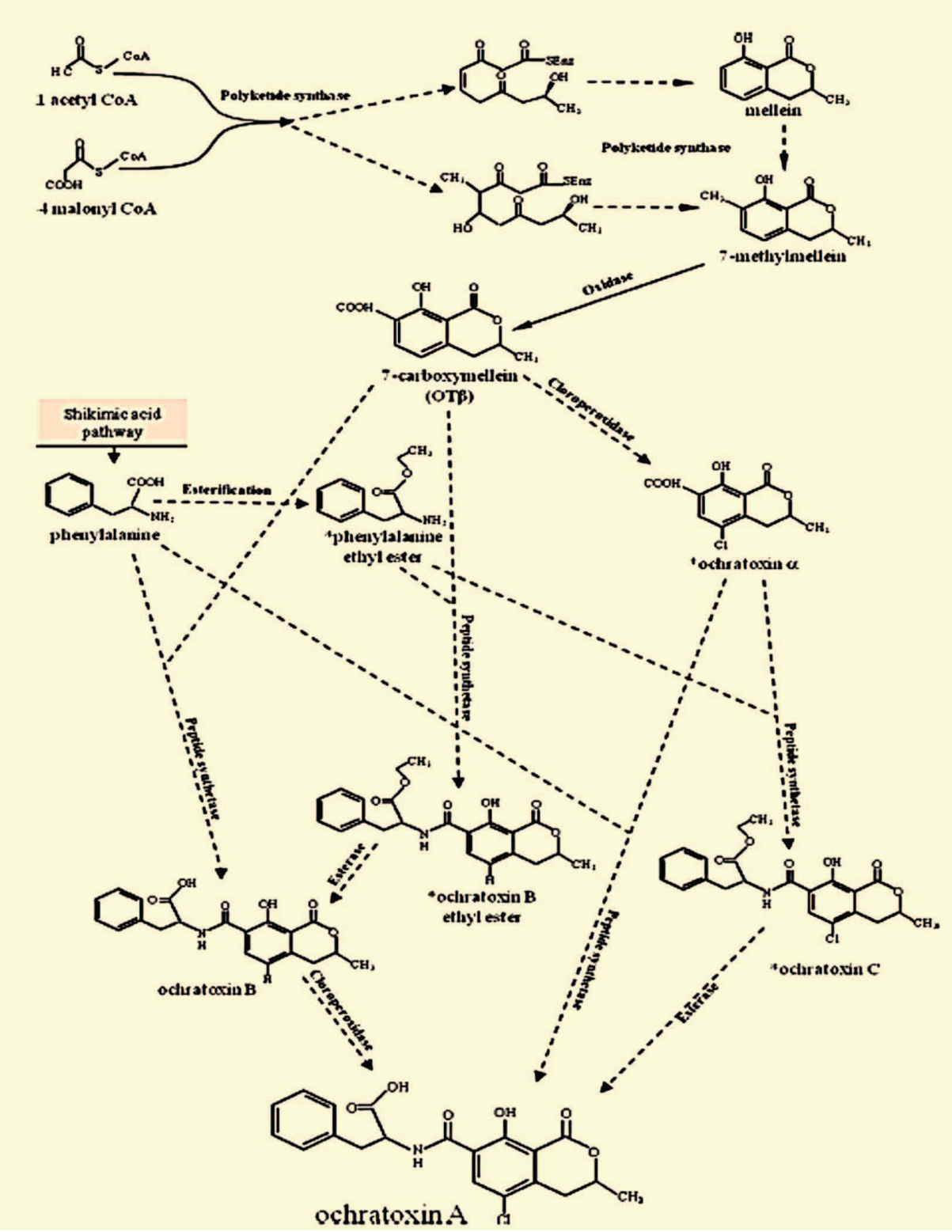



**Figure 1.11.: Scheme showing all the different hypotheses of the OTA biosynthesis pathway** (Gallo *et al.,* 2012).

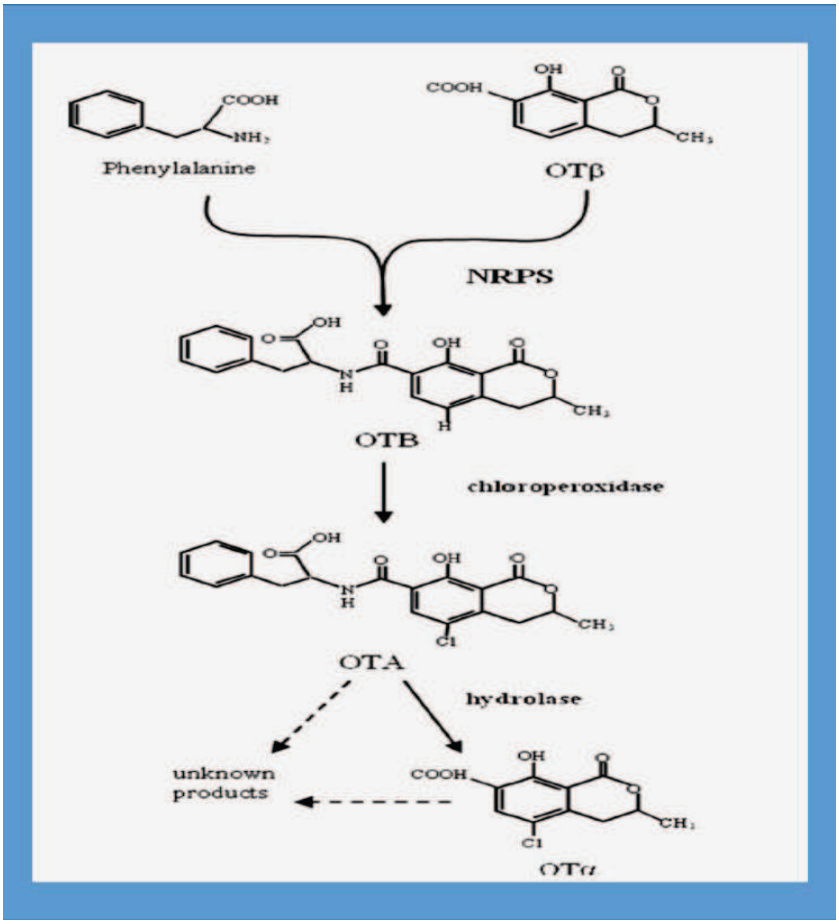



**Figure 1.12: New schematic representation of the last steps of the OTA biosynthetic pathway as proposed** (Gallo *et al*, 2012)

### 1.3.5. OTA occurrence in food

OTA is found in a variety of foods and beverages, including both plant-based products and animal products (Tab. 1.7). Among the first ones, its presence in cereal grains (corn, wheat, barley, flour, oats, rye, rice, etc.), beans (coffee, cocoa, soy, etc.), spices, and beverages like coffee and wine must be highlighted. In 1983, OTA was reported in olive oil (Letutour *et al.,* 1983) and recently it was detected again in this product (Papachristou and Markaki, 2004). OTA can be absorbed from contaminated feed by monogastric animals such as pigs, where it is accumulated in the blood and kidneys, and therefore it can be found in products made from them, such as black pudding, sausages, etc. Moreover, OTA has been detected in milk, cheese and other animal products. The presence of OTA in grape and its derivatives such as dried vines, grape juice, musts, wine, vinegar, etc. is detailed in the following chapter

**Table 1.7: Occurrence of OTA in several food and feed.**

| Food/feed | Reference |
|---|---|
| **Animal feed** | van Egmond and Speijers (1994); Höhler (1998); Dalcero *et al.* (2002); Accensi *et al.* (2004) |
| **Bee pollen** | Medina et al. (2004) |
| **Beer** | Scott and Kanhere (1995); Zimmerli and Dick (1995); Jørgensen (1998); Legarda and Burdaspal (1998); Ueno (1998); Degelmann *et al.* (1999); Bresch *et al.* (2000a); Tagni *et al.* (2002) |
| **Cereals (Rye, wheat, barley, oat, maize, etc.) & Cereal products (bread, muesli, breakfast cereals)** | Speijers and van Egmond (1993); Wood *et al.* (1996); Trucksess *et al*. (1999); Engel (2000); Wolff (2000); Legarda and Burdaspal (2001); Blesa *et al.* (2004) |
| **Cheese** | Shina and Ranjan (1991); Elsawi *et al.* (1994); Engel (2000) |
| **Chocolate & Cocoa** | van Egmond and Speijers (1994); MAFF (1999); Engel (2000); Serra-Bonvehí (2004) |
| **Coffee** | Levi *et al.* (1974); Zimmerli and Dick (1995); Nakajima *et al.* (1997); Bucheli *et al.* (1998); Burdaspal and Legarda (1998); Jørgensen (1998); Ueno (1998); Trucksess *et al.* (1999); Bresch *et al* (2000a); Joosten *et al.* (2001); Otteneder and Majerus (2001); Varga *et al.* (2001); Pardo *et al.* (2004); Suàrez-Quiroz *et al*. (2005); Mounjouenpou *et al.* (2013) |
| **Cow milk** | Engel (2000); Breitholtz-Emanuelsoon *et al.* (1993) |
| **Dried fig & Dried prunes** | Majerus *et al.* (1993); Zohri and Abdelgawad (1993); Doster *et al.* (1996); Engel (2000); Bayman *et al.* (2002) |
| **Fruit & Vegetal fruits** | Majerus et al. (2000) |
| **Grapes & Grape juices** | Majerus and Otteneder (1996); Majerus *et al*. (2000); Zimmerli and Dick (1996), Larcher and Nicolini (2001), Burdaspal and Legarda (1999), Filali *et al.* (2001); Dachoupakan *et al.,* 2009 |
| **Liquorice** | Bresch et al. (2000b) |
| **Meat &** | Gareis (1996); Jørgensen (1998); Gareis and Scheuer (2000) |

| | |
|---|---|
| **Meat products (pork, beef, sausages, etc.)** | |
| **Nuts (hazelnuts, peanuts)** | Engel (2000) |
| **Olive oil** | Letutour *et al.* (1983); Papachristou and Markaki (2004) |
| **Pulses** | Scott *et al.* (1972); Jørgensen (1998); MAFF (1999) |
| **Raisins** | Slayne (2001), Abarca *et al*. (2003); Leong *et al*. (2004); Magnoli *et al.* (2004); Valero *et al*. (2005) |
| **Sauces (ketchup, moustard, barbacue)** | Majerus *et al.* (2000) |
| **Seeds (sunflower seed, sesame, linseed)** | Engel (2000) |
| **Spices** | Patel et al. (1996); Hübner et al. (1998); Thirumala-Devi *et al.* (2001); Abdulkadar *et al.* (2004) |
| **Tea** | Bresch et al (2000a) |
| **Yoghurt** | Engel (2000) |
| **Vinegar** | Markaki *et al*. (2001); Majerus *et al.* (2000) |
| **Wine** | Majerus and Otteneder (1996); Visconti *et al*. (1999); Cerutti *et al*. (2000); Majerus *et al*. (2000); Soleas *et al*. (2001), Zimmerli and Dick (1996); Dachoupakan *et al.,* 2009 |

## 1.3.6. OTA occurrence in grapes and grape products

### 1.3.6.1. Grape Juices

In view of the suspected occurrence of OTA also in fruit-juices and their potential consumption by children, some researchers analyzed grape and other fruit-juices samples too. The number of grape juices containing OTA was also very high, especially for red grape juice samples (86% Majerus and Otteneder, 1996; 88% Majerus *et al.,* 2000). While, the OTA content of white grape juice samples lower (17% Majerus and Otteneder, 1996; 78% Majerus *et al*., 2000; 0% Zimmerli and Dick, 1996). Furthermore, 100% positives were found by Larcher and Nicolini (2001) in concentrated musts and by Burdaspal and Legarda (1999) in both white and red grape musts and grape juices, but due to the limited number of samples analyzed (n=17 and n=18, respectively) more data on the occurrence of OTA in grape juices are required. So, products made from red grapes were more contaminated with OTA,

probably due to the longtime of enzymatic treatment at high temperature of the crude juice and the berries to improve the deep color yield (Majerus *et al.,* 2000).

Grape juices also seem to contain more OTA than some table wines, this suggested that the toxin in the wines was likely present prior the wines were made (Majerus *et al.,* 2000). Furthermore, the comparison between grape juice and wine demonstrates that OTA has a relatively high stability against the alcoholic fermentation and the subsequent technological steps. Grape juice, apple (n=33) and orange juice (n=30) as well as black currant (n=19) and vegetable juices (n=78) were analyzed by Majerus *et al.* (2000) and found apple and orange juice free from OTA; levels slightly above the limit of detection were detected in black currant, tomato and carrot juices. Similar results were obtained by Filali *et al.* (2001) who did not found OTA in 13 fruit juices (cocktail, orange, mango, peach, pineapple, clementine) and the only sample analyzed containing OTA was a grape fruit juice (1.16 µg OTA/L). More data on the occurrence of OTA in grape juices are necessary because children are one of the main consumers and because juice consumption is more than that of wine.

#### 1.3.6.2. Raisins (dried vine fruits)

Raisins are dried grapes that can be eaten raw, used in cooking and baking or as ingredients in muesli, biscuits, cakes, etc. During summer, when grapes have attained their optimum sweetness, farmworkers carefully hand-pick the grape bunches and let them dry on rows of clean paper trays next to the vines. The grapes dry naturally in the sun for two or three weeks. The process can be done also indoor in industrial controlled driers. Raisins have high concentration of sugars, and if stored for a long period the sugar crystallises inside the fruit. This makes the fruit gritty, but does not affect the usability (Christensen and Peacock 2000). The unexpectedly high levels of OTA in dried vine fruits were of immediate concern. Slayne (2001) concluded that, based upon dietary consumption patterns, the levels of OTA in dried vine fruits are safe, but recommended that industry should reduce levels to the lowest technologically achievable. Dried grape fruits, which have low water activity ($a_w$), are generally resistant to microbial attack. However, different surveys of mycoflora in dried fruits have been carried out (Abarca *et al*., 2003; Leong *et al*., 2004; Magnoli *et al*., 2004; Valero *et al.*, 2005), all pointing out black *Aspergillus* and mainly *A. carbonarius*, as the responsible for the OTA levels detected in these fruits. Adaptation to environmental conditions of sun-drying, and a strong dominance of black aspergilli among the common mycobiota of grapes at these conditions, are suggested as the two main reasons supporting the prevalence of black aspergilli in sun dried grapes (Valero *et al.,* 2005).

#### 1.3.6.3. OTA in vinegar

Vinegar, from the French word 'vinaigre' meaning sour wine, is a sour liquid made from the oxidation of ethanol in wine, cider, beer, or similar alcoholic products. Vinegar is typically 3-5 % by volume acetic acid, and natural vinegars also contain smaller amounts of tartaric acid, citric acid, and others. Vinegar may be started by the addition of what is called 'mother of vinegar'. The oxidation is carried out by acetic acid bacteria, as was shown in 1864 by Louis Pasteur. Vinegar may also contain OTA. Few studies analyzed such as the study of Majerus *et al.* (2000) which was conducted in Germany on several kinds of vinegar, found that the Balasmic vinegar (produced from the white Trebbiano grape juice) had the highest OTA contamination (0.01-4.35 µg/L) followed by the wine vinegar (0.01-1.9µg/L) and the apple and fruit vinegar (0.01-0.02µg/L). These results agreed with Markaki *et al*. (2001) study on the Balsamic vinegar with high OTA concentration (0.1-25 µg/L).

#### 1.3.6.4. OTA in wine

The presence of OTA in wine has been reported by several authors from the entire world since its first description in 1996 (Zimmerli and Dick, 1996). Until now, OTA and its related compounds are the only mycotoxins detected in wine. Most of the studies carried out before 2001 were compiled in order to obtain a general overview of OTA occurrence in wines from different countries over the world

### 1.3.7. OTA occurrence in mediterranean region

There is a great concern on this metabolite in the Mediterranean countries because many results show that wines from the Mediterranean area contain higher levels of OTA than wines from Northern areas in Europe (Majerus and Otteneder 1996; Zimmerli and Dick 1996). Many surveys carried out in different countries from Southern Europe, with Mediterranean climate, have reported some geographic regions in Southern Europe are more prone to contamination with the toxigenic species and OTA (Battilani and Pietri, 2002). They have reported that *Aspergillus* section Nigri (black aspergilli), in particular *A. carbonarius*, is the main responsible for OTA production in grapes (Cabañes *et al.* 2002; Bellí *et al.* 2002; 2004; 2005; Battilani *et al.* 2003; 2006), and can also dominate the raisins ecosystem (Battilani *et al.,* 2003; 2006)

Serra *et al* (2003) evaluate the incidence of fungi producing-OTA in Portuguese wine grapes. They have reported that almost all ochratoxigenic strains (mainly, *A. carbonarius*)

were isolated at harvest time, with the highest incidence of these fungi mainly in the regions with a Mediterranean climate.

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 (Zimmerli & Dick, 1996; Abrunhosa *et al.,* 2001; Ostry *et al.*, 2004; Torelli *et al.,* 2003; Varga *et al.*, 2005).

Rousseau (2004) classified the viticulture in the northern mediterranean regions for the incidence OTA producing fungi and for their OTA production. They have reported that vineyards closed to the sea have the favorable conditions for the OTA-producing fungi and the highest OTA risk of OTA contamination (Fig. 1.13)

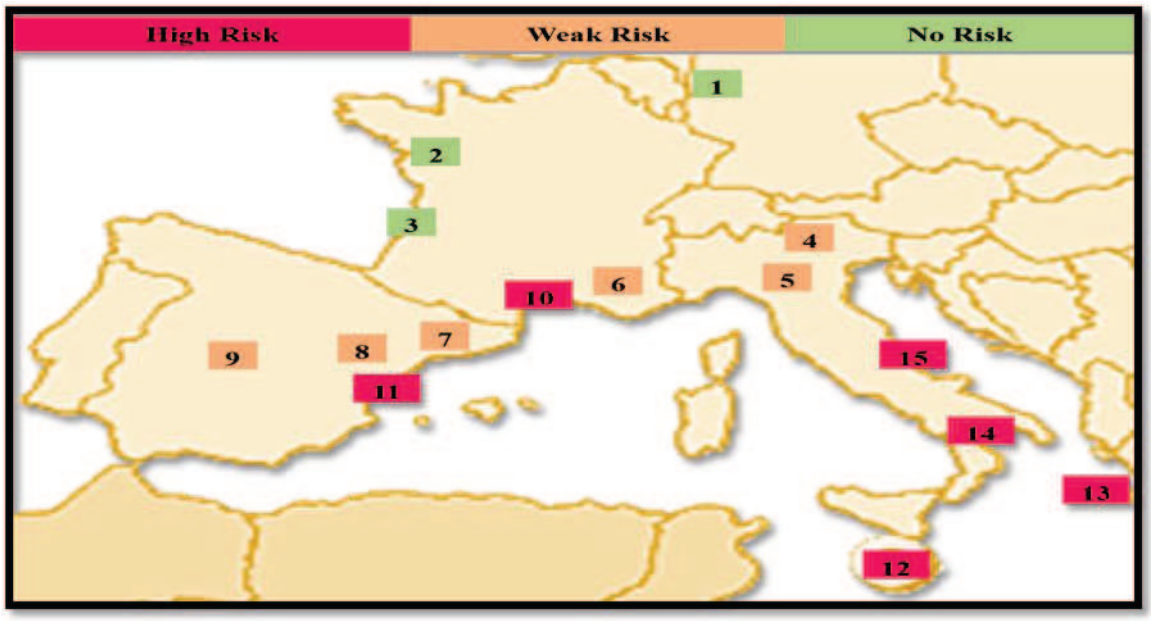



**Figure 1.13: Classification of the viticulture regions in the North-western Mediterranean according to their risk of OTA contamination p on the grape juice analysis** (Rousseau, 2004); **1:** Rheinplatz, **2:** Charentes, **3:** Gers/Sud-Ouest, **4:** Veneto, **5:** Reggio Emilia, **6:** Vaucluse/Rhône valley, **7:** Penedes, 8: Mancha, **9:** Utiel Requena, **10:** Languedoc-Roussillon, **11:** Valencia/Alicante, **12:** Sicily, **13:** Greece, **14:** Pouilles, **15:** Abbruzes.

## 1.3.8. OTA occurrence in France

A survey on the occurrence on grape of black Aspergillus species and their capability to produce ochratoxin A (OTA) has conducted in France over three years (2001–2003) by Bejaoui *et al.* (2006) in 10 vineyards from four winemaking regions with different

geographical locations and climatic conditions. The *Aspergillus* genus was essentially represented by Section Nigri (99%) and it was predominant (80%) when compared to Penicillium (20%). Regardless of sampling year, 32.5% of the fungal isolates were OTA producers and 93% belonging to black aspergilli. The ochratoxigenic potential of the isolates and their occurrence on grapes revealed that *A. carbonarius* was the main OTA producer (up to 37.5 µg/g). At harvest time, the fungal population was maximal and this was the most critical period influencing OTA contamination. Grapes from Languedoc-Roussillon region were most infested with ochratoxigenic fungi and had the highest concentrations of OTA (up to 2.8 ng/g).

### 1.3.9. Factors influencing growth and ochratoxin production

Different fungal species require different conditions for optimal growth (Pitt and Hocking, 2009). Microbial metabolism is significantly influenced by the physical and chemical environment. Thus, toxin-producing fungi may invade food at pre-harvesting period, harvest-time, during post-harvest handling and in storage. According to the site where fungi infest food, toxinogenic fungi can be divided into three groups (Suttajit, 1989): Field fungi [includes species of plant pathogenic fungi, usually with high requirements of water, such as *Fusarium*, *Alternaria* and *Cladosporium*], Storage fungi [with lower requirements of humidity, are principally the genus *Aspergillus* and *Penicillium*] and advanced deterioration fungi [normally do not infest intact food, but easily attack damaged one and require high moisture content. Some examples are some other aspergilli species, *Chaetomium*, *Scopulariopsis*, *Rhizopus*, *Mucor* and *Absidia*]

Mycotoxigenic fungi grow better under certain environmental conditions. However, the presence of these fungi does not mean the mycotoxin synthesis, as production of secondary metabolites is not essential to the synthesizing organism. In fact, the conditions in which these moulds produce their mycotoxins are very specific and independent of those required for fungal growth. Conversely, the visible absence of mould does not mean that no mycotoxins are present since these may remain in the product long after the producing fungus has disappeared. It is not possible to entirely prevent the formation of mycotoxins, but eliminating the conditions necessary for fungal growth helps prevent formation of the toxin.

The main factors that influence growth and production of mycotoxins include temperature, pH and moisture. But a part from environmental factors, chemical and biological factors clearly play a role (Fig. 1.10). Under some circumstances these effects are

additive. Under others, the implication is that synergistic interactions lead to a combined effect of greater magnitude than the sum of constraints applied individually. This has been described by Leistner and Rödel (1976) as the '**hurdle concept**'.

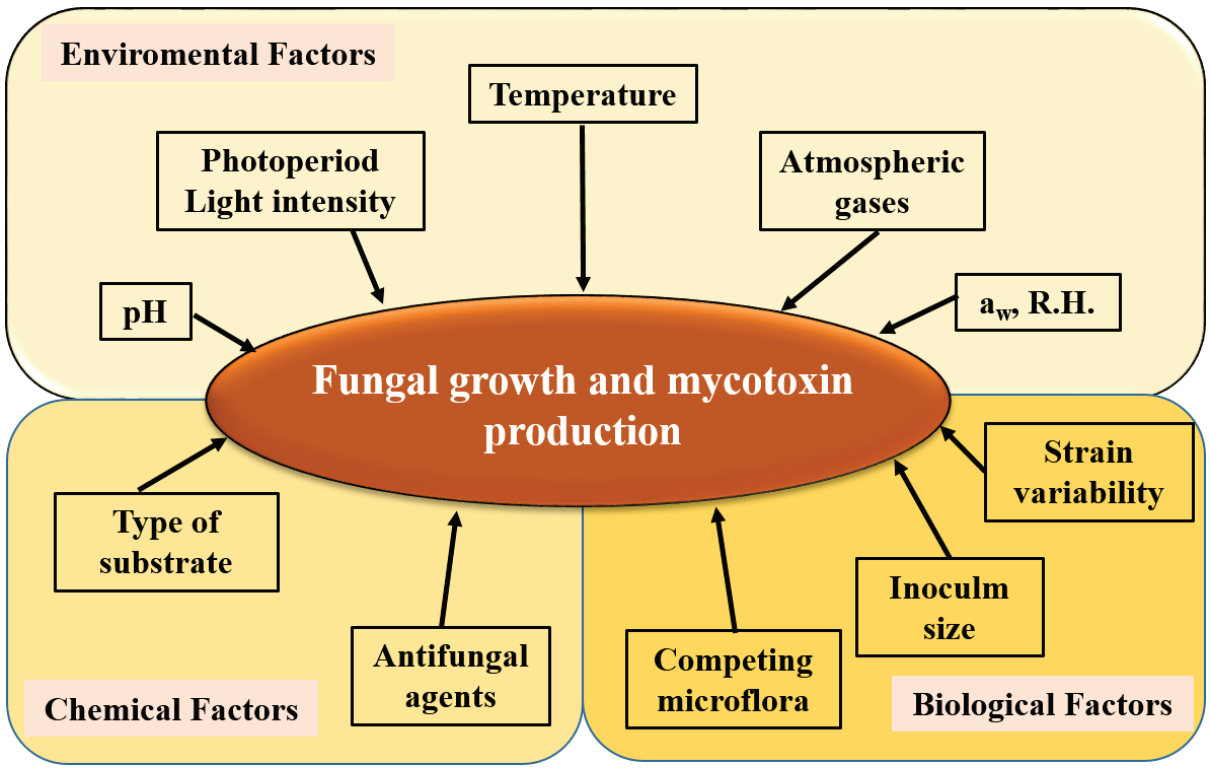



**Figure 1.14. Principal factors influencing fungal growth and mycotoxin production** (EC, 1994).

Paterson and Lima (2010) had mentioned that the favorable temperature and water activity are crucial factors for mycotoxigenic fungi and mycotoxin production (especially, ochratoxin A). Hence, fungal diseases of crops provide relevant information for pre-harvest mycotoxin contamination. However, the mycotoxin issue also involves post-harvest scenari. There are no data on how mycotoxins affect competing organisms in crop ecosystems. In general, if the temperature increases in cool or temperate climates, the relevant countries may become more liable to aflatoxins. Tropical countries may become too inhospitable for conventional fungal growth and mycotoxin production.

*Aspergillus* and *Penicillium* are able to develop under a wide variety of environmental conditions (Sweeney and Dobson, 1998).However, the synthesis of secondary metabolites is conditioned by several factors such as temperature, water activity ($a_w$) (Romero *et al.,* 2007; Amézqueta *et al.,* 2012) and medium composition, in particular its micronutrients, its pH and the presence of competitive agents (Valero *et al.,* 2007; Amézqueta *et al.,* 2012).

### 1.3.10. Sample origin influencing on OTA content

Most of the surveys show that the geographic region of origin of the wine has a strong influence on OTA contamination. The further Southern the provenance, the more common the occurrence and the greater the concentration of OTA found. Zimmerli and Dick (1996) were the first to point out a higher incidence of contamination in red wines from Southern Europe and Northern Africa, in comparison with the rest of wines they analyzed. Majerus and Otteneder (1996) results, also indicate a North-South gradient of OTA level in wines, whereby samples from the Mediterranean area were contaminated more frequently. Recently, Pietri et al. (2001) found that wines produced in Southern Italy were markedly more contaminated than those from the North. A study of red wines originated from Mediterranean sea countries (Markaki et al., 2001) revealed that the more contaminated samples were one from Greece (2.35 µg/L), four from France (2.62 µg/L, 1.59 µg/L, 3.21 µg/L, and 3.40 µg/L), and one sample labelled European Union (1.54 µg/L). Furthermore, one sample from Italy and two samples from Morocco were found to be contaminated with OTA at levels of 0.892 µg/L, 0.551µg/L and 0.554µg/L, respectively.

Majerus et al. (2000) also analyzed few samples from Tunisia and Algeria obtaining a range from 0.37 to 1.85 µg/L. Taking into account earlier results from Morocco [10.5-15.6 µg OTA/L by Ospital *et al.* (1998), 100% OTA contaminated wine samples by Filali *et al.,* (2001)], there seems to be a tendency that samples from Northern Africa have even higher OTA concentrations than those from regions in Europe. This could be because of the high temperature and humidity that characterise the South climate conditions. By contrast, Majerus *et al.* (2000) compared the OTA contamination of some European samples and observed higher OTA contamination in the North (Germany, Northern France, Northern Italy) than in the South (Southern France, Spain and Greece) regions in Europe. In addition, Soleas *et al.* (2001) analyzed the incidence of OTA in 942 commercial wines and found that European red wines was quite variable: Spain (33%), Portugal (22%), and Central Europe (22%) had higher percentages, while Greece (17%) and Italy (16%) had lower ones. Therefore, evidence for a North-South gradient in wine OTA concentrations is not clear from these data.

### 1.3.11. OTA-producing species

OTA was originally described as a metabolite of *A. ochraceus* (*Aspergillus* section *Circumdati*) from laboratory experiments (van der Merwe *et al.,* 1965). Later, the production of this toxin was repeatedly reported in Section *Nigri*, mainly by *Aspergillus carbonarius* and a low percentage of isolates of the closely related species *A. niger* (Téren *et al.*, 1996). Even recently, new ochratoxigenic species in this section are emerging (Samson *et al.* 2004; Medina *et al.,* 2005). Isolates from other subgenera usually produce only small amounts of OTA, or they ability to produce the toxin was not confirmed by other authors (Tab. 1.8).

Natural occurrence and practical importance of OTA, however, was first linked with *Penicillium* species (Ciegler *et al.*, 1972; Krogh *et al.*, 1973) (Tab. 1.9). Some of these, like *P. viridicatum* and *P. cyclopium*, have been found afterwards to do not produce OTA.

Later, Larsen *et al.* (2001), biochemically characterized several OTA-producing strains of the genus *Penicillium*, and separated them in two large groups: *P. verrucosum* and *P. nordicum*. The last deserve a special attention as they produced more OTA than *P. verrucosum* under laboratory conditions. Moreover, they suggested that *P. nordicum* could be the source of OTA of *Penicillium* contaminated meat-derived products as all the isolates they examined from these food belonged to this group, whereas *P. verrucosum* was only found in plant-derived material.

To sum up, the two main species of moulds that produce OTA are *P. verrucosum* and *A. ochraceus*, but a third major source, *A. carbonarius*, have been later identified and other members of the *A. niger* aggregate have been reported as possible sources of OTA contamination in grapes (Da Rocha Rosa *et al*., 2002; Sage *et al.,* 2002; Battilani *et al*., 2003; Magnoli *et al.* 2003; Serra *et al.,* 2003; Bau *et al.,* 2005; Bellí *et al*., 2004; Bellí *et al*., 2006a; Dachoupakan *et al*., 2009), wine (Cabañes *et al*., 2002; Dachoupakan *et al*., 2009 ) and raisins (Abarca *et al*. 2003; Heenan *et al*., 1998).

All three species differ in physiology and ecology, which in turn affect the types of foodstuffs in which these moulds are most commonly found.

**Table 1.8: OTA-producing *Aspergillus* species** (modified from Varga *et al*., 2001).

| Species | Section | References |
|---|---|---|
| *A. glaucus* | *Aspergillus* | Chelkowski *et al.* (1987) |
| *A. repens* | | El-Kady *et al.* (1994) |
| *A. sydowii* | | Ueno *et al.* (1991) |
| *A. albertensis* | *Flavi* | Varga *et al.* (1996); Peterson (2000) |
| *A. alliaceus* | | Ciegler (1972); Doster *et al*. (1996); Peterson (2000); Bayman *et al.* (2002) |
| *A. flavus* | | Atalla and El-Din (1993) |
| *A. auricomus* | *Circumdati* | Varga *et al.* (1996) |
| *A. melleus* | | Ciegler (1972) |
| *A. muricatus* | | Frisvad and Samson (2000) |
| *A. ochraceus* | | van der Merwe *et al.* (1965); Krivobok *et al*. (1995); Varga *et al.* (1996); Mühlencoert *et al*. (2004); Pardo *et al.* (2004). |
| *A. ostianus* | | Ciegler (1972) |
| *A. petrakii* | | Ciegler (1972) |
| *A. sclerotiorum* | | Ciegler (1972); Moss (1996); Varga *et al.* (1996) |
| *A. sulphureus* | | Ciegler (1972); Madhyasta *et al.* (1990) |
| *A. clavatus* | *Clavati* | Atalla and El-Din (1993) |
| *A. wentii* | *Cremei* | Varga *et al.* (1996) |
| *A. fumigatus* | *Fumigati* | Abarca *et al.* (1997); Atalla and El-Din (1993): Varga *et al.* (2000) |
| *A. awamori* | *Nigri* | Ono *et al.* (1995); Téren *et al.* (1996); Accensi *et al.* (2001) |
| *A. carbonarius* | | Heorie (1995); Téren *et al.* (1996); Wicklow *et al.* (1996); Heenan *et al.* (1998); Joosten *et al.* (2001) |
| *A. foetidus* | | Ueno *et al.* (1991); Téren *et al.* (1996); Magnoli et *al.* (2003) |
| *A. japonicus* | | Dalcero *et al*. (2002); Battilani *et al.* (2003) |

| | | |
|---|---|---|
| *A. lacticoffeatus* | | Samson *et al.* (2004) |
| *A. niger* | | Abarca *et al.* (1994); Ono *et al.* (1995); Téren *et al.* (1996); Nakajima *et al.* (1997); Heenan *et al.* (1998) |
| *A. sclerotioniger* | | Samson *et al.* (2004) |
| *A. tubingensis* | | Medina *et al.* (2005) |
| *A. usamii* | | Ono *et al.* (1995); Accensi *et al.* (2001) |
| *A. vadensis* | | De Vries *et al.* (2004) |
| *A. terreus* | *Terrei* | Ueno *et al.* (1991) |
| *A. ustus* | *Usti* | Ueno *et al.* (1991) |
| *A. versicolor* | *Versicolores* | Abarca *et al.* (1997) |

**Table 1.9.: OTA-producing *Penicillium* species** (modified from Varga *et al*., 2001).

| Species | Section | Reference |
|---|---|---|
| *P. cyaneum* | *Aspergilloides* | Ueno *et al.* (1991) |
| *P. implicatum* | | Ueno *et al.* (1991) |
| *P. montanense* | | Ueno *et al.* (1991) |
| *P. sclerotiorum* | | Ueno *et al.* (1991) |
| *P. spinulosum* (*P. purpurescens*) | | El-Banna *et al.* (1987); Ciegler *et al.* (1972) |
| *P. variabile* | *Biverticillium* | Ciegler *et al.* (1972); Krivobok *et al.* (1995) |
| *P. purpurogenum* | | Ueno *et al.* (1991) |
| *P. verruculosum* | | Ueno *et al.* (1991) |
| *P. canescens* | *Eladia* | Ueno *et al.* (1991) |
| *P. corylophilum* | | Ueno *et al.* (1991) |

| | | |
|---|---|---|
| *P. fascum* | | Ueno *et al.* (1991) |
| *P. hirayamae* | | Ueno *et al.* (1991) |
| *P. janczewskii* | | Ueno *et al.* (1991) |
| *P. melinii* | | Ueno *et al.* (1991) |
| *P. miczynskii* | | Ueno *et al.* (1991) |
| *P. raistrickii* | | Ueno *et al.* (1991) |
| *P. simplicissimum* | | Ueno *et al.* (1991) |
| *P. atramentosum* | *Penicillium* | Bridge *et al.* (1989) |
| *P. aurantiogriseum* (*P. solitum*) | | Bridge *et al.* (1989); Krivobok *et al.* (1995) |
| *P. commune* | | Ciegler *et al.* (1972) |
| *P. expansum* | | Bridge *et al.* (1989) |
| *P. nordicum* | | Larsen *et al.* (2001) |
| *P. verrucosum* | | Pitt (1987); Larsen *et al*. (2001) |
| *P. chrysogenum* | *Ramosum* | Turner and Aldridge (1983); Krivobok *et al.* (1995) |

The morphological and biochemical diversity of black aspergilli occurring on grapes is being summarized in Tab. 1.10. They differ both in their micromorphology and in extrolite profiles, but for some species like *A. niger*, *A. tubingensis*, *A. foetidus* and *A. brasiliensis* molecular data (chemical or DNA based) are needed for their correct identification. The most frequently occurring species, as underlined above, are the "biseriate" *A. niger*, *A. tubingensis* and *A. carbonarius*, together with the "uniseriate" *A. japonicus*, *A. aculeatus* and the new species *A. uvarum* currently found only on European grapes (Abarca *et al*., 2004) (Fig. 1.15).

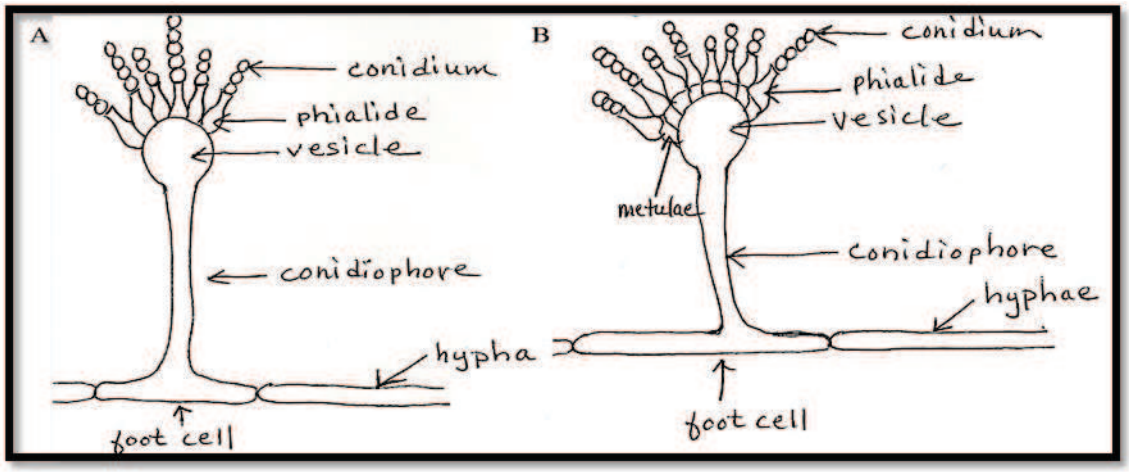



**Figure 1.15: Drawings of intact fungal conidiophores, showing morphological characteristics that are diagnostic for determining species of *Aspergillus*. A: uniseriate, B: Biseriate.** http://ldnissen30269.blogspot.fr/2012/09/lab-3.html

Samson *et al.* (2007) presented phenotypic methods to identify the accepted species of the black aspergilli (colony morphology, conidial size and ornamentation of the ex-type cultures is in a pictorial overviewed) (Fig. 1.16 & 1.17).

Surveys in 107 vineyards in the Mediterranean basin have identified four main *Aspergillus* populations: *A. carbonarius*, *A. tubingensis*, *A. niger*, and a group of *Aspergillus* 'uniseriate' isolates morphologically indistinguishable from *A. japonicus* and *A. aculeatus*. The latter could be clearly distinguished by molecular tools such as AFLP, RFLP and sequence analyses (Bau *et al.* 2006; Perrone *et al.* 2006a, 2006b).

In the past, it was very common that all *Aspergillus* isolates developing black colonies were identified as *A. niger* by non-taxonomists, because of the similarities in morphology, and sometimes the same isolate was preserved in culture collections under different species names. To solve this problem, a last review in the taxonomy of black aspergilli has been published by Abarca *et al.* (2004), who proposed an identification key to distinguish the most common taxa (Tab. 1.10). Cultures on Czapek agar (CZ), CYA or MEA (Pitt and Hocking, 1997) incubated at 25 ºC for 7 days were recommended. In a first step, *Aspergillus* section *Nigri* isolates were identified as uniseriates or biseriates.

**Table 1.10: Morphological and biochemical diversity of black aspergilli occurring on grapes** (Perrone *et al.,* 2007).

| Species | Conidial size (µm) | Color and size of sclerotia (mm) | Source | OTA |
|---|---|---|---|---|
| ***Biseriates*** | | | | |
| ***A. brasiliensis*** | 3.5-4.5 | Found only in some strain, white, 1-1.5 | Soil, grape | - |
| ***A. carbonarius*** | 7-9 | Pink to brown, 1 | Grape, cocoa, coffee, spices, palm oil, soil, air | + |
| ***A. foetidus*** | 3.5-4.5 | Found only in some strain, white, 1-1.5 | Tomato, grape, bottled fruits | - |
| ***A. ibericus*** | 5–7 | - | Grape | - |
| ***A. niger*** | 3.5-5 | - | Grape, cocoa, coffee, cereals, soil, paper, date palm | +/- |
| ***A. tubingensis*** | 3-5 | White to pink, 0.5-0.8 | Grape, cocoa, coffee, soil, cereals | +/- |
| ***Uniseriates*** | | | | |
| ***A. aculeatus*** | 4-5 | - | Grape, papaya, pistachio, rice, tomato | - |
| ***A. japonicus*** | 4-5 | white to cream, 0.5 | Grape, green coffee berries, pineapple, sesame seed | - |
| ***A. uvarum*** | 3-4 | dark brown to black | Grape | - |

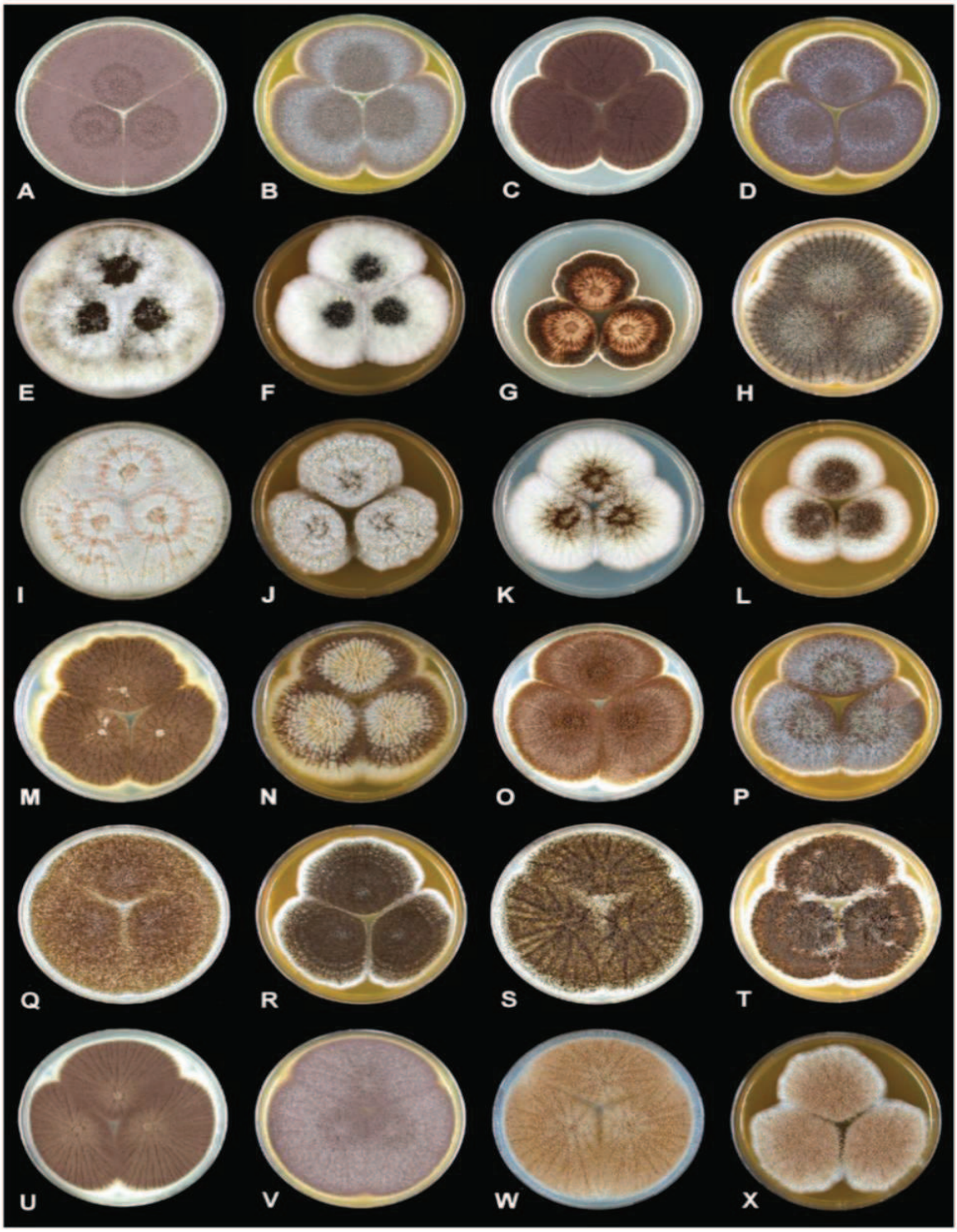



**Figure 1.16: Colony morphologies of type strains of species assigned to *Aspergillus* section Nigri grown on CYA and MEA plates at 25 °C for 7 d. (A-B)** *A. aculeatinus*, **(C-D)** *A. aculeatus*, **(E-F)** *A. brasiliensis*, **(G-H)** *A. carbonarius*, **(I–J)** *A. costaricaensis*, **(K–L)** *A. ellipticus,* **(M-N)** *A. foetidus*, **(O-P)** *A. japonicus*, **(Q-R)** *A. heteromorphus*, **(S-T)** *A. homomorphus*, **(U-V)** *A. ibericus*, **(W-X)** *A. lacticoffeatus* (Samson *et al.,* 2007)

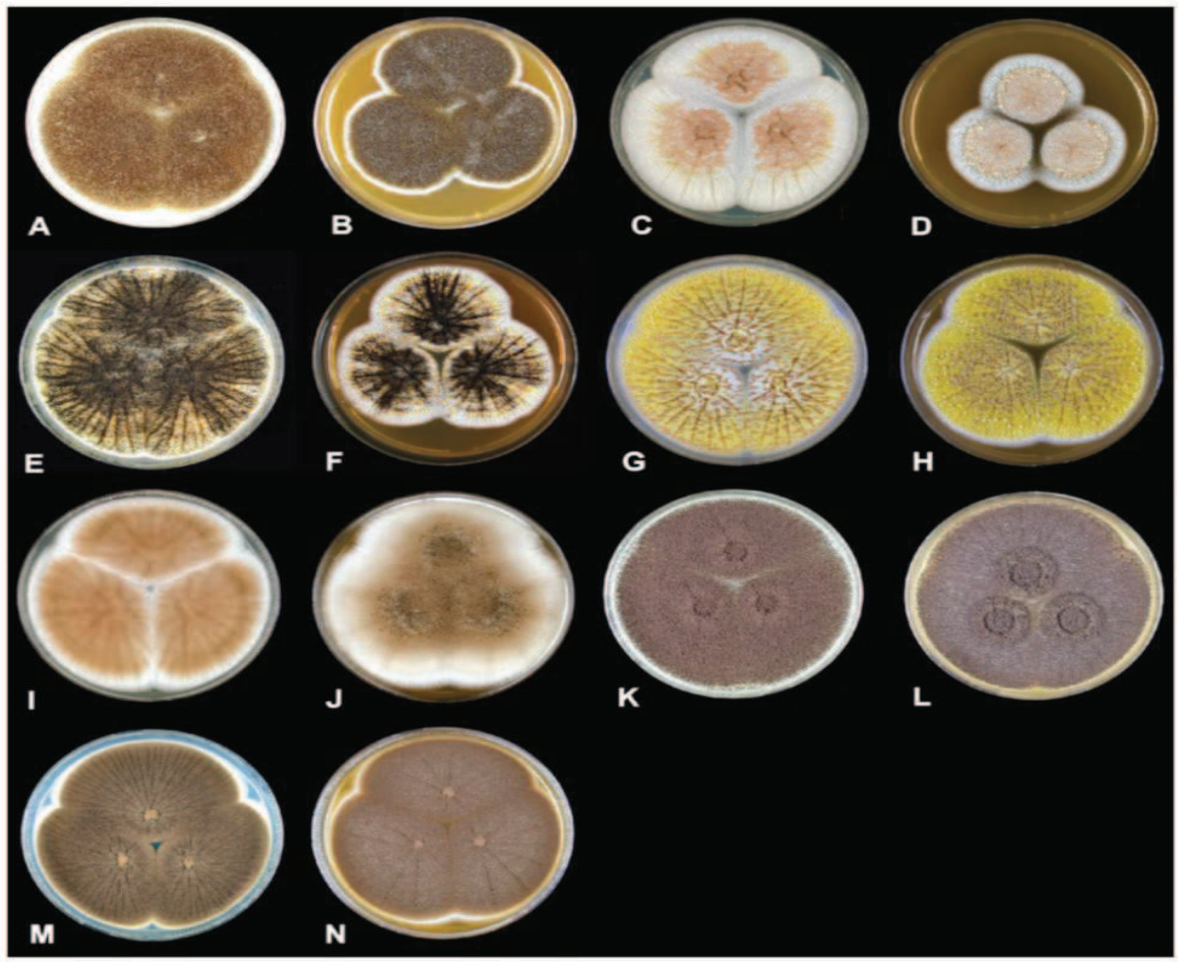



**Figure 1.17: Colony morphologies of type strains of species assigned to *Aspergillus* section *Nigri* grown on CYA and MEA plates at 25 °C for 7 d. (A-B)** *A. niger*, **(C-D)** *A. piperis*, **(E-F)** *A. sclerotiicarbonarius*, **(G-H)** *A. sclerotioniger*, **(I-J)** *A. tubingensis*, **(K-L)** *A. uvarum,* **(M-N)** *A. vadensis* (Samson *et al.,* 2007)**.**

Uniseriates were those with uniseriate conidial heads, while biseriates showed biseriates heads, and among these, *A. carbonarius* isolates were identified at species level. This classification was used throughout the mycoflora studies of grapes in this thesis. As it was based on morphological criteria, in some cases, further differentiation at species level was very difficult and required considerable expertise using conventional methods based on morphological features.

A provisional key of section *Nigri*, based on phenotypic characteristics, extrolites and β- tubulin sequencing, was also proposed by Samson *et al.* (2004). They accepted 15 taxonomic species in this section: *A. aculeatus*, *A. brasilensis*, *A. carbonarius*, *A. costaricaensis*, *A. ellipticus*, *A. foetidus*, *A. heteromorphus*, *A. homomorphus*, *A. japonicus*,

*A. lacticoffeatus*, *A. niger*, *A. piperis*, *A. sclerotioniger*, *A. tubingensis* and *A. vadensis*. Only four of them were recognized as OTA positives: *A. carbonarius*, *A. niger*, *A. lacticoffeatus* and *A. sclerotioniger*, the first two occurring on grapes and derivates and the last two isolated from coffee and reported as new species of section *Nigri*.

As some of the species remain difficult to differentiate using phenotypic methods, other techniques have been described to help in their differentiation: DNA sequences of the cytochrome b gene (Yokoyama *et al*. 2001), internal transcribed spacers (Parenicová *et al*., 2001), β-tubulin (De Vries *et al*., 2004) have been targeted using RFLP and other fingerprinting methods (Abarca *et al.,* 2004). Furthermore, several methods based on the PCR technique have been recently proposed to discriminate the main species included in the section *Nigri*: *A. carbonarius* and *A. japonicus* (Perrone *et al.,* 2004), *A. japonicus*, *A. heteromorphus*, *A. ellipticus* and *A. niger.*

### 1.3.12. OTA toxicity

OTA is nephrotoxic, mutagenic, carcinogenic, teratogenic and immunosuppressive in a variety of animal species. It is a mitochondrial poison causing mitochondrial damage, oxidative burst, lipid peroxidation and interferes with oxidative phosphorylation. In addition, OTA increases apoptosis in several cell types. Much has been written about the possible role of OTA in the etiology of these phenomena and detailed reviews on OTA toxicology have been published (Kuiper-Goodman and Scott, 1989; Dirheimer, 1996; Creppy, 1999; Petzinger and Ziegler, 2000; Mantle, 2002; O'Brien and Dietrich, 2005).

In 1993, the International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (Group 2B), based on sufficient evidence of carcinogenicity in experimental animal studies and inadequate evidence in humans (IARC, 1993). In the subsequent years since the IARC classification, studies have shown a tendency in the direction of group 2A toxicity (Kuiper-Goodman, 1996), as well as indicating the occurrence of synergistic multiple actions of diverse mycotoxins.

Despite of the second metabolites, black aspergilli are considered the third most common species associated with invasive pulmonary aspergillosis and it is also often a causative agent of aspergilloma (Sharma *et al*., 1985). Moreover, these species have been reported as responsible for a subcutaneous infection (Paldrok, 1965) and they were isolated of the tongue of a patient with respiratory illness (Williams, 1987). In immunocompromised patients, pulmonary infections or colorizations may occur, and are often characterized by

oxalosis, which is extensive production of microscopically conspicuous oxalic acid crystals in sputum (Kurrein *et al*., 1975). Oxalosis may also distinguish *A. niger* in some cases of invasive otomycosis in compromised patients (Landry and Parkins, 1993). Allergic responses are often common after inhalation of spores of black aspergilli and also of the enzymes they produced. For example, enzymes derived from *A. niger* present in baking additives have been identified as a causative allergen in baker's asthma (Quirce *et al.*, 2002).

## 1.4. Methods for measuring the growth of fungi-producing mycotoxins

Growth may be defined as the orderly increase in cell components leading to an increase in biomass (Prosser, 1995). The growth form of filamentous fungi is complex; extension of individual hyphae is localized at the tip, whereas biomass synthesis supporting that growth may take place throughout the mycelium. The growth of a fungus can be measured in various ways such as increase in colony diameter, increase in dry weight, rate of production of different type of metabolites, etc. The mechanisms involved in the control and regulation of mycelial growth are better studied on solid medium than in submerged cultures, as fungi are adapted to growth on solid substrates (Pitt and Hocking, 2009).

### 1.4.1. Direct methods

Some of the direct methods to determine fungal growth are stated afterwards:

#### 1.4.1.1. Growth of hyphal extension

Hyphal extension rate is measured microscopically on solid medium as an increase in length, e.g. measure of the increase in radii of circular colonies, daily or every other fixed period of time, after inoculating the mould in an agar plate and incubating it in the appropriate conditions. This is probably the most common technique for estimation of growth of filamentous fungi on solid media. A growth rate function can be derived by plotting colony diameter against time and measuring the slope of the straight part of the line (Pitt and Hocking, 2009). Analysis is now greatly facilitated by the increased availability of image analysis systems which enable automated measurement of hyphal lengths and subsequent kinetic analysis of data (Wiebe and Trinci, 1990; Gray and Morris, 1992).

#### 1.4.1.2. Measure of fungal biomass

Moulds are usually grown on the surface of a cellophane membrane, overlaying the agar, from which the biomass can be washer or otherwise removed for the determination of the dry weight. This measure is also possible in liquid cultures. In both solid and liquid media, separation of biomass from the growth medium is slow, tedious and requires relatively large amounts of biomass for accuracy. Turbidimetric techniques are less reliable due to the heterogeneous nature of liquid cultures of filamentous fungi (Pitt and Hocking, 2009).

### 1.4.2. Indirect methods

#### 1.4.2.1. Ergosterol

Fungal plasma membranes are similar to mammalian plasma membranes, differing in having the nonpolar sterol ergosterol, rather than cholesterol, as the principal sterol (Djajakirana *et al*, 1996; Miller and Young, 1997). The plasma membrane regulates the passage of materials into and out of the cell by being selectively permeable. Membrane sterols provide structure, modulation of membrane fluidity, and possibly control of some physiologic events. Fungal growth and biomass could therefore be estimated by measuring this specific component of fungi. Quantifying ergosterol production in foods has proved more difficult (Pitt and Hocking, 2009). Since now, ergosterol content has been mainly assayed in cereal samples. The determination of ergosterol is also valuable in correlating metabolites such as aflatoxins and OTA (Gourama and Bullerman, 1995; Saxena *et al.* 2001).

#### 1.4.2.2. Impedimentary and conductimetry

Metabolites produced by growth of microorganisms in liquid media alter the medium's impedance and conductance. The use of changes in these properties has been used to estimate fungal growth. A major problem of these techniques involves the selection of suitable media, but when the method is set up, this method results rapid and effective (Pitt and Hocking, 2009).

#### 1.4.2.3. Adenosine triphosphate (ATP)

Another measure of microbial biomass is the measure of the bioluminescence emitted by the molecules of fungal ATP (Bååth *et al.,* 1991). However, living plant cells contain also high levels of ATP and fungi are often very difficult to separate from food materials (Pitt and Hocking, 2009).

#### 1.4.2.4. Pectinesterase

The fundament of this technique is that gas liquid chromatography is used to determine the amount of methanol released from pectin by the fungal enzyme pectinesterase. This is considered a rapid method for detecting viable spores of spoilage fungi, but it needs some improvements before practical application. (Maldonado and Callieri, 1989; Maldonado and Strasser de Saad, 1998).

#### 1.4.2.5. Fungal volatiles

It consists in measuring the effects of fungi on foods, rather than fungi *per se*. Fungi produce chemical volatiles during growth and particular chemicals can be detected and therefore measure fungal growth in an indirect way (Pitt and Hocking, 2009). Adamek *et al.,* (1992) identified methylfuran, 2-methylpropanol and 3-methylbutanol as the most important metabolites from *Eurotium amstelodami, A. flavus, P. cyclopium* and *F. culmorum* growing on wheat. Volatile profiles have also been used to differentiate between toxigenic and non-toxigenic *Fusarium* strains (Keshri and Magan, 2000; Demyttenaere *et al*., 2004), to identify mycotoxins (aflatoxins, ochratoxin A and deoxynivalenol) in durum wheat (Tognon *et al.,* 2005) and to detect and quantify ochratoxin A and deoxynivalenol in barley (Osslon *et al*., 2002).

Several commercial gas sensor array instruments are now available on the market covering a variety of chemical sensor principles, system design and data analysis techniques. A serie of different detection principles can be used in chemical gas sensors: heat generation, conductivity, electrical polarization, electrochemical activity, optical properties, dielectric properties and magnetic properties. In principle, the results obtained from a gas-sensor array represent qualitative and quantitative information of the composition of the headspace gas mixture of a sample. The technique should therefore have a great potential in a number of applications related to food. Numerous **electronic nose** studies related to food already have been published, but the electronic nose technology applied on food must be regarded as being in its early stage. A goal of this technology is to explore the use of an electronic nose for rapid detection of food spoilers and pathogens via development of a standard curve of some potential volatile compounds that can be used to develop some specific aroma-labeled substrates (Schnürer *et al.,* 1999; Kuske *et al*., 2005; Paolesse *et al*., 2006; Berna, 2010; Campagnoli *et al.*, 2011).

#### 1.4.2.6. Immunological techniques

Fungal cell wall proteins produce antigens, which can be detected by immunological methods. Some antigens are derived from components common to a wide range of fungi, and hence are indicative of general fungal growth, while others are genus or even species specific (Pitt and Hocking, 2009). Recently, Wang *et al*. (2012) has developed an immunochip for quantifying the concentrations of six mycotoxins: aflatoxin B1, aflatoxin M1, deoxynivalenol, ochratoxin A, T-2 toxin, and zearalenone, which were added to drinking water.

#### 1.4.2.7. Molecular methods

They are based on nucleic acid sequences that are specific to the target fungi. The most known method is called **nucleic acid hybridization** and it involves the selection, cloning and chemical labelling of sequences specific to the target organism. These are then used as probes to detect RNA or DNA of the pathogen in extracts of the substrate. DNA may be specific at almost any taxonomic level (Schmidt-Heydt and Geisen, 2007). Wataru *et al*. (2005) improved the condition for hybridization to obtain high sensitivity in oligonucleotide microarray systems for the detection of mycotoxins producing fungi.

In some instances the detection and identification of the causal agent(s) may be secondary to other consideration. For example, it may be more important to quantify the amount of pathogen present rather than just determine its identity. Several approaches have been taken to develop diagnostic assays, and are divided into immunological and DNA-based systems, this last generally being **polymerase chain reaction** (PCR). In contrast to hybridization, PCR-based assays for detecting mycotoxins in fungi have been widespread in the last years. PCR is an extremely sensitive technique and involves the enzymatic amplification of a target DNA sequence by a thermo-stable DNA polymerase. Patiño *et al*. (2005) developed PCR method using designed specific primers on the basis of ITS (internal transcribed spacers of rDNA units) sequence to detect *A. carbonarius* and *A. ochraceus*.

## 1.5. Genomics and secondary metabolism in *Aspergillus*

### 1.5.1. Polyketide synthase (PKS gene)

Until now, the genomes of eight *Aspergillus* species have been published *A. clavatus, A. flavus, A. fumigatus, A. oryzae*, *A. nidulans*, *A. niger*, *A. terreus* (Brakhage and Schroeckh, 2011). Sequencing of several other species and further strains of the above named Aspergilli are under progress, e.g. of *Aspergillus parasiticus* (Univ. Oklahoma), *A. aculeatus* or *A. carbonarius* (DOE Joint Genome Institute). The availability of the genome sequences has led to rapid progress in identifying genes putatively responsible for secondary metabolite production. An exciting result of the subsequent genome annotations was the high number of secondary metabolite gene clusters in Aspergilli, typically between 30 and 40 per species. The vast majority of the genetically encoded metabolites, however, are unknown and the corresponding gene clusters were therefore referred to as ‘‘cryptic” or ‘‘orphan”.

Fungal polyketide synthases (PKSs) have been demonstrated to be involved in OTA biosynthesis in both *Penicillium* and *Aspergillus* species. Gallo *et al.* (2009) had reported the identification and characterization of part of a novel polyketide synthase gene, ACpks from *A. carbonarius*.

Polyketide synthases are large multi-enzyme protein complexes that contain a coordinated group of active sites or domains i.e. β-keto-acyl synthase (KS), acyl transferase (AT), ketoreductase (KR), dehydratase (DH), enoyle reductase (ER), methyl transferase (MT); acyle carrier protein (ACP) and thioesterase (TE). However AT, KS and ACP are the three principal domains while the remaing domains are optional (Fig. 1.18). The. KS domain is the most conserved domain in PKS gene (Simpson and Cox, 2008)

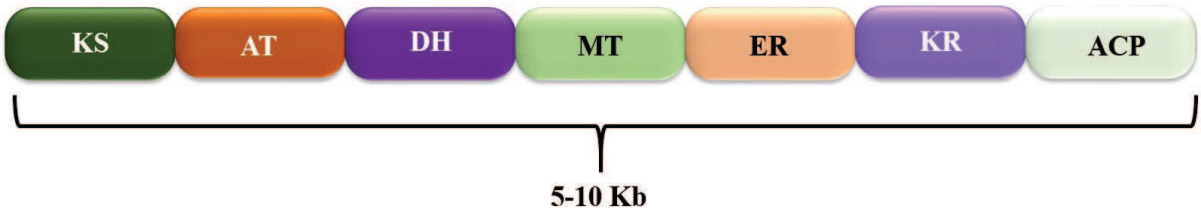



**Figure 1.18 : Structure of typical polyketide synthase (with different functional domains)**

Several specific primers were designed for *A. carbonarius* identification and quantification using real time PCR such as the primers designed by Atoui *et al.* (2007) (Ac12RL_OTAF/Ac12RL_OTAF) directed to the AT domain of PKS from *A. carbonarius*

delimiting a 141 bp fragment. And another primer set from the domain KS of PKS from *A. ochraceus* (Atoui *et al.,* 2006) which could detect the both ochratoxigenic *Aspergilli* (*A. carbonarius* and *A. ochraceus*).

Specific primers (Ac*pks*) for were designed by De Rossi *et al.* (2010) at PKS gene using quantitative-PCR which had early discriminated of *A. carbonarius* in wine and table grapes (especially the non-symptomatic berries) that could be used to predict the potential risk of contamination by potentially ochratoxigenic strains of *A. carbonarius*.

Selma *et al.* (2008a) had developed RTi-PCR assay as a promising tool in the prediction of potential ochratoxigenic risk, even in the case of *A. carbonarius* low-level infections, and suitable for a rapid, automated and high throughput analysis. Using the designed two specific primers (AcKS10L/AcKS10R) and a probe directed to the β-ketosynthase domain of a polyketide synthase from *A. carbonarius* delimiting a 161 bp fragment

Another primer set was designed by Spadaro *et al.* (2011) in the domain KS (AcPKS-F1/AcPKS-R1) amplified all the *A. carbonarius* strains tested specifically, and did not amplify any other species of *Aspergillus* or *Penicillium* normally found on grapes or involved in OTA biosynthesis.

Specific primer set (amplified 120 bp fragments) and a probe (directed to the PKS of *A. ochraceus*) detecting the ochratoxin A-producing strains of the *A. niger aggregate* were designed by Castellá and Cabañes (2011).

Degenerate primers (directed for the sequence an15g07920) targeted the PKS gene designed by Storari *et al.* (2010) indicated that the absence of this gene is apparently related to a failure to produce OTA. The work provides a step for the development of molecular methods to detect the producers in vineyards.

### 1.5.2. Internal transcripted spacer (ITS region)

The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis (Martin and Rygiewicz, 2005). The ITS region includes the ITS1 and ITS2 regions, separated by the 5.8S gene, and is situated between the 18S (SSU) and 28S (LSU) genes in the nrDNA repeat unit (Bellemain *et al.,* 2010) (Fig. 1.19)

Schoch *et al.* (2012) have found that the internal transcribed spacer (ITS) region, among studied six different regions of the ribosomal cistron (LSU, SSU, RPB1, ITS, RPB2

and MCM7) , has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation.. ITS will be formally proposed for adoption as the primary fungal barcode marker to the Consortium for the Barcode of Life, with the possibility that supplementary barcodes may be developed for particular narrowly circumscribed taxonomic groups

Accensi et al. (2001) had screening OTA-producing isolates belonging to the *A. niger aggregate* according to their ITS-5.8S rDNA RFLP patterns. The distribution of the isolates into the two species corresponding to *A. tubingensis* and *A. niger*. Patiño *et al*. (2005) had developed PCR assays using designed specific primers on the basis of ITS which were highly sensitive and specific and represent a good tool for an early detection of OTA-producing *Aspergillus* species (*A. carbonarius* and *A. ochraceus*). Other PCR assays using specific primers designed by González-Salgado *et al.* (2005) on the basis of ITS sequence, were highly sensitive and specific and represent a good tool for an early detection of OTA-producing *A. carbonarius* and *A. niger* species.

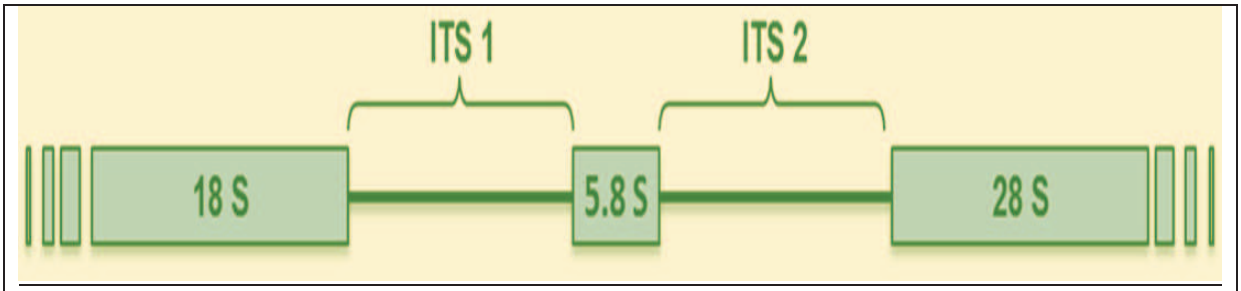



**Figure 1.19: Structure of the ITS regions (The two ITS regions (ITS 1 and ITS 2) are situated between the 18S and 28S rRNA genes and are separated by the 5.8S rRNA gene).** http://www.gatc-biotech.com/en/products/inview-applications/inview-microbiome-profiling.html

Martínez- and Ramón (2007) proposed The Restriction endonuclease digestion of the ITS assay (ITS-RFLP) as a rapid and easy method to identify black *Aspergillus* species isolated from grapes, especially in studies that involve a large number of isolates (A. niger, A. tubingensis, A. carbonarius and A. aculeatus species) using different restriction enzymes (HhaI, NlaIII and RsaI).

Nilsson *et al.* (2012) observed the probably, in nearly all cases, severely compromised ITS sequences can be detected manually using just a few simple guidelines (Table 1.11), without the assistance of technical software packages or access to significant computational power in order to identify new species of fungi.

**Table 11. Overview of the guidelines identifying new fungal species using the ITS online database** (Nilsson *et al.,* 2012).

| Target of guideline | Way of getting there |
|---|---|
| 1. Establish that the sequences come from the intended gene or marker | Do a multiple alignment of the sequences and verify that they all feature some suitable, conserved sub-region (here the 5.8S gene) |
| 2. Establish that all sequences are given in the correct (5' to 3') orientation | Examine the alignment for any sequences that do not align at all to the others; re-orient these; re-run the alignment step; and examine them again |
| 3. Establish that there are no (bad cases of ) chimeras in the dataset | Run the sequences through BLAST in INSD/UNITE and verify that the best match comprises more or less the full length of the query sequences |
| 4. Establish that there are no other major technical errors in the sequences | Examine the BLAST results carefully, particularly the graphical overview and the pairwise alignment, for anomalies |
| 5. Establish that any taxonomic annotations given to the sequences make sense | Examine the BLAST hit list to see that the species names produced make sense |

## 1.6. Analytical analysis of OTA

Hayat *et al*. (2012) designed a scheme for the recently developed innovative analytical methods used in the analysis and detection of OTA-producing fungi and OTA itself in food matrices which is shown in Fig. 1.20.

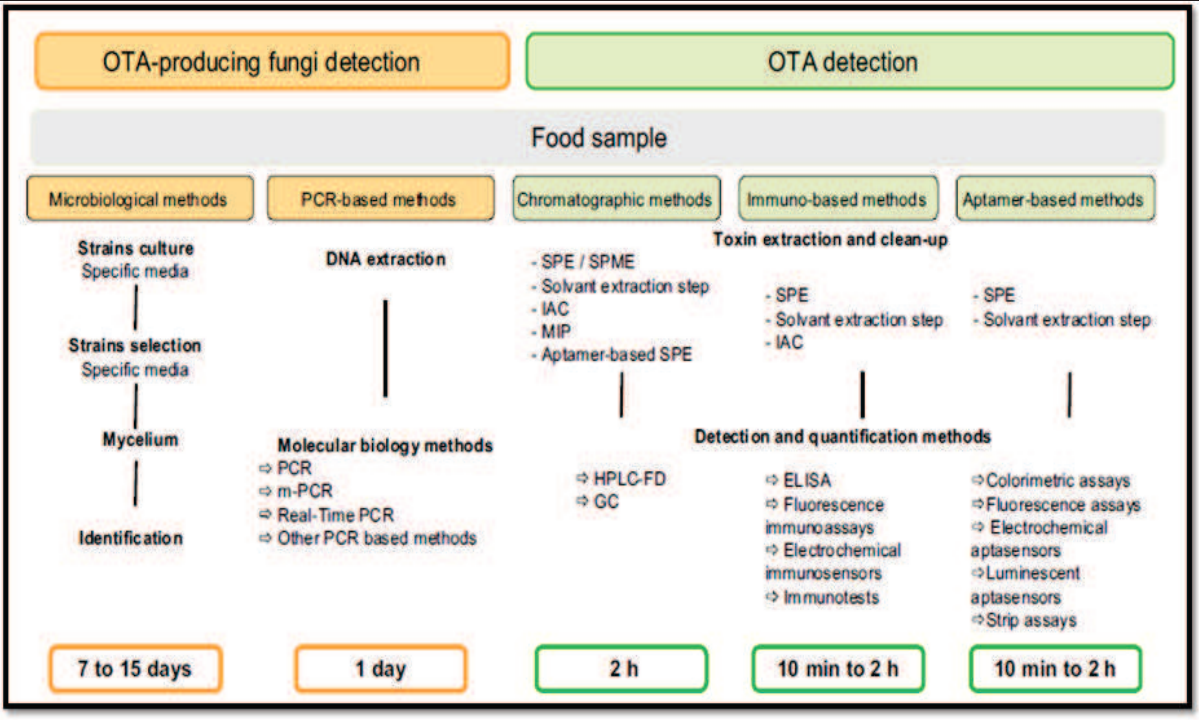



**Figure 1.20: Analytical methods for OTA-producing fungi and OTA detection** (Hayat *et al*., 2012)

### *1.6.1. Chromatographical methods*

The analysis of OTA in most foodstuffs is relatively straightforward and generally reliable results are obtained, as evidenced by the results from proficiency testing. Samples need to be acid- or alkaliextracted from the foodstuffs, with alkaline extraction from most matrices showing generally better recoveries (Senyuva *et al*., 2005).

#### 1.6.1.1. Thin layer chromatography (TLC)

TLC is featured in earlier AOAC methods (Nesheim *et al*., 1973), which use a silica gel adsorbent and an acidic solvent system. Since then, this method has been very commonly used in many laboratories around the world for identifying and quantifying the OTA in foodstuffs (Masoud and Kaltoft, 2006; Turner *et al*., 2009; Amézqueta *et al*., 2012). The TLC consists of an OTA visual detection by its greenish fluorescence under long wave ultraviolet light (at 336 nm), which changes to blue fluorescence after spraying the chromatographical

plate with methanolic sodium bicarbonate solution or exposing it to ammonia fumes; scanning densitometric analysis may also be carried out. Taniwaki *et al.* (2003) had successfully evaluated the OTA contamination of coffee cherries and beans in certain regions in Brazil using TLC and agar plug technique. Recently, a new TLC method has been developed using photosensor detection (De Rossi *et al.*, 2011). TLC is fast and appropriate in preliminary screenings as it provides semi-quantitative data (El-Khoury and Atoui, 2010; Amézqueta *et al*., 2012).

#### 1.6.1.2. Liquid chromatography (LC)

Conventionally liquid/liquid extraction and solid phase clean-up have been used prior to HPLC (High Pressure Liquid Chromatography) determination with fluorescence detection (El Adlouni *et al.*, 2006; Turner *et al*., 2009). However, over the past 10 years, most laboratories have tended to move towards using immuno-affinity column clean-up because they considered it is relatively simple to carry-out and provides sample extracts generally free of interferences (Meulenberg, 2012).

Good methods are available for dried fruit (Mac Donald *et al.,* 1999), beer (Legarda and Burdaspal, 1998), coffee (Leoni *et al.,* 2000; Stegen *et al.,* 1997), dried figs (Senyuva *et al*., 2005), milk (Valenta and Goll, 1996) and wine (Zimmerli and Dick, 1996; Dachoupachan *et al.,* 2009). Roasted coffee tends to be the most problematic of foodstuffs to analyze for OTA and in some methods an additional clean-up step is advocated prior to the affinity column stage (Entwistle *et al*., 2001).

### 1.6.2. Immunological methods

The use of ELISA (Enzyme linked immuno sorbent assay) for OTA analysis is considered as an important and very rapid method, because it is easy to use and due to the large number of samples that can be processed at the same time (up to 90 samples for each ELISA kit in 45 minutes), and because this method do not require any clean-up procedure (El-Khoury and Atoui, 2010). This technique is fast, cheap, it does not require high-skilled technician and is usually employed for screening studies. Compared to HPLC-FLD, it provides lower LODs (Díaz *et al.*, 2009) and often demands the false-positive confirmation (Amézqueta *et al*., 2012).

ELISA methods have been applied to quantify OTA in cereals, food, feed, animal tissues and serum (Dragacci and Frémy, 1998). However, an important consideration with

ELISA should be taken: the specificity of the antibody. Cross reactivity with related molecules can vary widely given over-estimated values.

Radio-immunoassay (RIA) for OTA has been applied to surveys of cereals, cereal products, feedstuffs, pig serum and tissues (Fukal and Marek, 1991). However, these methods do not appear to have been used recently (El-Khoury and Atoui, 2010). Sauceda-Friebea *et al*. (2011) develop a rapid, efficient immunoassay format detection chip by chemiluminescence detection for OTA determination in coffee.

Commercial immunoaffinity (IAC) columns are available for many target compounds, including OTA (Fig. 1.21), and used as a purification means before further analysis (HPLC, MS, ELISA) (Senyuva *et al*., 2005; Meulenberg, 2012). Roasted coffee tends to be the most problematic of foodstuffs to analyze for OTA and in some methods an additional clean-up step is advocated prior to the affinity column stage (Scott and Trucksess, 1997; Entwistle *et al*., 2001).

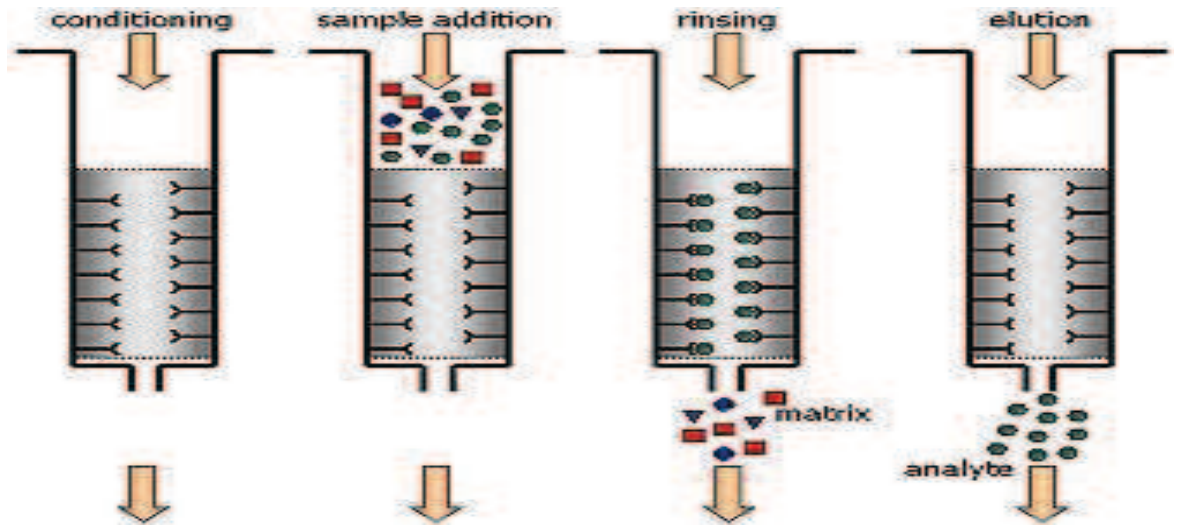



**Figure 1.21: Basic principle of immunoaffinity columns (IFC)**
http://services.leatherheadfood.com/eman/FactSheet.aspx?ID=61

### 1.6.3. Detection of ochratoxigenic species by PCR

Early and rapid detection of potential OTA producing fungi is important to reduce the negative impacts of OTA. Usual identification and quantification methods of food-borne fungi require multiple steps. Morphological and physiological tests were time-consuming and often, mycological expertise was necessary (Farber and Geisen, 2004; El-Khoury and Atoui, 2010). Within the last 10 years PCR systems have been developed for the detection and differentiation of major species and groups of mycotoxigenic fungi. One of the most

important factors in the setup of such method is the reliability of the primer set designed and the targeted DNA sequence of interest organism (El-Khoury and Atoui, 2010). Recently, various pairs of PCR primers were developed to set up novel diagnostic approaches for OTA producers in the *Aspergillus* and *Penicillium* genera.

#### 1.6.3.1. AFLP, RFLP, RAPD markers primers based

Many PCR techniques are used for assessing the genetic diversity of different species, which have largely been circumvented by the development of DNA markers such as restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNAs (RAPD) and amplified fragment length polymorphisms (AFLP). However, these molecular markers have technical differences in terms of cost, speed, amount of DNA needed, technical labor, degree of polymorphism, precision of genetic distance estimates and the statistical power of tests (Garcia *et al.,* 2002) (Fig. 1.22).

Pelegrinelli-Fungaro *et al.* (2004) described the development of a primer pair with high specificity to *A. carbonarius*, the main OTA producer in grapes. In their study, 29 strains of *A. carbonarius*, 16 *A. tubingensis* strains and 29 strains of *A. niger* were subjected to random amplified-polymorphic DNA (RAPD) analysis using 471 random oligonucleotides. One particular fragment of 809 bp differentiated *A. carbonarius* from the other species. SCAR primers designed from this fragment sequence enabling differentiation of *A. carbonarius* strains from other *Aspergillus* spp. by PCR.

Dachoupakan *et al.* (2009) discriminate the biodiversity of *A. carbonarius* and *A. niger* strains using eight different primers at the PKS regions which isolated from chemically and biologically treated grapevine against OTA producing fungi (especially the black aspergilli strains). However, primers could not distinguish between toxigenic and non-toxigenic isolates of *A. carbonarius*.

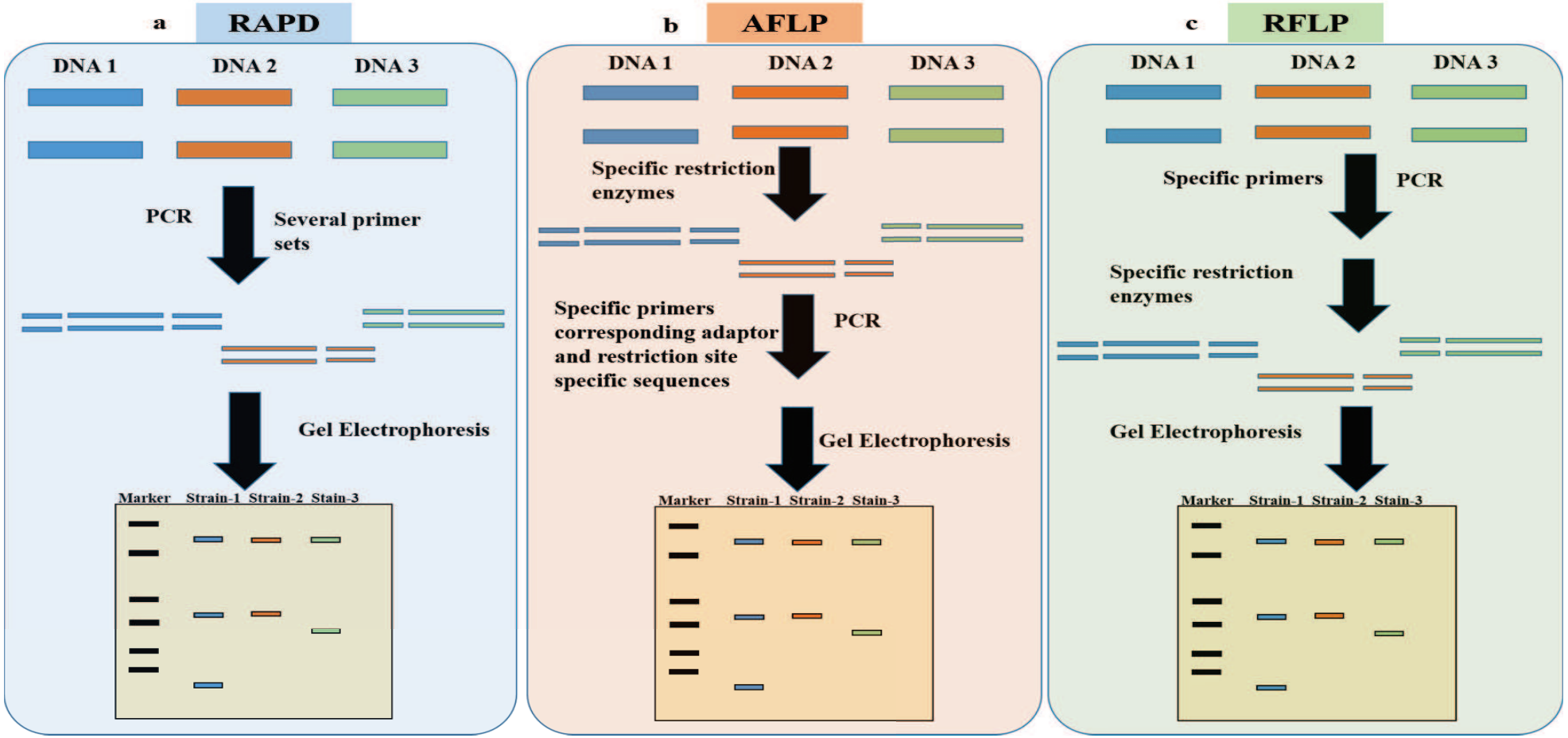



**Figure 1.22: Basic principles for discriminative PCR techniques: a: Random Amplified Polymorphic DNA (RAPD-PCR); b: Amplified Fragment Length Polymorphism PCR (AFLP-PCR); c: Restriction fragment length polymorphism (RFLP-PCR).**

Amplified fragment length polymorphism (AFLP) marker-based primers for *A. ochraceus* and *A. carbonarius* have also been applied (Vos *et* al., 1995; Schmidt *et al.,* 2003; 2004). AFLP revealed high similarity of banding patterns between the *A. ochraceus* strains (Schmidt *et al.,* 2003) and also between strains of *A. carbonarius* (Schmidt *et al.,* 2004). However, both OTA producers and non-producers were scattered randomly in both species analyzed. These findings were in strict contrast with results obtained by Castella *et al.* (2002). In their study, Castella *et al.* (2002) classified 66 strains of *P. verrucosum* in two distinct genotypes based on AFLP and RFLP analysis. The two RAPD as well as the two AFLP groups were completely coincidental. Strains in the two groups differed in their ability to produce OTA, with group I containing mainly high producing strains, and group II containing moderate to non-producing strains.

Perrone *et al. (2007)* reported the identification of four main populations separated molecularly using AFLP, RFLP and sequence analyses for the discrimination of the species diversity and the potential toxigenic strains in the Mediterranean basin. Hence, a large survey in 2001–2002 of black aspergilli occurring on grape from 107 vineyards in different European countries was performed within the EU project Wine-Ochra Risk (QLK1-CT-2001-01761) was conducted. These populations included *A. carbonarius*, *A. tubingensis*, *A. niger*, and a group of *Aspergillus* "uniseriate" isolates morphologically indistinguishable from *A. japonicus* and *A. aculeatus* but clearly separated by molecular techniques (Fig. 1.23). The genetic variability of these four populations observed by AFLP polymorphisms ranged from 15 to 35 % in *A. carbonarius*, *A. tubingensis* and the *Aspergillus* "uniseriate" group and 45–55 % in the *A. niger* group. The higher genetic diversity encountered in *A. niger* reflect the complexity of this taxon/group and the difficulties of identification at species level. The main OTA producer was *A. carbonarius* (95–100 % of strains), while the production of OTA was limited to a smaller proportion of strains in *A. niger* and *A. tubingensis* (10–15 % of the strains). No OTA production was observed in strains belonging to *Aspergillus* "uniseriate" group.

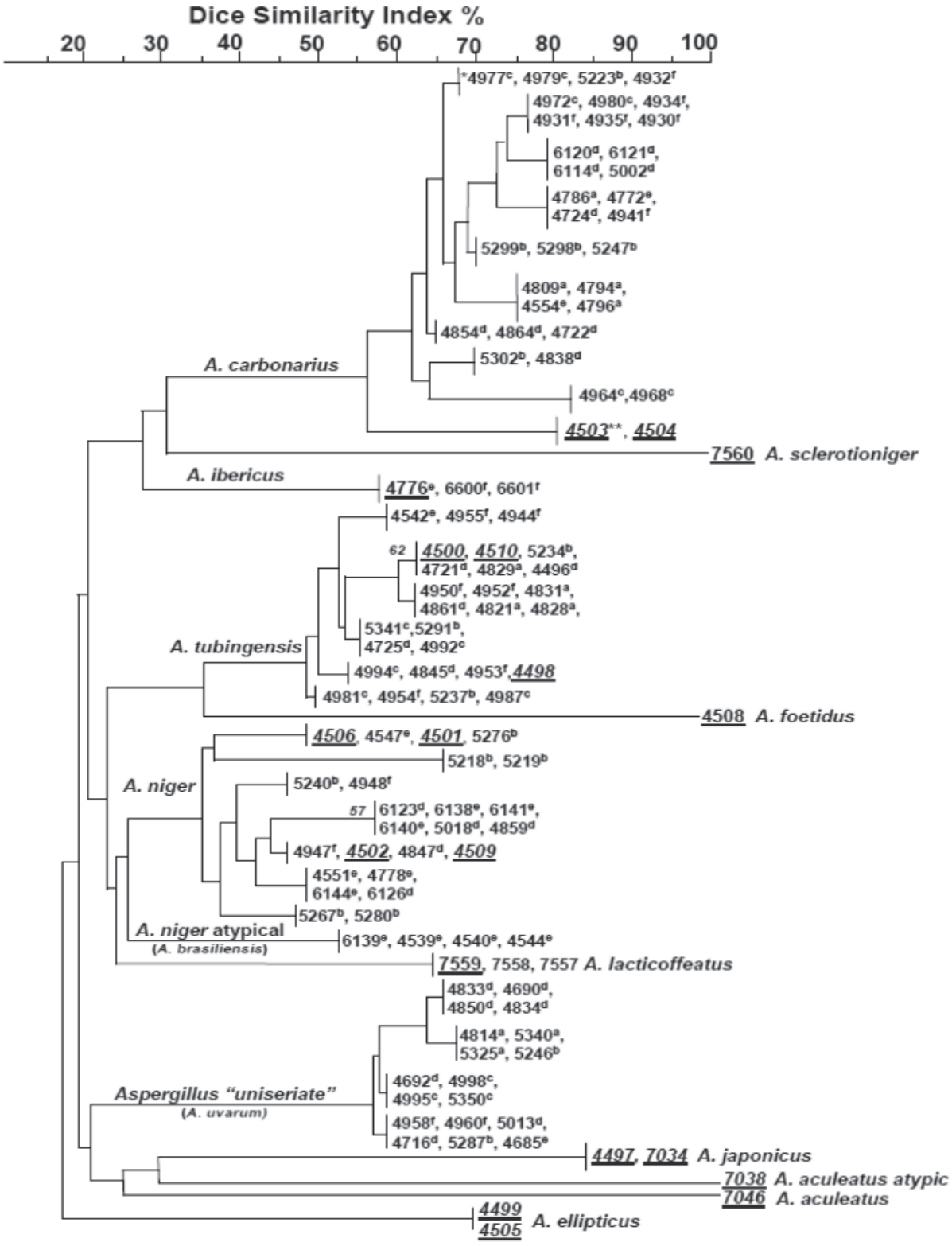



**Figure 1.23: AFLP dendrogram evidencing molecular biodiversity of representative black aspergilli isolated from grape in Europe** (Perrone *et al.,* 2007)

### 1.6.3.2. Real time PCR (Q-PCR)

Real time PCR (qPCR) is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process. This technique became possible after introduction of an oligonucleotide probe, which was designed to hybridize within the target sequence. Cleavage of the probe [TaqMan® (Livak *et al.,* 1995)] or attached primer with fluorescent chemical [SYBR® Green I dye (González-Salgado *et al.,* 2009)] during PCR because of the 5' nuclease activity of Taq polymerase can be used to detect amplification of the target-specific product (Fig. 1.24).

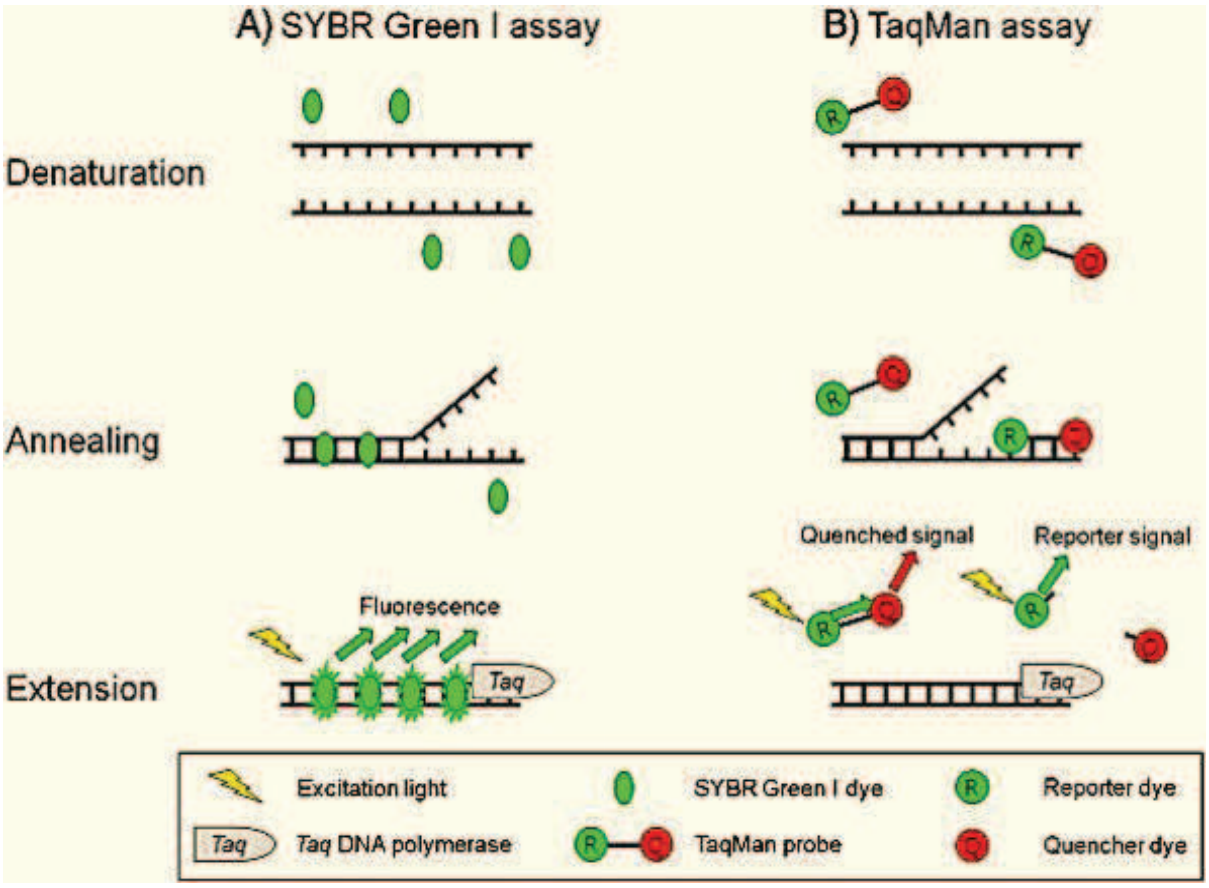



**Figure 1.24: Detection chemistries for qPCR: (A) SYBR Green I assay and (B) TaqMan assay** (Kim *et al.,* 2013)

Both systems have proven useful in monitoring and quantification of OTA fungal producer in many food commodities (Mulè *et al.,* 2006; Atoui *et al.,* 2007).

One of the major motivations for the development of PCR based detection systems in many publications is the prospect of using this kind of analysis to estimate OTA concentrations in sample material. One might therefore anticipate that assays based on OTA

biosynthetic genes might better fit that purpose as compared to systems based on genes unrelated to their biosynthesis. By using real-time PCR, a positive correlation between OTA content and DNA quantity has been indicated for *A. ochraceus* (Farber and Geisen, 2004) and more recently, in *A. carbonarius* (Mulè *et al.,* 2006; Atoui *et al.,* 2007) With regards to food safety, Atoui *et al.* (2007) established, according to their correlation, that *A. carbonarius* DNA content has to be lower than 10 ng DNA/g grape berry to fulfill the maximum.

Within the last years, PCR-based methods have been set up for the detection, differentiation and the identification of OTA-producing fungi. Hayat *et al.* (2012) described a brief description of those methods reported to analyze the OTA-producing fungi in food samples is given below and summarized in Tab. 1.12.

With the available systems of PCR-based detection and quantification of OTA described above, the choice of the best method depends on the goal of the study to be conducted. For example, AFLP and RAPD could be the best choice when the purpose of the study is (1) to show the polymorphism of some isolated strains belonging to the same species or (2) to discriminate between relevant OTA producer species. Concerning the application of the described molecular techniques in food, AFLP and RAPD show limitations such as fungal isolation and preparation of DNA of very high quality. For this reason Real time PCR technology provides an insight into the mycotoxigenic status of food sample as well as it has the power to estimate its mycotoxin content (El-Khoury and Atoui, 2010).

#### 1.6.3.3. PCR-DGGE

Double stranded DNA fragments of same length but different base composition can be separated in a polyacrylamide gel, which has a linear gradient of denaturing chemicals: urea and formamide (Denaturing gradient gel electrophoresis, DGGE) or temperature (Temperature gradient gel electrophoresis, TGGE). The DNA fragments migrate in the gel until they are partially denatured. The separation of DNA fragments is enhanced by using a GC-clamp (a long sequence rich in guanine (G) and cytosine (C)), which is incorporated to one of the primers. The denaturation of the GC-rich sequence is much harder than that of the rest of the sequence and therefore the DNA fragments are denatured only partially in the gel. The difference in the denaturation tendency of GC- rich versus AT-rich regions is caused by the different amount of hydrogen bonds holding the bases together (Fig. 1.25).

The (PCR-DGGE) fingerprinting was recently introduced into food microbiology. Applications of PCR-DGGE in several fields of food microbiology: the identification of

microorganisms isolated from food, the evaluation of microbial diversity during food fermentation, and microbiological and commercial food quality assessment. (Ercolini, 2004)

Laforgue *et al*. (2009) report that PCR-DGGE represents a useful tool to compare environmental samples for the study of the dynamics of grape fungal communities (which involved in wine defects), but co-migrations represent a limit in its use to describe the species present.

**Table 1.12: PCR, m-PCR and qPCR methods for detection of OTA-producing species in food matrices.** (Hayat *et al.,* 2012)

| Producer fungal species | Gene target | PCR-based method | Food sample |
|---|---|---|---|
| ***A. carbonarius*** | pks gene | PCR | Grapes |
| | pks gene: KS domain | PCR | Vineyards |
| | pks gene and ITS | m-PCR | Wines |
| | pks gene: AT domain | qPCR | Grapes |
| | pks gene: KS domains | qPCR | Wine grapes |
| | pks gene: KS and AT domains | qPCR | Fruits, wine |
| | ITS region of the rDNA | PCR | Wine grapes |
| | | PCR | Chilli, paprika |
| | | m-PCR | Coffee beans |
| | | qPCR | Grapes |
| | Calmodulin gene | PCR | Grapes |
| | | qPCR | Grape berries |
| ***A. niger*** | pks gene: AT domain | PCR | Vineyards |
| | pks gene | qPCR | Wines, corn, soya |
| | ITS region of the rDNA | PCR | Chilli, paprika, Wine grapes Grapes, wheat |
| | | m-PCR | Coffee beans |
| ***A. ochraceus*** | pks gene | PCR | Foodstuffs |
| | ITS region of the rDNA | PCR | Coffee beans, grapes, chilli, paprika |
| | | m-PCR | Coffee beans |
| | | qPCR | Coffee beans, grapes |
| ***A. steynii*** | ITS region of the rDNA | PCR | Chilli, paprika |
| | | PCR | Grapes, coffee |
| ***A. westerdijkiae*** | ITS region of the rDNA | PCR & qPCR | Coffee beans, grapes, chilli, paprika |
| ***P. nordicum & P. veruscosum*** | pks gene | PCR | Cured meats |
| | | qPCR | Wheat |

(**AT:** Acyltransferase, **KS:** Ketosynthase, **ITS**: Intergenic Transcribed Spacer, **m-PCR**: multiplex PCR, **q-PCR**: real time PCR)

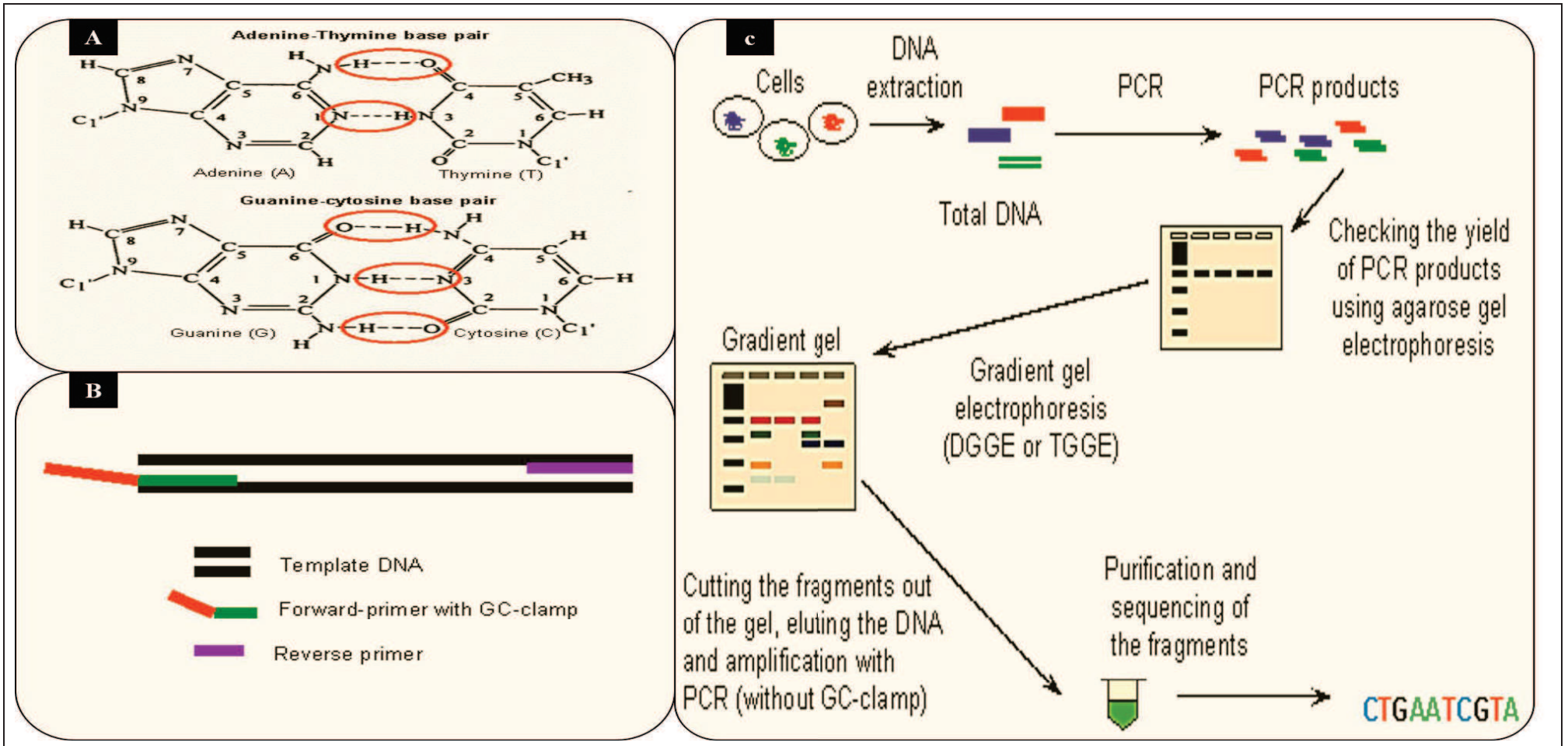



**Figure 1.25: PCR-DGGE principle; A: Adenine-Thymine and Guanine-Cytosine base pairs. Hydrogen bonds are shown with red ovals, B: GC-clamp on the forward primer, C: Denaturing gradient electrophoresis (DGGE) or temperature gradient electrophoresis (TGGE) of PCR amplified DNA.** http://wiki.biomine.skelleftea.se/biomine/molecular/index_11.htm

Prakitchaiwattana *et* al. (2004) reported that PCR-DGGE was less sensitive than culture on MEA for determining the yeast ecology of grapes and could not reliably detect species present at populations less than $10^4$ cfu/g. However, this method detected a greater diversity of species than agar plating.

## 1.7. Control methods for OTA contamination in grapes and its products

Varga and Kozakiewicz (2006).demonstrated the ochratoxin contamination of grapes and its products by black aspergilli. OTA contents of wine can reach high levels in some parts of Europe including Southern Italy, Greece, some parts of Spain and France. Attempts to reduce fungal colonization and OTA content of grapes include agronomic practices and biological and chemical treatments met with varying degrees of success, and the data obtained are sometimes controversial. Further studies are necessary to find suitable practices for lowering fungal contamination and OTA levels in grapes and grape derived products.

Recently, several scenari were created to avoid the grapes and its products contamination by OTA. Tirado *et al*. (2010) conclude that there is a need for intersectoral and international cooperation to better understand the changing food safety situation and in developing and implementing adaptation strategies to address emerging risks associated with climate change. Amézqueta *et al.* (2012) summarized certain efforts have to be made in order to avoid OTA contamination in the most susceptible products, by an efficient pre- and post-harvest management that prevents mold growth and OTA production. In cereals, post-harvest measures are crucial, whereas in grapes and coffee emphasis has to be put in both pre- and post-harvest strategies. In general, unpacked products must be maintained at 5-10 °C and packed products at 15-20 °C and water activity must be under 0.75-0.80.

Visconti *et al.*, (2008) have listed the main critical points suggested to prevent and reduce the OTA contamination in grapes and wine (Tab. 1.13).

Good agricultural practices (GAPs)

Murphy *et al.* (2006) have summarized the first line of defense against the introduction of mycotoxins that is at the farm level and starts with implementation of good agricultural practices to prevent infection. (Listed below):

a- Preventive strategies should be implemented from pre- through postharvest. Preharvest strategies include

- Maintenance of proper planting/growing conditions (i.e. soil testing, field conditioning, crop rotation, irrigation),

- Antifungal chemical treatments (for example, proprionic and acetic acids),

- Adequate insect and weed prevention.

b- Harvesting strategies include:

- Use of functional harvesting equipment,

- Clean and dry collection/transportation equipment,

- Appropriate harvesting conditions (low moisture and full maturity).

- Postharvest measures include use of drying as dictated by moisture content of the harvested grain, appropriate storage conditions, and use of transport vehicles that are dry and free of visible fungal growth

The most promised practices reducing and avoiding the incidence of OTA producing fungi and their OTA production ability will be described below:

### 1.7.1. Detoxification

#### 1.7.1.1. Biological method

Numerous microorganisms as bacterial, yeast and filamentous fungal species in nature are able to degrade and detoxify OTA. Notably, some of them have very strong degradation ability and could be a promising strategy to reduce the contamination of OTA in food and feed (Wu *et al.,* 2011).

Several bacteria species with OTA degradation activity have been isolated in the past; *Phenylobaterium immobile* and *Acinetobacter calcoaceticus* (Heidler and Schatzmayr, 2003). Other two bacterial species (related to *Clostridium sporogenes* and *Lactobacillus vitulinus*) were isolated from rumen fluid which were able to cleave OTA into the non-toxic metabolite ochratoxin α and theamino acid phenylalanine (Fig. 1.26).

Schatzmayr *et al.* (2003) report *Trichosporon sp. nov* (yeast strain) which can be fermented and stabilized. In a feeding trial with broilers lyophilized *Trichosporon*-cells could compensate performance losses caused by OTA. Schatzmayr *et al.* (2006) show that microbial detoxification by using stabilized microorganisms is a method to counteract these mycotoxins in a very specific and gentle way during the digestion of feed. For deactivation of trichothecenes a bacterial strain (*Eubacterium* BBSH 797) gave promising results; in the case

of ochratoxin A the yeast strain *T. mycotoxinivorans* (MTV-115) has the best properties to be used as detoxifying agent.

Abrunhosa *et al.* (2010) have described the yeast *Saccharomyces cerevisiae* as OTA biodegradation agents, since most of the effects detected and reported are from wall adsorption mechanisms.

**Table 1.13. Main critical control points plus suggested preventive and corrective measures to reduce ochratoxin A contamination in grapes and wine** (Visconti *et al.*, 2008).

| Risk factors | Preventive and corrective measures |
|---|---|
| **Field and pre-harvest** | |
| - Mediterranean basin, closeness to the sea<br>- High temperature and relative humidity from veraison to harvest<br>- Rainfall during ripening period (berry splitting)<br>- Berry damage (high risk with grape berry moth infestation)<br>- Grape training system susceptibility (high risk with espalier)<br>- High nitrogen fertilization, frequent tillage<br>- Grape variety susceptibility | -Monitoring climatic conditions from veraison to harvest<br>-Monitoring with trap system the *Lobesia botrana* pressure in the vineyards<br>-Monitoring black aspergilli rot berries from veraison to ripening<br>-Avoid excess of vigor and vegetation favoring aeration of bunches<br>-Avoid tillage from veraison to harvest<br>-Combined fungicide/insecticide treatments (1 or 2) when favorable climatic conditions occur |
| **Harvest–wine making** | |
| - Mechanical harvest without selection of bunches<br>- High incidence of rot bunches<br>- Long grape storage after harvesting (48 h) | -Anticipate harvest time in high OTA risk areas when favoring conditions occur<br>-Segregate rot bunches at harvesting<br>-Minimize storage time before processing<br>-Control OTA contamination in must<br>-Use carbon preparations to reduce OTA contamination during fermentation |

Abrunhosa *et al*. (2010) have listed the enzymes produced by certain microorganisms and affected the OTA degradation in food materials (Tab. 1.14)

### 1.7.1.2. Physical methods

Physical methods consist of segregation, sorting, cleaning, peeling and shelling processes that aim to remove the most contaminated fractions of the commodities. They also may involve the utilization of sorbents as nutritional additives that absorb OTA hence reducing bioavailability (Abrunhosa *et al.,* 2010).

### 1.7.1.3. Chemical methods

Chemical methods consist of the utilization of compounds to destroy OTA: some processes use ammonium (ammoniation), alkaline hydrolysis (nixtamalization), bisulphites and ozone (ozonation). These are reported generally as effective in the elimination of OTA and other mycotoxins (Karlovsky, 1999; Abrunhosa *et al.,* 2010)

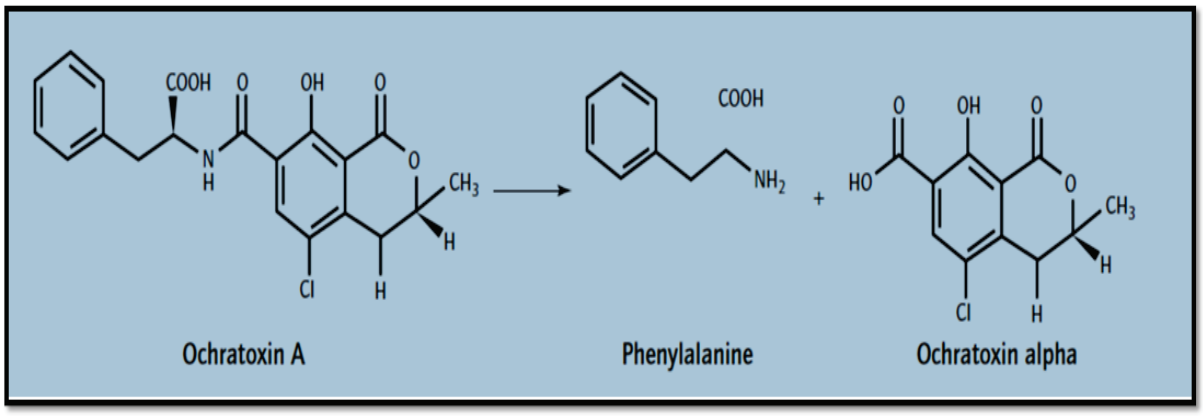



**Figure 1.26: Detoxification of ochratoxin A by cleavage of a phenylalanine moiety** (Heidler and Schatzmayr, 2003)

Bejaoui *et al.* (2006) have studied 40 isolates representing the black aspergilli species *Aspergillus carbonarius*, *A. niger aggregate* and *A. japonicus*, isolated on French grapes, for their OTA degradation capacities in CZAPEK yeast extract broth (CYB) and in a synthetic grape juice medium (SGM) contaminated with OTA at 2 mg/L (5 µM). It was clearly observed that in both media these fungi had the ability to degrade OTA to OTα. However, there were differences between the media used and species tested during OTA degradation. Despite a better growth on SGM, specific OTA degradation was higher on CYB for most of the isolates. Kinetic studies carried out on SGM with three black *Aspergillus* isolates all showed different OTA degradation rates. After 9 days of incubation, OTα had decreased, whereas an unknown compound appeared. *A. niger* could be the first interesting species for OTA detoxification processes, followed by *A. japonicus*.

**Table 1.14: Reported pure enzymes and enzyme formulations that hydrolyze OTA.** (Abrunhosa et al., 2010) (- data not available)

| Commercial name | Origin | Main activity | Supplier |
|---|---|---|---|
| Carboxypeptidase A | *Bovinus bovis* | exopeptidase | Boehringer |
| Carboxypeptidase Y | *Saccharomyces cerevisiae* | exopeptidase | Sigma |
| Lipase | *Aspergillus niger* | Lipase | Amano Inc. |
| Enzyme preparations | - | Proteolysis | - |
| Protease A | *Aspergillus niger* | Acid protease | Amano Inc. |
| Prolyve PAC | *Aspergillus niger* | Acid protease | Lyven |
| Pancreatin 4XNF-P211P | Porcine pancreas | Amylase, lipase and protease | Biocatalysts |
| Crude extract | *Aspergillus niger* | OTA-hydrolase | - |

### 1.7.2. Chemical fungicides

Tjamos *et al*. (2004) have performed a vineyard survey on a Corinthian raisin cultivar carried out during 2002 in the Greek region of Peloponnesus demonstrated that application of the commercial product Switch (*i.e.* fludioxonil and cyprodinil) at 1-2 and 3-4 weeks before veraison and two weeks before harvesting significantly reduced the occurrence of OTA-producing *Aspergillus* spp. and controlled incidence of rot. On the contrary, vineyard applications with other fungicides such as carbendazim and cyprodinil (c.p. Chorus) were ineffective in controlling the fungus in the Corinthian raisin cultivar. The study demonstrated that fludioxonil was the active ingredient responsible for the effectiveness of the synthetic botryticide, Switch.

The effect of pre-harvest fungicide treatments (Chorus and Switch) was evaluated on *Aspergillus* infection and on the OTA content of dehydrating grapes (Valero *et al.*, 2007). The study showed that there were no differences between single or double applications of the two fungicides; both products remained active on grapes during the drying process, reducing fungal colonization and OTA synthesis. Also artificial inoculation of fungicide-treated grapes with *A. carbonarius* OTA-producing strain, OTA-negative *A. niger* aggregate, *Eurotium amstelodami* and *Penicillium janthinellum* in different combinations confirmed the effectiveness of both fungicides, but their efficacy probably depended on the mycobiotic composition of the grapes. *E. amstelodami* has been previously shown to favor OTA accumulation when interacting with *Aspergillus* spp. (Valero *et al*., 2007; Covarelli *et al*., 2012).

Other studies have been conducted to set up the suitable fungicide/pestcide controlling the incidence of OTA producing fungi and reducing the OTA contamination of grapes and its products. Some of these researches are illustrated in Tab. 1.15.

**Table 1.15: Pesticides used controlling the OTA-producing fungi in grapes and its products** (Ponsone *et al*., 2012).

| Pesticide | Active compounds | Efficacy | Reference |
|---|---|---|---|
| **Azoxystrobin** | Strobilurin derivative | Decrease the OTA contamination in wines | Lo Curto *et al*.(2004) |
| **Dinocap + sulfar** | dinitrophenyl derivative | | |
| **Carbendazim and Chorus** | cyprodinil | Inactive against sour rot | Tjamos *et al*.(2004) |
| **Switch,** | That contains cyprodinil (pyrimidine) and fludioxonil (pyrrolnitrin) | Active in reducing the OTA contamination | Chernin *et al*.(1996) |
| **Switch, Scala** | Containing pyrimidine fungicide pyrimethanil | Effective for lowering OTA content in wines | Molot and Solanet (2003) |
| **Mikal** | containing fosetyl-Al and the dicarboximide folpel | | |

In general, fungicides that contained copper or strobilurins reduced both, growth and OTA production, contrary to sulfur fungicides. Among the fungicides that inhibited *A. carbonarius* growth in synthetic medium, cyprodinil seemed to be the most effective active ingredient to stop fungal growth when reduced doses were tested. When these fungicides were tested on grapes, the effect was similar to that observed on synthetic medium (Bellí *et al.,* 2006b). It is important to remark that fungicides must be applied with care since some of them, such as carbendazim, have been found to reduce fungal flora but stimulate OTA production (Lo Curto *et al*., 2004).

## 1.7.3. Biological control

Ponsone *et al.* (2011) have illustrated three yeast strains among the twenty-eight evaluated yeast isolated with the most effective isolates of *Kluyveromyces thermotolerans* which were able to control both growth and ochratoxin A production. This could be due to

ochratoxin A degradation by the toxigenic strains or by the antagonistic yeasts, using the toxin as an alternative carbon source. The efficacy of *K. thermotolerans* RCKT4 and RCKT5 to diminish OTA accumulation in detached berries is promising but it is necessary to test these isolates under greenhouse and field conditions. It has been showed that the efficacy of biological control agents can be variable, depending upon the pathogen's inoculum level and local environmental conditions

Sharma *et al*. (2009) reported only a few of the microbial antagonists reported to control postharvest diseases of fruits and vegetables under laboratory conditions were commercialized (listed in tab. 1.16). Those have biological activity against grapevine postharvest-pathogens)

**Table 1.16: Biocontrol products developed for control of postharvest diseases of grapevine** (modified Sharma *et al*., 2009)

| Product | Microbial agent | Target disease(s) | Manufacturer/distributor |
|---|---|---|---|
| **AQ-10 bio fungicide** | *Ampelomyces quisqualis* | Powdery mildew | Ecogen, Inc., USA |
| **Serenade** | *Bacillus subtilis* | Powdery mildew, late blight, brown rot | Agro Quess Inc., USA |

Yeasts are considered one of the most potent biocontrol agents due to their biology and non-toxic properties. The mechanism most probably involved in filamentous fungi biocontrol by yeast is competition. Competition among microorganisms for essential factors, such as nutrients and space, is expected to have a dramatic effect on the secondary metabolism of filamentous fungi. On the other hand, parasitism and production of fungal growth inhibiting compounds have been also described. Nowadays, several yeast species included in different genera are considered potential biocontrol agents towards ochratoxigenic *Aspergillus* (Ponsone *et al.*, 2012) (Tab. 1.17)

Bleve *et al.* (2006) have found 6 yeast isolates belonging to 5 species, namely 2 isolates of *Issatchenkia orientalis* and one each of *Metschnikowia pulcherrima*, *Kluyveromyces thermotolerans*, *Issatchenkia terricola* and *Candida incommunis*, among total of 144 yeast tested isolates with the ability to inhibit infection by ochratoxigenic moulds (*Aspergillus carbonarius and A. niger*). The best antagonistic activity was shown by the two *I. orientalis* isolates. Results suggest that antagonist yeasts with the potential to control *A.*

*carbonarius* and *A. niger* on grape can be found among the microflora associated with the berries.

Results of field trials conducted by Cozzi *et al.* (2013) in Italy evaluating bio insecticide control strategy (using a commercial *Bacillus thuringiensis* formulate and an experimental *Beauveria bassiana* formulate) against *Lobesia botrana, grape moth,* showed that *B. bassiana* is a valid bioinsecticide against *L. botrana* and that grape moth biocontrol is a strategy to reduce OTA contamination in vineyard in seasons with heavy natural infestation.

**Table 1.17: Some of the yeast strains with the antifungal activity against the OTA-producing fungi**

| Biological control agent | Efficacy | Reference |
|---|---|---|
| ***Aureobasidium pullulans*** | Effective in reducing sour rot infection, A. carbonarius presence on berries at harvest and OTA contamination in must | Dimakopoulou *et al.* (2008) |
| **two epiphytic strains of *Lanchancea thermotolerans*** | Control the growth and OTA accumulation of ochratoxigenic fungi both "*in vitro*" and "*in situ*" | Ponsone *et al.* (2011) |
| ***Issatchenkia orientalis*** | Have a strong antagonistic action against ochratoxigenic species | Bleve *et al.* (2006) |
| ***Metschnikowia pulcherrima* and *Candida incommunis*** | Significantly reduced *A. niger* and *A. carbonarius* growth both *in vitro* and *in situ* | |
| ***Candida guillermondii* and *Acremonium cephalosporium*** | Efficient in reducing decay caused by *Botrytis*, *Rhizopus* and *Aspergillus*. | Ponsone *et al.* (2012) |

## 1.7.3. Induced control strategies

### 1.7.3.1. Induced stibenes

Stilbenes can be constitutive compounds in the woody part of the plant or induced metabolites in soft tissues such as fruits and leaves. These phytochemical compounds, which

act as part of the plant's defensive arsenal (phytoalexins), can be induced by biotic and abiotic elicitors (Bavaresco and Fregoni, 2001). Previous studies have described the induction of resveratrol-related compounds in grapes (Goyal *et al.*, 2012) (Tab. 1.18) as a response to infection of *Botrytis cinerea* (Jeandet *et al*., 1995), *Rhizopus stolonifer* (Sarig *et al*., 1997), and ochratoxigenic aspergilli (Bavaresco *et al*., 2003) including *A. carbonarius* (Bavaresco *et al.,* 2008). Stilbenes can also be induced by abiotic elicitors such as ultraviolet-C (UV-C) illumination, ozone, methyl jasmonate as well as other chemicals (Selma *et al*., 2008b). In particular, postharvest illumination with UV-C light has been proposed as a valuable method to increase the *trans*-resveratrol content of table grapes, wine grapes, and red wines by induction of stilbenoid biosynthesis. Moreover, UV-C illumination has been extensively used in a broad range of antimicrobial applications, including surface disinfection of vegetable commodities (Selma *et al*., 2008b).

Selma *et al*. (2008b) have investigated the efficacy of ultraviolet-C (254 nm) and induced stilbenes to inhibit *Aspergillus carbonarius* and *A. tubingensis* and control ochratoxin A production in grapes. In addition, the stilbene synthesis as a response to UV-C treatment and to infection of ochratoxigenic Aspergillus was compared. UV-C elicited the biosynthesis of *trans*-resveratrol, while microbial infection and tissue damage triggered the biosynthesis of *trans*-piceid. *Trans-Resveratrol* was not synthesized as a consequence of ochratoxigenic *Aspergillus* contamination. However, when *trans*-resveratrol was synthesized by UV-C, it contributed to inhibiting the development of ochratoxin A producing aspergilli. Furthermore, UV-C treatment also contributed to decrease ochratoxin A production by ochratoxigenic aspergilli. Therefore, UV-C is a promising emerging technology either for reducing the potential ochratoxigenic risk in grapes, which is of particular interest to the wine industry, and also for increasing *trans*-resveratrol content of grapes, which would provide an added value to the wine.

**Table 1.18: Effect of elicitors on the stilbenes content of in-vitro grown plants** (Goyal *et al*., 2012)

| Plant | Culture types | Elicitors used | Products |
|---|---|---|---|
| ***Vitis rupestris* and *Vitis vinifera* cvs** | In vitro plants | UV irradiation, aluminum chloride, and *Botrytis cinerea* | Resveratrol |
| ***Vitis* spp.** | Non-embryogenic callus | UV-C irradiation | Resveratrols and piceids |
| ***Vitis vinifera*** | Cell cultures | Methyl jasmonate | trans-Resveratrol and piceids |
| | | Cyclodextrins | Resveratrol |
| | | Chitosan | |
| | | Methyljasmonate, cyclodextrins | |
| | | 9 Dimethyl β-cyclodextrin | |
| | | Salicylic acid, Na-orthovanadate, jasmonates, chitosan and the monomers d-glucosamine and N-acetyl-d-glucosamine, ampicillin and rifampicin | |
| | | UV irradiation | trans-Resveratrol |
| ***Vitis vinifera* cv. Barbera** | Cell culture | Jasmonic acid, methyljasmonate and Na-orthovanadate | trans- and cis-resveratrol; trans-resveratrol and piceids |
| ***Vitis vinifera* cvs Michele Palieri and Red Globe** | Cell cultures | Methyl jasmonate | trans-Piceid and β-viniferin |

#### 1.7.3.2. Elicitors (plant extracts)

Induced resistance (IR) is a new strategy for managing plant diseases. It is an alternative procedure to protect plants against disease by activating plants' own defense mechanisms using specific biotic or abiotic elicitors (Walters *et al.,* 2005; Kumar and Purohi, 2012). The basic tenet of IR lies in enhancing resistance in response to an extrinsic stimulus without altering the genome. The protection is based on the stimulation of defense mechanisms by metabolic changes that enable the plants to defend themselves more efficiently. A number of publications with different host-parasite systems have proven the efficacy of IR against fungi, bacteria and viruses through the manipulation of the host plant's physical and biochemical properties (Kumar and Purohi, 2012; Thakur and Sohal, 2013). The elicitors secreted through bio-agents are non-specific and therefore, can be effective against a

wide range of pathogens. These elicitors work by bringing about certain metabolic changes in plants to fight against infections. Thakur and Sohal (2013) have defined the elicitor IR mode of action against certain plant pathogens. Eventually, the induction of defense responses may lead to enhanced resistance. This broader definition of elicitors includes both substances of pathogen origin (exogenous elicitors) and compounds released from plants by the action of the pathogen (endogenous elicitors). Elicitors are classified as physical or chemical, biotic or abiotic, and complex or defined depending on their origin and molecular structure (Fig. 1.27. A).

Recent studies have indicated remarkable similarities between the defense mechanisms triggered by general elicitors and the innate immunity of animals, and it is tempting to speculate that the recognition of general elicitors subsequently leads to plant innate immunity. Elicitors act as signal compounds at low concentrations, providing information for the plant to trigger defense, distinguishing elicitors from toxins, which may act only at higher concentrations and/or affect the plant detrimentally without active plant metabolism. Elicitor signal transduction mechanism which activates plant primary immune response is shown in Fig.1.27.B (Thakur and Sohal, 2013).

Several SIR of various origins have recently been discovered and some of them have been commercialized (Tab.1.19) with wide range of crops and fruits. Among these SIR, the regular use of Stifénia® product in vineyards could reduce the incidence of Aspergillus spp. producing OTA (Benhamou and Rey, 2012) while its mode of action is unknown till now.

Elicitors were primary designed to improve plant resistance against pathogens. These compounds do not kill pathogens but trigger plant defense mechanisms, among them, the production of increased levels of phenolic compounds. The effect of the application of different elicitors to plants also proved a useful technique for improving their phenolic content (Ruiz-García and Gómez-Plaza, 2013). A summary of the effects of different elicitors on grapevine phenolic composition and phenol-related enzymes is shown in Tab. 1.20.

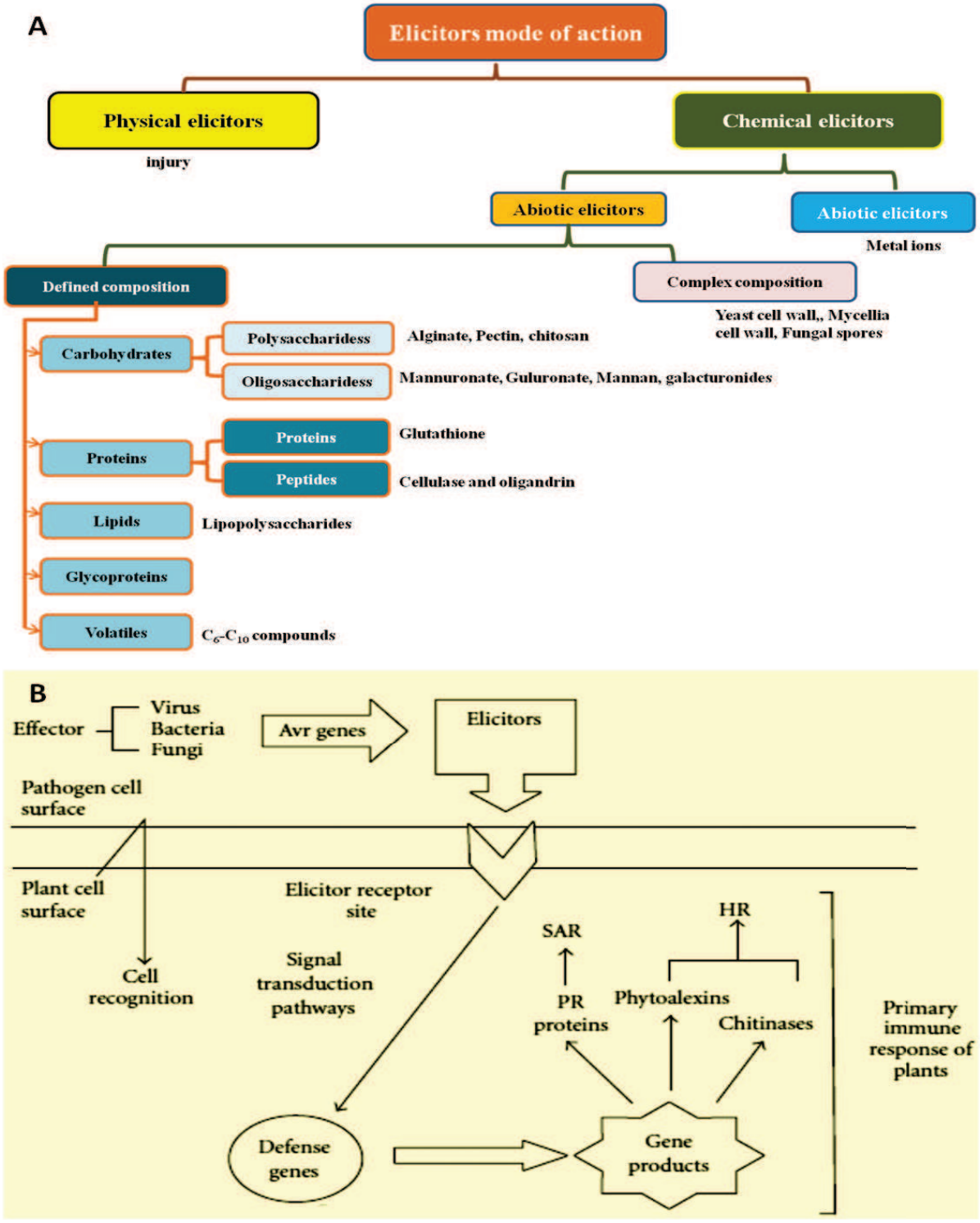



**Figure 1.27: Elicitors, A: mode of actions; B: Primary immune response of plant in plant-pathogen interaction** (Thakur and Sohal, 2013 (*in press*)).

**SAR:** Systemic Acquired Resistance, **HR:** Hypersensitive Response; **PR:** Related Proteins, **Avr genes:** Avirulence gene.

**Table 1.19: List of SIR currently on the market** (modified from Benhamou and Rey, 2012).

| Product | Composition | Plant | Disease | Company |
|---|---|---|---|---|
| **Iodus 40® and Iodus ™ 2cerials** | Laminarine = β-1,3- glucans extract of brown algae (Laminaria digitata) | cereals | Powdery mildew, septoria spot | Goëmar (France) |
| **Elexa ™** | Chitosan = N - glucosamine | Pearl millet | Sclerospora graminicola | Glycogenesys Inc. (U.S.) |
| **Messenger®** | Harpin = peptide produced by the bacterium Erwinia amylovora | Cotton, tomato, cucumber, pepper, tabac, rice and strawberry | Different pathogens | Eden Bioscience (U.S.) |
| **Stifénia®** | extracts of fenugreek seeds | grapevine | Powdery mildew | Occitan Manufacturing Company Technology (SOFT) (France) |
| | | peach | Taphrina deformans | |
| **Milsana®** | extracts Reynoutria | tomato | Powdrey mildew | KHH BioSci Inc. (U.S.) Dr. sacchalinensis Shaette (Germany) |
| **Sil-MATRIX™** | Potassium Silicate | Melon, cherry and strawberry | Fungicige, insectcide, arachnicide | PQ Corporation (EU) |
| **Neem oil (NeemAzal®, Soluneem® and many others)** | Azadirachtin extracted from the seeds of Azadirachta indica (Neem) | | Several defoliating lepidopteran larvae and against the pine weevil | Ultrateck (Canada) Lawn & Garden Products Inc. (USA) and several other companies in the world |
| **Cerebrosides** | Glycosphingolipids extracts the membrane of fungi | Pearl millet | Downy mildew | - |
| **Brassinostéroïdes** | Plant hormones type stéroïde | cabbage | Abiotic as toxic effect of cadmium | - |

The use of elicitors in crop protection and pest management is still in the very early stages of use as a new control method, and thus the current experiences come from experimental trials, and not yet from large scale agricultural use. At least the following

advantages of using elicitor treatments have been reported or can be expected: (1) reduced damage from insects, fungi, pests, and herbivores; (2) reduced environmental hazards as elicitors affect directly the crop plant, and their acute toxicity to other organisms is lower than that of pesticides; (3) as protective agrochemicals, elicitors can be applied with the current spraying technology; (4) elicitor treatments could be an alternative to genetically modified (GM) plants for better attraction of natural enemies of pest organisms on cultivated plants; (5) elicitor-treated plants bear lower ecological risks than GM plants (Goyal *et al.,* 2012; Thakur and Sohal, 2013).

**Table 1.20: Summary of the effects of the preharvest application of different elicitors on grapevine polyphenol content and activity of polyphenol-related enzymes** (Ruiz-García and Gómez-Plaza, 2013).

**BTH:** benzothiadiazole; **MeJ:** methyl jasmonate; **CHS:** chalcone synthase enzyme; **STS:** stilbene synthase enzyme; **UPGT:** UDP glucose: flavonoid-*O*-transferase (UPGT).

| Elicitor | Activated enzyme and/or increased compound | Reference |
|---|---|---|
| **BTH** | resveratrol, anthocyanins; proanthocyanidins; flavonols | **Iriti *et al.* (2004; 2005); Fumagalli *et al.* (2006)** |
| **MeJ** | CHS, STS, UPGT, stilbenes and anthocyanins | **Belhadj *et al.* (2008)** |
| | flavonoids | **Ruiz-Garcia *et al.* (2012)** |
| | resveratrol and viniferins | **Esna-Ashari and A. Pour (2011); Vezzulli *et al.* (2007)** |
| **Chitosan** | total phenols in grapes and wine | **Meng *et al.* (2012)** |

In cereals, grapes, cocoa and feed, OTA-producing species are well-known (Amézqueta *et al*., 2012). In grapes, *Aspergillus* section Nigri species strains are frequently isolated, being *A. carbonarius* ones the most efficient in producing the mycotoxin (Battilani *et al*., 2006; Amézqueta *et al*., 2012). And is the major implication of *A. carbonarius* in ochratoxin A contamination in the Languedoc region (French southern region) at harvest time (Sage *et al*., 2004).

OTA quantification is generally carried out by HPLC coupled to a fluorimeter detector, after an extraction of the toxin from culture medium (Amézqueta *et al*., 2012). To prevent OTA contamination in foodstuffs, recently several methods mostly based on PCR-based assays have been developed for identifying and quantifying OTA-producing fungi in food samples. PCR including its different formats remains the technique of choice, because its ability to detect even small amounts of fungal DNA in raw materials and processed foods (Hayat, *et al*, 2012).

Many efforts have to be made in order to avoid OTA contamination in the most susceptible products, by an efficient pre- and post-harvest management that prevents mold growth and OTA production. In cereals, post-harvest measures are crucial, whereas in grapes, coffee and coffee emphasis has to be put in both pre- and post-harvest strategies (Amézqueta *et al*., 2012). Several researchers have reported on the biocontrol activity of *Saccharomyces. cerevisiae* against different pathogens, such as *Botrytis cinerea* in grapes (Nally *et al*., 2012). However, thus far there are no reports about *S. cerevisiae* as biocontrol agent of filamentous fungi isolated from sour rot-damagedgrapes. Despite the high antifungal potential of *S. cerevisiae*, thereis presently no commercial biocontrol product containing this species (Nally *et al*., 2013).

In conclusion, an integrated strategy based on the combination of biological control agents with natural compounds or reduced dosage of fungicides appears to be one of the most reliable options for large-scale utilization of microbial antagonists in the control of ochratoxigenic fungi and reduction of the entry of OTA to the food chains (Lima *et al.,* 2008*;* Droby *et al.,* 2009*;* Ponsone *et al.,* 2012)

# Chapter 2

# Material and Methods

# 2. MATERIAL AND METHODS

## 2.1. Biological material

### 2.1.1. Vineyard and experiment

Six different treatments were applied on experimental viticulture orchard in IFV (*Institut Français de la vigne et du vin*), Narbonne (Languedoc-Roussillon region), France (Fig. 2.1), Vines were not treated nor contaminated with *Aspergillus carbonarius* [previously isolated from vines; OTA producing fungus (OTA-PF) (Sánchez-Hervás *et al.,* 2008)] for the first treatment (Control 1). And the other five treatments were contaminated with OTA-PF. One of them was not treated (Control 2) while the other four treatments were treated each by one of the treatments shown in Tab.1 (one was treated by Scala®; Stifénia® and the other by biological agents) as shown in table 2.1.


**Figure 2.1.: Location of sampling vineyard in IFV experimental vineyard.**

### 2.1.1. Sampling

Grapes were harvested in late September 2010 (Fig. 2.2). Grape bunches (5 kg/ treatments) were randomly collected for each treatment and placed in two previously sterilized bags, which were kept at about 4°C until analysis. Mycological analysis was immediately done and the remaining samples were kept frozen at -20°C.

**Table 2.1: The different treatments applied on the cultivar *Mourvèdre* at the experimental field of IFV, Narbonne, France; during the year 2010.**

| | Name | Contamination by OTA-PF | | Treatment | | |
|---|---|---|---|---|---|---|
| | | Case | Time | Type | Frequency | Time |
| 1 | **Nocont** | ---- | ---- | ---- | | ---- |
| 2 | **Cont** | Artificially contaminated by OTA-PF | Contaminated at the veraison stage | ---- | | ---- |
| 3 | **Scala** | Artificially contaminated by OTA-PF | Contaminated at the veraison stage | Chemical fungicide: Commercial name; SCALA®, active ingredient; Pyriméthani 400g/L | Twice | One at the green tip stage, and the other at leaves output stage |
| 4 | **Tricho.** | Artificially contaminated by OTA-PF | Contaminated at the veraison stage | Fungal bio-agent using the fungi *Trichoderma atroviride*. | Twice | One at the green tip stage, and the other at leaves output stage |
| 5 | **Saccharo.** | Artificially contaminated by OTA-PF | Contaminated at the veraison stage | Yeast bio-agent using the yeast *Saccharomyces cerevisiae*. | Once | At the leaves output stage |
| 6 | **Stifénia** | Artificially contaminated by OTA-PF | Contaminated at the veraison stage | Plant extracts FEN 560 (fenugreek seed powder) (Stifénia®). | Multiple | Starts at the green tip stage and followed with15 days intervals ones |

**OTA-PF; OTA producing fungi (*Aspergillus carbonarius*)**

**Figure 2.2: OIV experimental lines** (*Appendix 2*)

## 2.2. Culture media

### 2.2.1. Solid culture media for isolation, enumeration of fungi and evaluation of their toxicity, and antagonistic tests

**2.2.1.1. PDA: Potato dextrose agar;** universal cultural medium for isolation and enumeration of fungi and yeasts.

***Composition:*** extract of potatoes, 4 g; dextrose, 20 g; agar, 15 g.

***Preparation:*** 39 g of PDA medium powder (Biokar diagnostics, Beauvais, France) in 1 liter of distilled water and autoclaved at 121°C for 15 minutes. The pH could be adjusted to 3.5 after autoclaving using 12 mL of sterilized 10% lactic acid or 10mL of 10% tartaric acid.

**2.2.1.2. CYA: Czapeck Yeast extract Agar;** is a reference medium, used by certain laboratories for the fungal and yeast enumeration in foods (Deak *et al.,* 2001).

***Composition:*** Sodium nitrate ($NaNO_3$): 3 g; di-Potassium Phosphate ($K_2HPO_4$): 1 g; Potassium Chloride (KCl): 0.5 g; Magnesium Sulfate ($MgSO_4.7H_2O$): 0.5 g; Ferrous sulfate ($FeSO_4.7H_2O$): 10 mg; Yeast extract: 5 g; Sucrose: 30 g; bacteriological agar: 20 g; distilled water: 1 liter.

***Preparation:*** after adding all the compositions in the distilled water, the pH is adjusted to 6.0-6.5 then autoclaved at 121°C for 15 minutes.

### 2.2.2. Liquid media

**2.2.2.1. Yeast - Malt extract medium (YM) for molecular biology**

***Composition:*** Yeast extract: 3 g; Malt extract: 3 g; Peptone: 5 g; Glucose: 5 g; distilled water: 1 liter.

***Preparation:*** autoclaved at 121°C for 15 minutes.

**2.2.2.2. Czapeck Yeast extract Broth medium (CYB) for antagonistic tests;** ***Composition:*** Sodium nitrate ($NaNO_3$): 3 g; di-Potassium Phosphate ($K_2HPO_4$): 1 g; Potassium Chloride (KCl): 0.5 g; Magnesium Sulfate ($MgSO_4.7H_2O$): 0.5 g; Ferrous sulfate ($FeSO_4.7H_2O$): 10 mg; Yeast extract: 5 g; Sucrose: 30 g; distilled water: 1 liter.

***Preparation:*** after adding all the composition in the distilled water, the pH is adjusted to 6.0-6.5 then autoclaved at 121°C for 15 minutes.

**2.2.2.3. PDA: Potato dextrose broth medium (PDB);**

***Composition:*** extract of potatoes, 4 g; dextrose, 20 g.

***Preparation:*** 24 g of PDB medium powder (Difeco™ lab, Becton, Dickinson and company, Le pont de Claix, France) in 1 liter of distilled water and autoclaved at 121°C for 15 minutes with a final pH 5.1± 0.2.

## 2.3. Methods of mycological study of grapes

### 2.3.1. Enumeration

Enumerations were done using the Petri plates that contained between 15-150 colonies. The results were expressed in colony forming unites (CFU)/g grapes. The number *N* (population estimation) was calculated using the following equation (ISO 7218: 1996/Amd. 1: 2001, AFNOR, 2002).

$$N = \frac{\sum C}{V(n_1 + 0.1n_2)d}$$

Where;

**∑C:** the sum of the numbers of colonies on all plates used two successive dilutions and which contains at least 15 colonies;

**V:** inoculation volume that was applied in each plate, in mL;

**$n_1$:** number of plates used in the first dilution;

**$n_2$:** number of plates used in the second dilution;

**d:** dilution ratio corresponding to the first dilution used.

Each count is accompanied by a confidence interval (IC) of 95% calculated using the following equation;

$$IC = N \pm \frac{1.96\sqrt{\sum C}}{V(n_1 + 0.1n_2)d}$$

#### 2.3.1.1. From grape juice

Decimal dilutions of grape juices (about 150 g of randomly grape clusters parts were homogenized in a stomacher (France) without external water and filtered under pressure) were spread on PDA (pH 3.5) in Petri plates and incubated at 25°C for 5 to 7 days in the dark. After incubation, the number of CFU of filamentous fungi per milliliter of juice homogenate was evaluated (AFNOR, 2002) (Fig. 2.3.).

#### 2.3.1.2. From grape stalks (GS)

To estimate the microbial population in the grape stalks (GS), the same protocol described above was used using 25-35 g of GS mixed with 100 mL of sterile physiological water and homogenized by stomacher for 2 min and decimal dilutions with sterile physiological water (NaCl; 8.5 g/L (Fig. 2.3.)).

### 2.3.2. Isolation and identification

#### 2.3.2.1. Isolation

During enumeration process, the majority of morphologically different colonies had purified on to PDA Petri plates.

#### 2.3.2.2. Identification

Culture characteristics for the black Aspergilli isolated strains on PDA were observed by naked eye and lope under the binocular. The studied culture characteristics were: the color, colony density, the colony appearance (the border of the colony and its pleated appearance or umbilical), and the color of the colonies and to the media of color changes. (*Appendix* 3-5)

Microscopic observations were conducted for the fresh cultures using the objective x10 and x40 for the conidial black head observation (head uniseriate or biseriate), the conidiophores and the conidia (Samson *et al*., 1995; 2004; 2007; Dachoupakan *et al*., 2009) (*Appendix* 6).

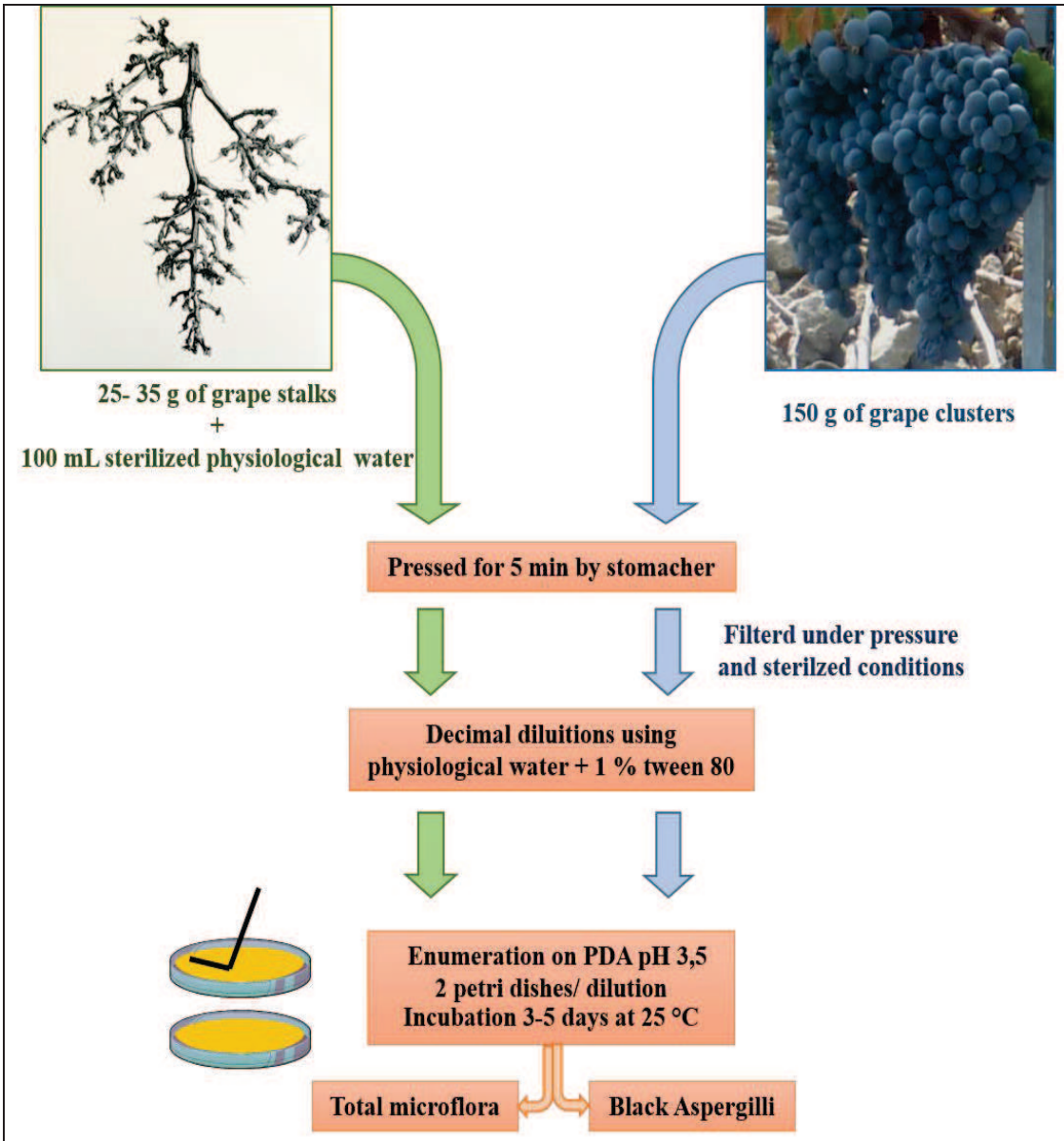



**Figure 2.3.: Enumeration and isolation protocols using different plant resources.**

### 2.3.3. Induce sporulation

Black *Aspergillus* cultures isolated were grown on PDA medium at 25°C for 5 days. Conidia suspensions of each isolates were prepared in 1% (v/v) Tween 80 in physiological water. They were adjusted to $10^5$ conidia/mL and numerated by using a Thoma haemocytometer (0.0025 $mm^2$ x 0/1mm) and 5 µL of adjusted spore suspensions were inoculated in 250mL Erlenmeyer flasks containing 20 mL of PDA solid medium. Cultures were inoculated at 25°C without shaking. After 6 days of inoculation, spores of each culture were collected by 100mL of physiological water with Tween 80 at 1% (v/v) after stirring par magnet and counted on haemocytometer (Arzumanov *et al.,* 2005).

Then the biomass of these *Aspergillus* strains were assessed by inoculate 5µL of spore suspension adjusted to $10^5$ spores/mL on Petri dishes containing the PDA medium and a sterile filter paper pre-weighted covering the surface. After incubation during 6 days at 25ºC, filter paper was removed and the biomass collected and placed in a previously dried and weighed filter paper. Biomass was dried during 24 h at 105°C, and weighed.

## 2.4. Molecular biology methods

### 2.4.1. DNA extraction

#### 2.4.1.1. Extraction of DNA from grape berries

The method described by El-Sheikha *et al*. (2009; 2012) was used for the DNA extraction from grape berries as shown in Fig. 2.4. About 5-8 berries was grounded by hand in 5-8mL oh peptone water then 2 mL were added to sterile eppendorf tubes with 0.3g of 0.5 mm diameter acid wash glass beads 425 - 600 µm (Sigma, France). The mixture was vortexed vigorously for 30 min in bead beater instrument (Vortex Genie 2 SI-A256, USA) then centrifuged at 12000 rpm/15 min and the suspension discarded. The cell pellet was re-suspended in 300 µL of breaking buffer [2% Triton X-100 (Prolabo, France), 1% SDS( sodium dodecyl sulphate; Sigma, France), 100 mM NaCl (Sigma), 10mM Tris, pH 8.0, 1mM EDTA, pH 8.0 (Promega, France)]. 100 µL TE [10 mM Tris – HCl, 1mM EDTA, pH 8.0 (Promega), 100 µL lysozyme solution (25 mg/mL, Eurobio, France) and 100 µL proteinase K solution (20 mg/mL, Eurobio, France) were added and the mixture was incubated at 42°C for 20 min. then 50 µL 20% SDS were added to each tube, then incubated at 42°C for 10 min. The tubes were vortexed vigorously for 5 min.400 µL MATAB (mixed alkyltrimethyl ammonium bromide; Sigma) were added to each tube, then incubated at 65°C for 10 min. The lysates were then purified twice by repeated extraction with 700 µL phenol: chloroform: Isoamyl alcohol (25:24:1; Carlo Erba, France) and the tubes were handily vortexed for 5 min and then centrifuged at 12000 rpm/15 min. The aqueous layer was transferred to an eppendorf tube and the residual phenol was removed by extraction with 600 µL chloroform: Isoamyl alcohol (24:1) and centrifuged for 15 min at 12000 rpm. The aqueous phase was collected and the DNA was stabilized with 30 µL sodium acetate (3M, pH 5), followed by precipitation by adding equal volume of ice cold isopropanol, and stored at -20°C for 12 h (overnight). After centrifugation at 12000 xg for 15 min, the supernatant was eliminated, DNA pellets were washed with 500 µL 70% ethanol and the tubes were centrifuged at 12000 g for 15 min. The ethanol was then discarded and the pellets were air-dried at room

temperature for 45-60 min. finally, the DNA was re-suspended in 50 µL ultrapure water and stored at -20°C until analysis.

#### 2.4.1.2. Production of fungal mycelia for different strains

Black *Aspergillus* strains had grown on PDA medium at 25°C for 5 days. Conidia suspensions of each isolates were prepared in 1% (v/v) Tween 80 in physiological water. They were adjusted to $10^6$ conidia/mL and numerated by using a Thoma haemocytometer (0.0025 $mm^2$ x 0/1mm) and 8 mL of adjusted spore suspensions were inoculated in 250mL Erlenmeyer flasks containing 100 mL of YM liquid medium ( yeast extract, 3 g/L; malt extract, 3 g/L; peptone, 5 g/L; glucose, 5 g/L). Cultures were inoculated at 25°C without shaking. Mycelia from 3 day cultures were harvested by filtration throughout a filter paper, kept at -20°C till use (Dachoupakan *et al*., 2009).

#### 2.4.1.3. Extraction of DNA from fungal mycelia

The same protocol that used for DNA extraction from grape berries with some modifications. The filtrated-fresh 3days old fungal mycelia grown on YM liquid media (as described in section 2.4.1.2.) was grounded using liquid nitrogen (Atoui *et al.,* 2007), in sterilized mortals. Grounded mycelia were divided into two replicate eppendorf tubes.

The reactions used for the DNA extraction were illustrated in *Appendix* 7.

#### 2.4.1.4. DNA extraction verification

##### 2.4.1.4.1. By nano-spectro

A drop of the DNA extraction was tested at Nano-drop spectrophotometer (Shimadzu Corporation, Kyoto, Japan) in order to discriminate the ratio of $OD_{260}/OD_{280}$ (Glasel, 1995).

##### 2.4.1.4.2. By gel electrophoresis

###### 2.4.1.4.2.1. Gel preparation

0.8% Agarose (Seakem LE Agarose, Cambrex Bio Science Rockland, Inc., USA), gels were prepared to verify the DNA extraction. Agarose quantity (depending upon gel plate size) was melted in TAE (1X) (Tris: 2M; EDTA: 0.05M) into a microwave. Gels were re-cooled to 50°C, then quantity (depending upon the gel quantity) of 10 mg/mL ethidium bromide solution (Sigma, Steinheim, Germany). The obtained gel was then slowly poured into the gel plaques (MIDIGEL2, MINIGEL2) (Apelex, Massy, France) with a thick of 7-8 mm where a comp (16 teeth; 1mm) (n° 320161, Apelex, Massy, France) was previously added. Gels were left to solidify for about 30 min;

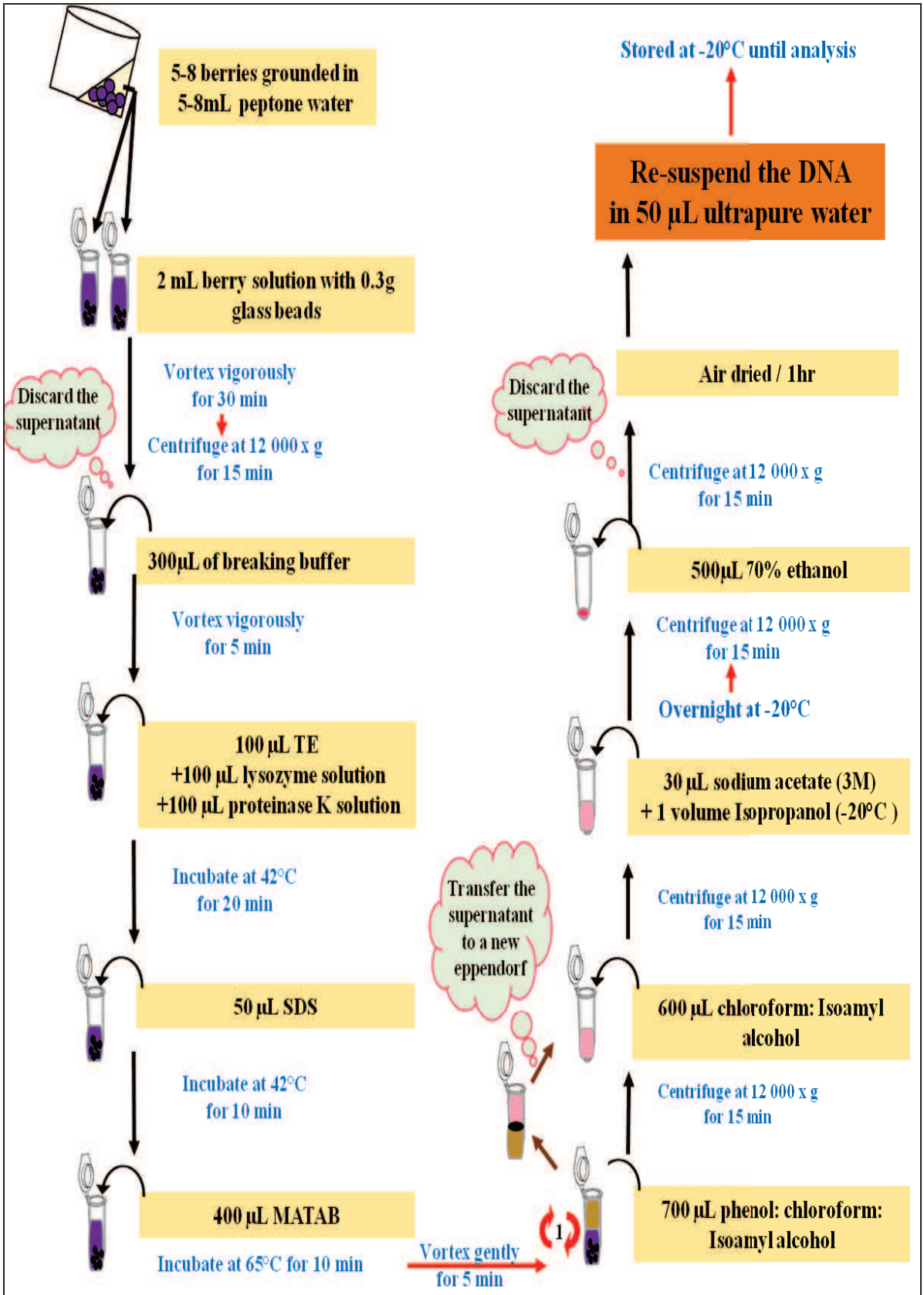



**Figure 2.4.: DNA protocol used for DNA extraction from grape berries** (El-Sheikha *et al.*, 2012)

#### 2.4.1.4.2.2. Gel analysis

After electrophoresis and ethidium bromide revelation, the DNA band size of our samples that appear orange under UV light were defined regarding the size marker bands.

### 2.4.2. PCR amplifications

#### 2.4.2.1. DGGE

##### 2.4.2.1.1. Gel preparation

Sixteen of the gradient agarose solutions with different concentrations (30%, 40%, 50% and 70%) (Tab.2.2.) were prepared (in the two tubes with fifty mL capacity of the Bio-Rad apparatus (Model 485, USA).50µL of 10% ammonium persulfate (p:v) (Promega, France) and 50 µL of tetramethylethylenediamine (TEMED) (Promega, France) were added.

**Table 2.2.: DGGE gel composition for gradient denaturation gels**

| Constituents | Gradient denaturation gels (concentration of the denaturant) | | | |
|---|---|---|---|---|
| | 30% | 50% | 40% | 70% |
| **40% Achrylamide/bisacrylamide** | 20 mL | 20 mL | 20 mL | 20 mL |
| **Formamide** | 12 mL | 20 mL | 16 mL | 28 mL |
| **Urea** | 12.6 g | 21.0 g | 16.8 g | 29.4 g |
| **TAE 50x** | 2 mL | 2 mL | 2 mL | 2 mL |
| **Distilled water** | 100 mL | 100 mL | 100 mL | 100 mL |

The glass plates sandwich (the thickness of glass plates are 200 mm with 0.75 mm spacer between) is then filled with the gel material using the Percom-1 pump (Watson-Marlon, USA) that adjusted to 20 turn/min which permits the transfer of the gradient gel in the spacer. A comb of 0.75 mm thick is placed between the plates from the top. The gel is placed to dry for at least 1 hour.

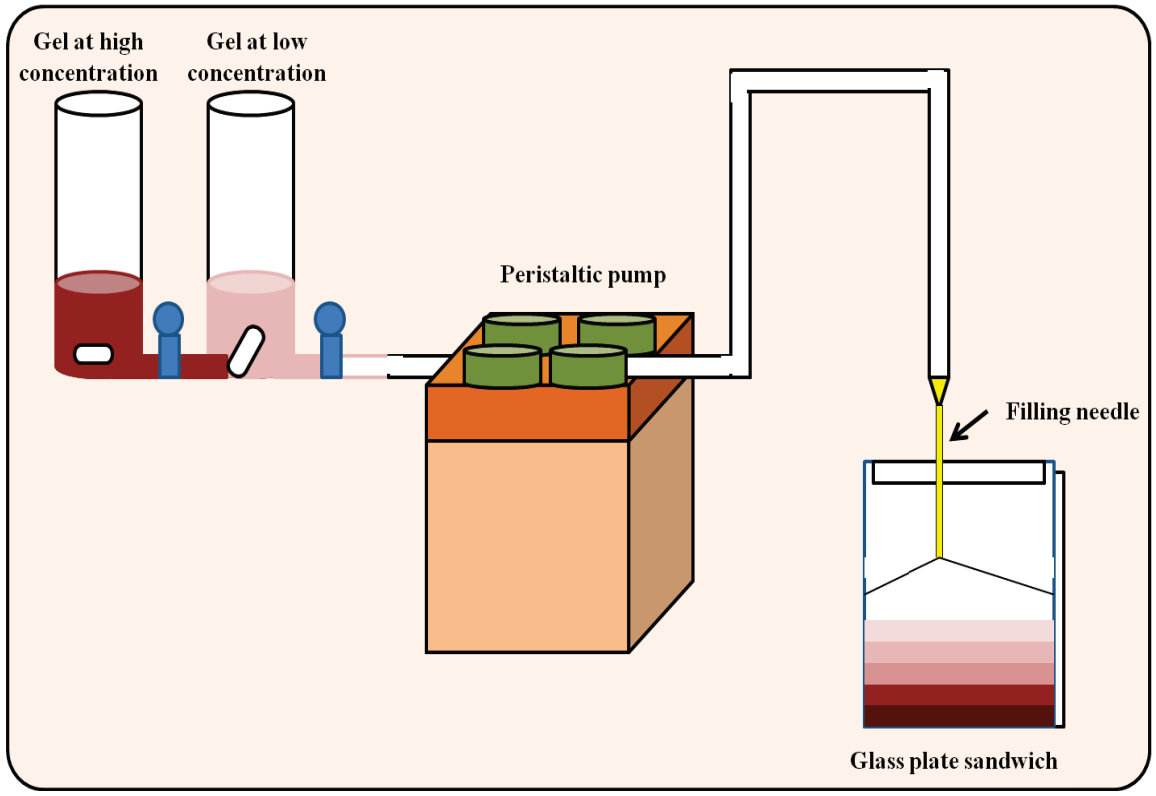



**Figure 2.5.: DGGE gel preparation procedure**

#### 2.4.2.1.2. PCR amplification

Two sets of primers were used for PCR amplifications of the grape berries of different treatments DNA (Tab. 2.3.). A 30-bp GC-clamp (Sigma, France) was added to the forward primers (the GC-clamp is underlined).

**Table 2.3.: Primer sets used for the PCR-DGGE amplification**

| Primer sets | | Sequences | Amplified Region | Amplified PCR product size |
|---|---|---|---|---|
| 1 | U1f-GC | 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA-3' | 28S region | 260 bp |
| | U2r | 5'-GAC TCC TTC GTC CGT GTT-3' | | |
| 2 | P65f-GC | 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG CAA CCT CCC ACC CG- 3' | ITS1 region and 5.8S | 515 bp |
| | P580r | 5' -CTA CCT GAT CCG AGG- 3' | | |

The first set of primers (U1f-GC and I2r) (Sigma, France) is eukaryotic universal primers (Sigma, France) (El Sheikha *et al.,* 2012). PCR was performed in a final volume of

25 µL containing 2 µL of each primers, 0.5 µL dNTPs mixture, 2 µL $MgCl_2$, 5 µL of 10X of reaction Taq buffer $MgCl_2$ free (Promega, France), 1.25 U of Taq DNA polymerase (Promega, France), and 2 µL of the extracted DNA (30 ng). PCR was run for 30 cycles with annealing at 50 °C for 50s, extension at 72 °C for 90s, and denaturation at 95 °C for 45 s (El Sheikha *et al.*, 2012).

The second set of primers was designed using online software and databases (NCBI database, Multalin, and oligonucleotide properties calculator) (*Appendix 8-9*). PCR was performed in a final volume of 25 µL containing 2.5 µL of 10x of reaction Taq buffer $MgCl_2$ free buffer (10x, Promega, France), 1 µL of each primers (10 pmol/µL), 1 µL dNTPs mixture (Promega, France), 2.5 µL $MgCl_2$ (Promega, France), 0.25 µL of Taq DNA polymerase (Promega, France), and 2 µL of the extracted DNA (30 ng) (Dachoupakan *et al.*, 2009). PCR was run for 30 cycles with annealing at 57 °C for 25s, extension at 72 °C for 35s, and denaturation at 95 °C for 30 s

#### 2.4.2.1.3. Migration conditions

The tank of electrophoresis (Bio-Rad DCode System, USA) was filled with TAE 1x buffer solution (acetate-Tris, EDTA, pH 8.3). This solution was pre-heated for approximately 1 h - 1 h 30 before placing the gradient gel containing the tested samples at 60°C (Díez *et al.,* 2001; El-Sheikha *et al.*, 2012)

The PCR products were analyzed by DGGE by using a Bio-Rad Dcode™ universal mutation detection system (Bio-Rad Laboratories, USA) using the procedure first described by El Sheikha *et al.* (2009). Samples containing approximately equal amounts of PCR amplicons (30 µL) were loaded into 8% (w/v) polyacrylamide gels (acrylamide/N,$N^1$-methylene bisacrylamide, 37.5/1, Promega, France) in 1x TAE buffer (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM $Na_2$-EDTA) (Fig. 2.5. and 2.6.). All electrophoresis experiments were performed at 60°C using a denaturing gradient ranging from 40% to 70% (100% corresponded to 7 M urea and 40% [v/v] formamide, Promega, France) for the first set of primers and ranging from 30% to 50% for the second set of primers. The gels were electrophoresed at 20 V for 10 min and then at 80 V for 16 h.

#### 2.4.2.1.4. Gel development

After electrophoresis, the gels were stained for 30 min with ethidium bromide and rinsed for 20 min in distilled water and then photographed on a UV transilluminator with the Gel Smart7.3 system (Clara Vision, Les Ulis, France).

#### 2.4.2.1.5. Gel statistical analysis

Banding patterns were standardized with the two control treatments as reference patterns included in gel. This software permitted to identify the bands relative positions compared with the standard patterns. In DGGE analysis, the generated banding pattern is considered as an image of all of the major fungi in the populations. Gel images were stored by using The Imager system (Ampligene). Quantification of ethidium bromide-stained bands for competitive PCR experiments was performed with the ImageQuant TL software v.2003 (GE Healthcare, USA).

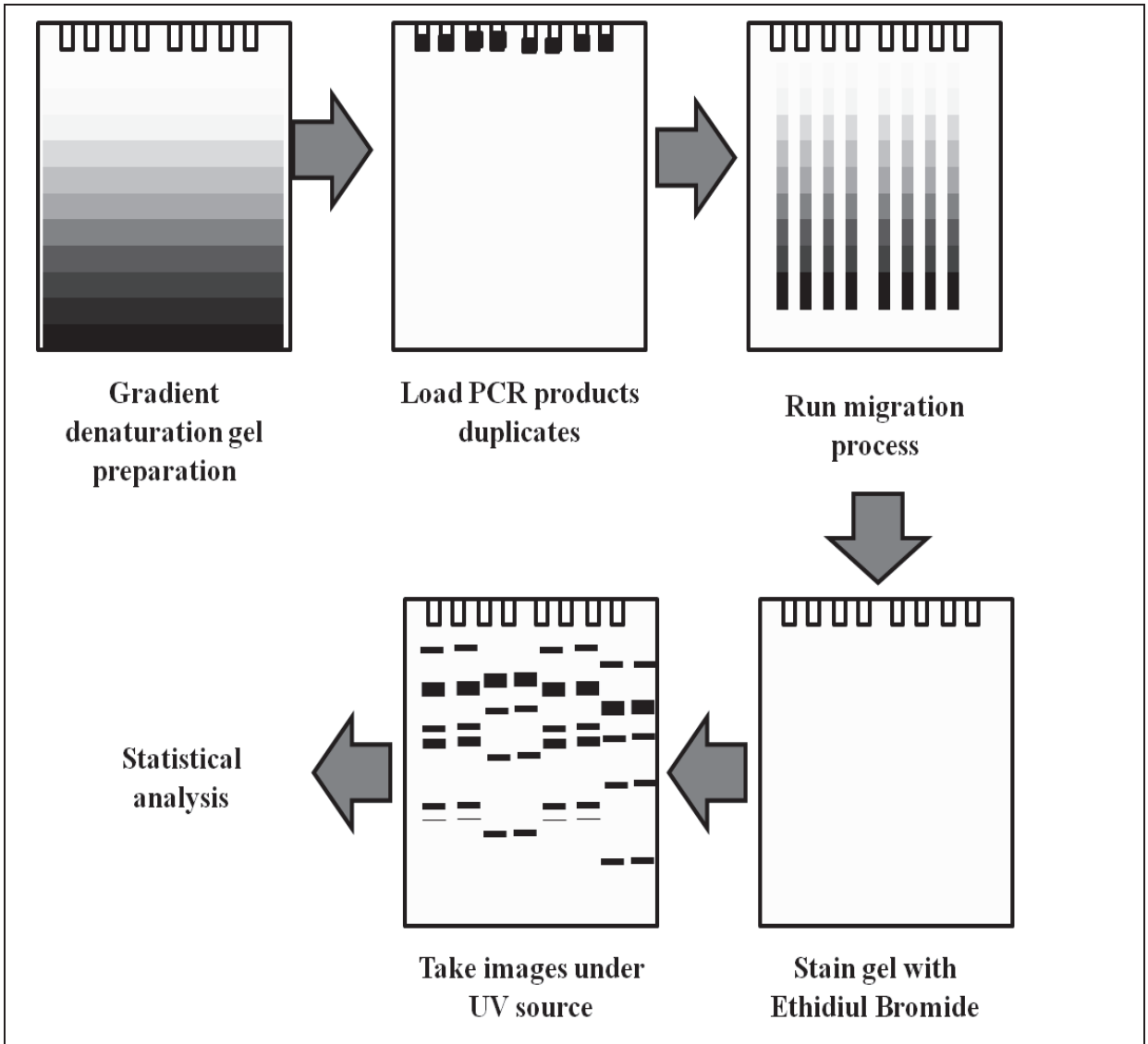



**Figure 2.6.: DGGE gel migration and development process**

An individual discrete band refers to a unique ‘‘sequence type’’ or phylotype (Muyzer *et al.*, 1995; van Hannen *et al.,* 1999). This was confirmed by Kowalchuk *et al.* (1997) who showed that co-migrating bands corresponded to identical sequence. The DGGE fingerprints

were manually scored by the presence and absence of co-migrating bands, independent of intensity. Pair wise community similarities were quantified using the Dice similarity coefficient (SD) (Heyndricky *et al*., 1996).

#### 2.4.2.2. Real time PCR

##### 2.4.2.2.1. Primers

Two quantitative PCR were conducted using two sets of primers (Tab. 2.4) according to the target searched fungi.

**Table 2.4.: A description of the primers group used for the real time PCR assays**

| | Primer | | | Amplified length | Ref. |
|---|---|---|---|---|---|
| | **Name** | **Sequence (5'-3')** | **Region** | | |
| **Specific primers for *A. carbonarius*** | **Ac12RL-OTAf** | aatatatcgactatctggacgagcg | Non-conserved regions in the AT domain of PKS gene (Ac12RL3) | 141 bp | Atoui *et al.*, 2007 |
| | **Ac12RL-OTAr** | ccctctagcgtctcccgaag | | | |
| **Universal primers for all fungi** | **ITS1f** | tccgtaggtgaacctgcgg | 5.8 S | 300 bp | Fierer *et al.*, 2005 |
| | **5.8S** | cgctgcgttcttcatcg | | | |

##### 2.4.2.2.2. PCR conditions

Real time-PCR amplifications and detections were carried out using The LightCycler® 480 Real-Time PCR instrument (Roche, USA). Q-PCR was prepared in duplicates of 6 µL reaction mixtures in MicroAmp optical 384-well reaction plates and sealed with optical adhesive covers. Each reaction well contains 2 µL of template DNA, 3 µL of 2× Quantitect SYBRR® Green I Mix (Qiagen, Courtaboeuf, France), and 0.6 pmol/µL (final concentrations) of each forward and reverse primers. Real-time QPCR was conducted the conditions shown in Tab. 2.5. To generate the standard curve, a: for the specific primers 10-fold dilutions (ranging from 1 µg to 1 pg) of *A. carbonarius* (CBS 120167) DNA whose concentration was previously determined were subjected to real-time PCR. The standard curve is a plot of the threshold cycle (Ct) versus log DNA concentration (*Appendix 10*). Then, DNA grape samples were subjected to real-time PCR and the amount of *A. carbonarius* DNA was calculated according to the generated standard curve. Control sample without DNA template was included in the experiment runs (Fig.2.7.). B: for the universal primers decimal dilutions were made for the two control treatments.

**Table 2.5.: Real time PCR conditions**

| | Pre-cycles steps | | 40 cycle | | |
|---|---|---|---|---|---|
| | 1 | 2 | Denaturation | Annealing | Extension |
| ITS1/5.8s | 50°C/ 2 min | 95°C/ 15 min | 95°C/15s | 58°C/30s | 72°C/30s |
| Ac12RL-OTAf/r | --- | | 95°C/ 1 min | 53°C/ 30 s | 72°C / 1 min |

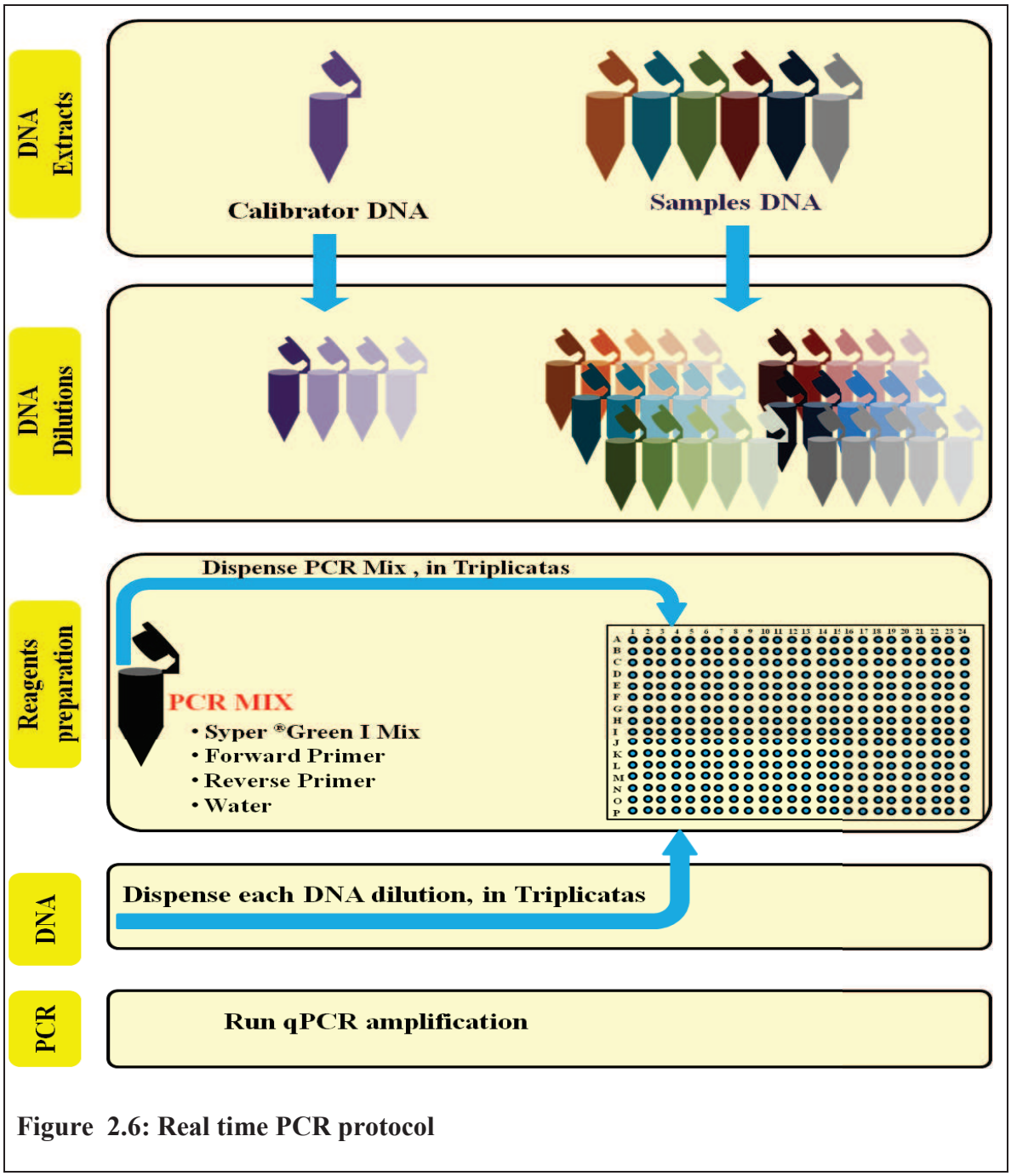



**Figure 2.6: Real time PCR protocol**

## 2.5. Chromatography methods

### 2.5.1. for OTA measuring

#### 2.5.1.1. OTA extraction

##### 2.5.1.1.1. from media cultures

Colony diameters were measured and three agar plugs of 5 mm in diameter were removed from the *A. carbonarius* colonies (in the area that confronting the other tested fungus for its antagonist activity) at 3, 7 and 10 days of incubation. The plugs were weighed, introduced into 3 mL vials and extracted with 2.5 mL of solvent (methanol/formic acid, 25:1) under sonication for 15 min. The solvent was then evaporated under a nitrogen stream at 40 °C. The dried extracts were re-suspended in the mobile phase of the subsequent HPLC (49.5% deionized water/49.5% acetonitrile/1% acetic acid) then filtered with a syringe (Minisart SRP 4 of diameter 0.45 µm, Sartorius, Germany) (Suarez-Quiroz et al., 2004) (*Appendix 11A*).

##### 2.5.1.1.2. From the whole plates

The same protocol described in the section 2.5.1.1. was used with modification: that the OTA content was extracted from all the agar into the plates in a 100 mL vials and extracted by 40 mL of (methanol:formic acid, 25:1) under sonication for 15 min.

##### 2.5.1.1.3. from grape juices

Specific grape juice was prepared from each treatment in a blender using 50 g of grape berries with 200 mL of 70% methanol (VWR, PROLABO, 20837.320) that was diluted with deionized water by two steps (30 mL water, then 140 mL methanol and 320 mL water),and then filtered (by a cellulose acetate filter 0.2 µL, Sartorius®, Germany). In total, 10 mL of the juice was diluted in 40 mL of PBS (8 g NaCl, 1.2 g $Na_2HPO_4$, 0.2 g KCl, 0.2 g $KH_2PO_4$, pH 7.0) (Sigma Aldrich, France, P4417) + 0.01% Tween 20 (Sigma Aldrich, France, P-1379). Then 20 mL of the diluted juice was passed through an *OCHRAPREP*® affinity column (R-Biopharm Rhone, France) (1 drop/sec). Next the column was washed by 10 mL of PBS/0.01% Tween-20, followed with 10 mL purified water. The OTA were collected by 1.5 mL methanol:acetic acid (VWR, PROLABO, 84-528.290) (98:2, v:v) twice (at the flow rate of 1-2 drop/sec) (Skarkova *et al.*, 2013). Then the extracts were stored at -20°C until measured (*Appendix 11B*).

#### 2.5.1.2. OTA measuring by HPLC

OTA was analyzed by HPLC (Pump, Shimadzu LC/9A, Japan) with fluorescence detection ($\lambda_{exc}$333 nm; $\lambda_{em}$460 nm, Shimadzu RF-10AXL, Japan), using a C18 column (25 x 4.6 mm, 5 µm, Phenomenex®, USA). The mobile phase [49% acetonitrile (Carlo Erba Reagent, France, P00637G21), 49% Milli-Q water (Quantum® Ex, MILLIPORE), and 1% acetic acid] was pumped at 1 mL/min. The injection volume was 20 µL, and the retention time was approximately 16.3 min. The calibration curve was prepared with OTA solution (Sigma Aldrich, France, 34037-2ML-R) from 0.05 to 5 ng/ mL. The limit of detection (LOD) and limit of quantification (LOQ) of this method which was applied to grape juice samples, were established at 0.023 µg/L and 0.076 µg /L, respectively (Dachoupakan *et al*., 2009).

### 2.5.2. TLC analysis identifying the metabolic compounds in fungal culture grown on liquid media

#### 2.5.2.1. *P. adametzioides* culture filtrates

Eight milliliters of $10^6$ spore suspension of *P. adametzioides* are used for inoculation of 100 mL of PDB in 250 mL conical flasks. These cultural media were incubated at 25°C with shaking (150 rpm) for three, seven and 10 days. Fungal cultures were filtered using nitrocellulose membranes (0.2 µm) under vacuum and sterile conditions. Ten millilitres of these filtrates were concentrated using hot water-bath at 40°C with 40 rpm/min under vacuum. The concentrated filtrates were re-filtered with sterile 0.2 µm non pyrogenic filters.

#### 2.5.2.2. Solvents

Several solvents were tested with different polarities. Apolare solvents (Cyclohexane, Toluene), Polare solvents (methanol, choloroforme), and mixtures of soulvents with different polarity [two solvents (acitonitril: methanol, cyclohexane: methanol, acetic acid: methanol, chloroforme: methanol) and three soulvents (cyclohexane: toluene: methanol)] with different concentrations.

#### 2.5.2.3. Separation conditions

The TLC were left for 30 min or 2h.

## 2.6. Morphological analysis of grape berries

One hundred randomly chosen berries from each treatment were analyzed using a precision measuring tool (Fisher Darex, France). The average of each treatment was compared with the average of other treatments.

### 2.6.1. Berry size

One hundred randomly chosen berries from each treatment were analyzed using a precision measuring tool (Fisher Darex, France) (Ojeda *et al*., 2001). The average of each treatment was compared with the average of other treatments.

### 2.6.2. Skin thickness

Thin sections of berry skin (approximately 0.5 x 1.0 cm) were taken with a scalpel, air dried at room temperature (Pathan *et al*., 2008) and measured under a scanning electron microscope (Quanta FEG, Type 200, D8399, FEI Company™, USA). In total, 100 measurements were completed at various zones of skin from 50 berries/treatment (5 berries /bunch).

### 2.6.3. Wax and cuticle layers

The method for cuticle and wax quantification, as described by Gabler et *al.,* 2003 was used (Fig. 2.8). Fifty berries were selected from the middle portion of the clusters and kept frozen at –20°C until analyzed. Several epidermal disks, which were 5 mm in diameter, were cut with a cork borer from different parts of each berry. The cuticle and wax contents of three replicates, each containing 50 disks, were determined. Cuticle and wax layers were separated from underlying tissues by soaking the disks for 24 h at 30°C in 2.5 ml of a digestion solution containing 0.5% cellulase (from *Aspergillus niger*, Sigma Chemical C1184-5KU) and 0.1% pectinase (from *Aspergillus niger*, Sigma Chemical P4716-5KU), which was dissolved in 0.1 M sodium acetate buffer (pH 4) (Sigma Aldrich, France, S2889-250G).

The disks of the digestion resistant material (cuticle plus epicuticular wax) were collected on a metal screen and rinsed with deionized water until clean. The disks were air-dried and the epicuticular wax was dissolved by immersing the disks in 2 ml of 100% chloroform (VWR, PROLABO, 22711.290) for 24 h to separate the wax from the cuticle. The wax which was dissolved in chloroform was transferred to pre-weighed beakers. Separate beakers containing either cuticle or wax were placed in an oven at 100°C for 24 h, and their weights were recorded.

## 2.7. Analysis of Physical-chemical characteristics and biochemical principles in grape juices.

### 2.7.1. Juice acidity

#### 2.7.1.1. Total pH

The pH of each juice was measured using a Series pH720 pH meter; (WTW® InoLab, Germany). All analyses were performed in duplicate at 20°C.

#### 2.7.1.2. Juices content of Tartaric and Malic acids

The grape organic acids were separated according to the method that was demonstrated by the International Organization of Vine and Wine (OIV, 2009), which uses HPLC after filtration with a filter syringe (0.45 µm, MiniSart, Sartorius®, Germany). The HPLC system was composed of a pump (Shimadzu LC-10A, Japan), which was connected to a UV detector (Shimadzu SPD-6A, Japan) that was set at 210 nm (Castellari *et al.,* 2000), with the following conditions: loop injection of 20 µL; C610H column (9 µm, 300 mm x 7.8 mm) (SUPELCO®, USA). The column was kept at a constant temperature of 40°C. The mobile phase was composed of ultra-pure water containing 0.5% of sulfuric acid (VWR, PROLABO, 220685.330) with a flow rate of 0.6 mL/min. Concentrations were determined according to standard curves for malic acid (Sigma Aldrich, France, M-0875) (0.1 to 0.6 g/ L) and tartaric acid (MERK Eurolab, 20-716.293) (0.1 to 0.4 g/L) (*Appendix 12*).

### 2.7.2. Juice Sugar concentration (Glucose and Fructose).

A grape juice from each treatment was diluted 1000 times to match the calibration curve. Sugars (glucose and fructose) were determined by HPLC (Castellari *et al.,* 2000). The same HPLC system was used for the organic acid determination, with the same column and conditions, coupled to a refractometer (Shimadzu RID-6A, Japan). Concentrations were determined according to standard curves for glucose (Sigma Aldrich, France, G0350500-1EA) and fructose (Sigma Aldrich, France, F-0127) (0.1 to 1 g of each sugar/L) (*Appendix 13*.

### 2.7.3. Juice Color Evaluation by colorimeter L*a*b*

This method determines 3 parameters for the juice color: *L* (lightness) with values ranging from 0 (black) to100 (white), point *a* on the green-red axis, and point *b* on the blue-yellow axis. Two milliliters of juice were centrifuged at 8,000 g and filtered (0.45 µm, MiniSart, Sartorius®, Germany). The analyses were performed using the Tri-Stimulus program

(Konica-Minolta), and the three color parameters ($L^*a^*b^*$) were recorded. Each sample was measured three times and the average values were calculated (León *et al*., 2006).

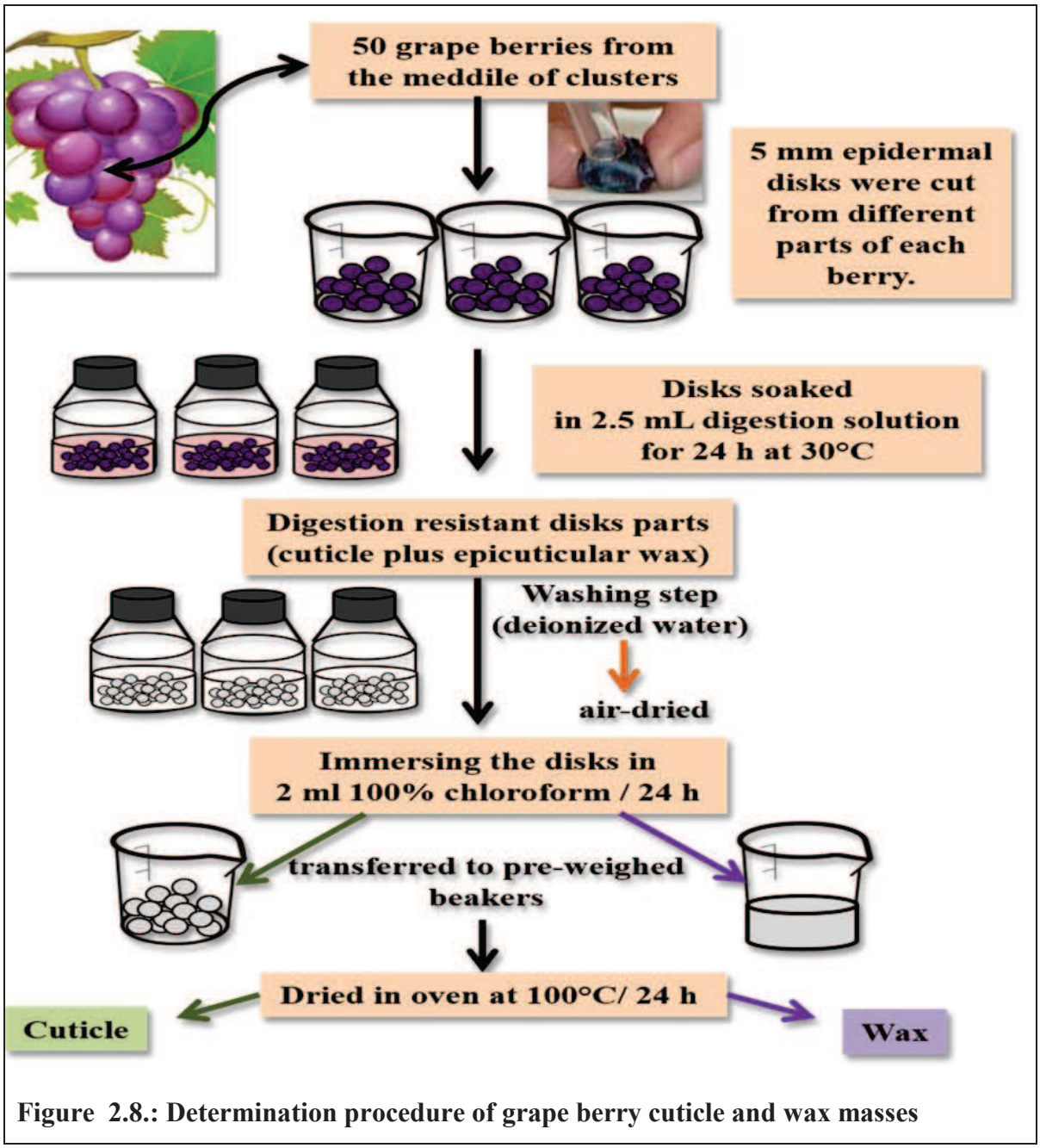



**Figure 2.8.: Determination procedure of grape berry cuticle and wax masses**

### 2.7.4. Juice content of polyphenols (galic acid)

The total concentration of polyphenols in grape juice was estimated with reagent Folin-Ciocalteau (MERCK, Germany, 1.09001.0100) (Singleton *et al.,* 1999; Tawaha *et al.,* 2007; Soto-Vaca *et al.,* 2012). A sodium carbonate (Sigma Aldrich, France, S7795) solution (0.25 g/mL) was filtered through a nitro-cellulose membrane (0.2 µm, Sartorius®, Germany) and added to 200 mL of water. For the analysis 20 µL of each juice sample, which was pre-diluted 10-fold, was used following the Folin-Ciocalteau method. Concentrations were

determined according to the standard curve of gallic acid (50 to 500 mg/L) (Sigma Aldrich, France, G-7384) (*Appendix 14*).

### 2.8. *In-vitro* direct effect Stifénia powder on OTA-PF

Two concentrations of Stifénia ® solutions; 15 mg/mL (the used concentration for the *in-vitro* treatment) and 150 mg/mL were examined for their antifungal activity against the fungus *A. carbonarius*.

#### 2.8.1. Direct effect of Stifénia powder on the OTA-PF mycelial growth.

Agar dilution method was performed by adding different concentration of Stifénia® (UV sterilize) solution to PDA (Biokar diagnostics, Beauvais, France) media before pouring (at 45°C). Consequently, after cultural media solidification, five µl of $10^6$ spore/mL of OTA-PF were inoculated at plate centers (Fenner *et al.,* 2005 and Cabañas *et al*., 2009). Normal PDA culture media inoculated by OTA-PF were used as control. Triplicates were performed for all the treatments. (Fig. 2.8. A)

#### 2.8.2. Direct effect of Stifénia powder on the produced OTA by OTA-PF

Saturated filter membranes (SFM) of six millimeter diameter (Filter Durieux n°268) with thirty microlitres of pre-sterilized sterilized Stifénia® solutions were centrally placed on pre-inoculated PDA (Biokar diagnostics, Beauvais, France) plates with 100 µL of *A. carbonarius* spore suspension ($10^6$ conidia/mL) (Ramamurthy *et al*., 2012). Two controls were used; SFMs with 35 µg/L of 5-flourocytosine (Sigma Aldrich, France) solution as positive control (Waldorf and Polak, 1983; Ribeiro *et al*., 2006), and SFMs with sterilized distilled water as a negative control. Triplicates were performed for all the treatments. (Fig. 2.8.B).

## 2.9. Study of volatile compounds of grape leaves

#### 2.9.1. Identification of vine leaf volatile compound composition using SPAM-GS-MS

The volatile compounds of grape leaves that treated with the two OTA-PF control treatments (elicitor (Stifénia®) and chemical (SCALA®) treatments) and the volatile compounds present naturally in the Stifénia® powder were identified using the HS-SPME-GC-MS technique [the best SPME technique when target analyses are volatile organic compounds (Marengo *et al*. 2001)] as performed by Tesniere *et al.* (2006) with certain

modifications. SPME parameters were optimized: fiber type was 4 CAR/PDMS (Supelco, Bellefonte, Pennsylvania, USA); trans-2-Nonenal (Sigma, France) as internal standard; 60°C for incubation; 10 min as equilibrium period and 20 min for the extraction. (Sánchez-Palomo *et al*., 2005 and Haddada *et al*., 2007). The mass spectrum of the volatile compounds which were found at the HS was compared with the Wiley mass spectral data, consequent by their calculated relative retention (CRR). The CRR were calculated according to the retention time of the separated compounds and the alkaline negative control of the standard solution (Retention Index Standard, Sigma). The analyses of free and bound compounds were performed in triplicate (Pozo-Bayón *et al.,* 2007; Cayot *et al.,* 2008).

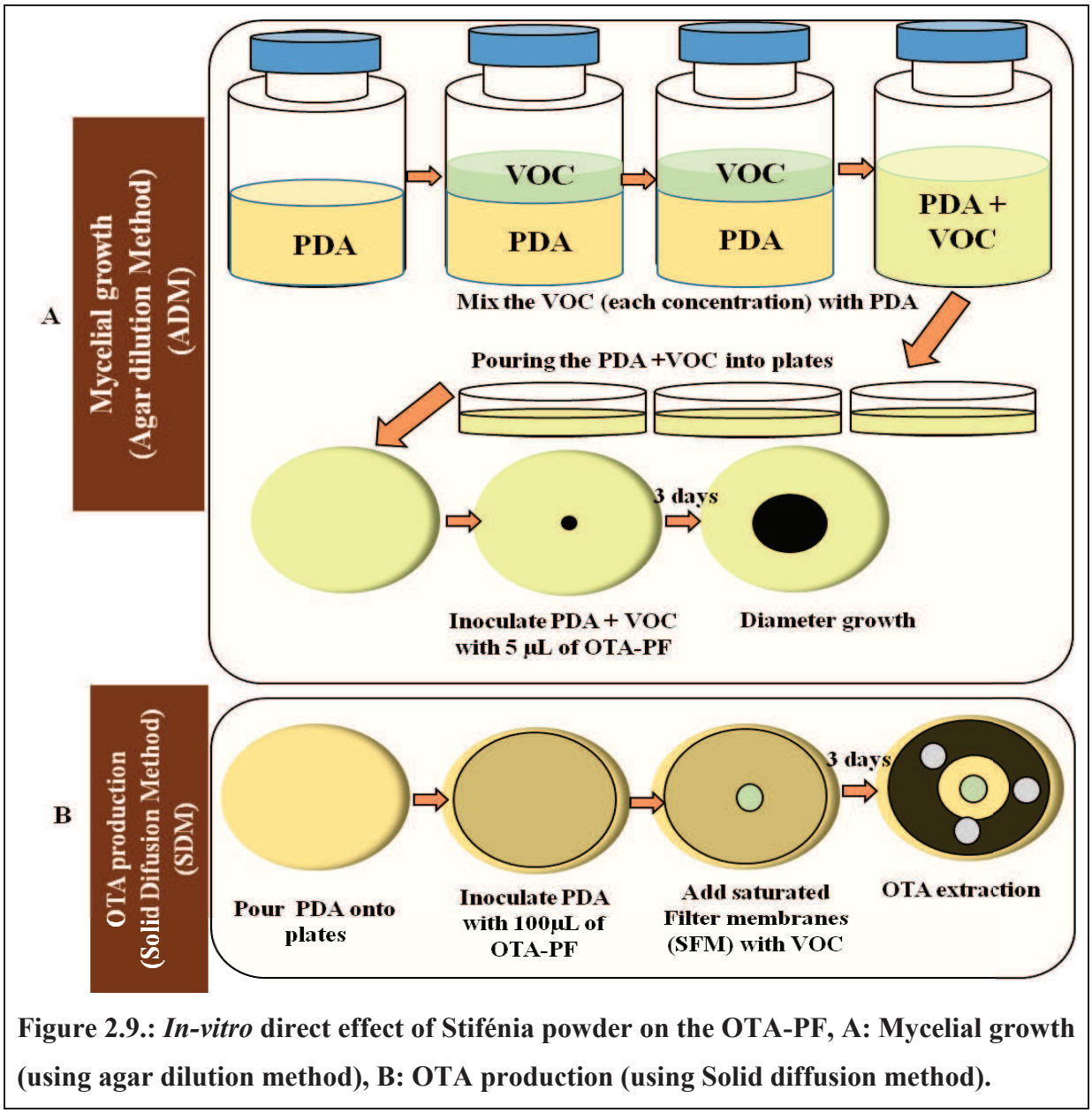



**Figure 2.9.: *In-vitro* direct effect of Stifénia powder on the OTA-PF, A: Mycelial growth (using agar dilution method), B: OTA production (using Solid diffusion method).**

### 2.9.2. Antifungal activities of the most relevant volatile compounds in the Stifénia leaves

The antifungal activity of three volatile compounds was measured against the strain used for contamination (*A. carbonarius*). Two of them were highly concentrated in the elicitor treatment grape leaves [Trans-6-Nonenal (13) (Sigma, France) and Trans-2-Octenal (6) (Fulka, France)] and the last Trans-2-Hexenal (Fulka, France) was chosen as a bibliographic study reference (Utto *et al.*, 2008). Four concentrations of each (100, 1, 0.5 and 0.05 µg/mL in alcoholic water) were examined. The 10% ethanol solution was used as a negative control and 130 ng/L of 5-fluorocytosine as a positive control for its recognized antifungal activity (Waldorf and Polak, 1983).

Three different methods were conducted to assess the antifungal activity; solid diffusion method (SDM), vapor diffusion method (VDM), and agar diffusion method (ADM).

#### 2.9.2.1. Solid Diffusion Method (SDM)

The SDM was conducted as Ramamurthy *et al*. (2012) described by centrally placing a SFM of 6 mm diameter with different concentration to pre-inoculated PDA Petri dishes with 100 µL of $10^6$ spore/mL of OTA-PF) (the VOCs efficiency on mycelial growth was assessed according to the presence of an inhibition zone or not, and on the OTA production according to OTA extraction from 3 cultural plugs of 5mm as described above in 2.8.2. section (Fig 2.8.B))

#### 2.9.2.2. Vapor Diffusion Method (VDM)

VDM was performed by adding a SFM of 13 mm diameter with different concentration to the plate covers of PDA plates with pre-weighted and sterilized filter membranes (that cover all the medium surface), which were consequently inoculated with 100 µl of $10^6$ spore/mL of OTA-PF) (Lopez *et al.,* 2005) (Fig..2.10.).

#### 2.9.2.3. Agar Diffusion Method (ADM)

ADM was carried out by mixing the different concentration of the tested volatile compounds to PDA media before pouring (at 45°C). Consequently, after cultural media solidification, sterilized pre-weighted filter membranes were placed onto the agar surface, then 100 µl of $10^6$ spore/mL of OTA-PF were inoculated) (Fenner *et al.,* 2005; Cabañas *et al*., 2009). VOCs affecting OTA-PF mycelial growth and OTA production were expressed for VDM and ADM corresponding to the dry weight of the OTA-PF in each treatment.

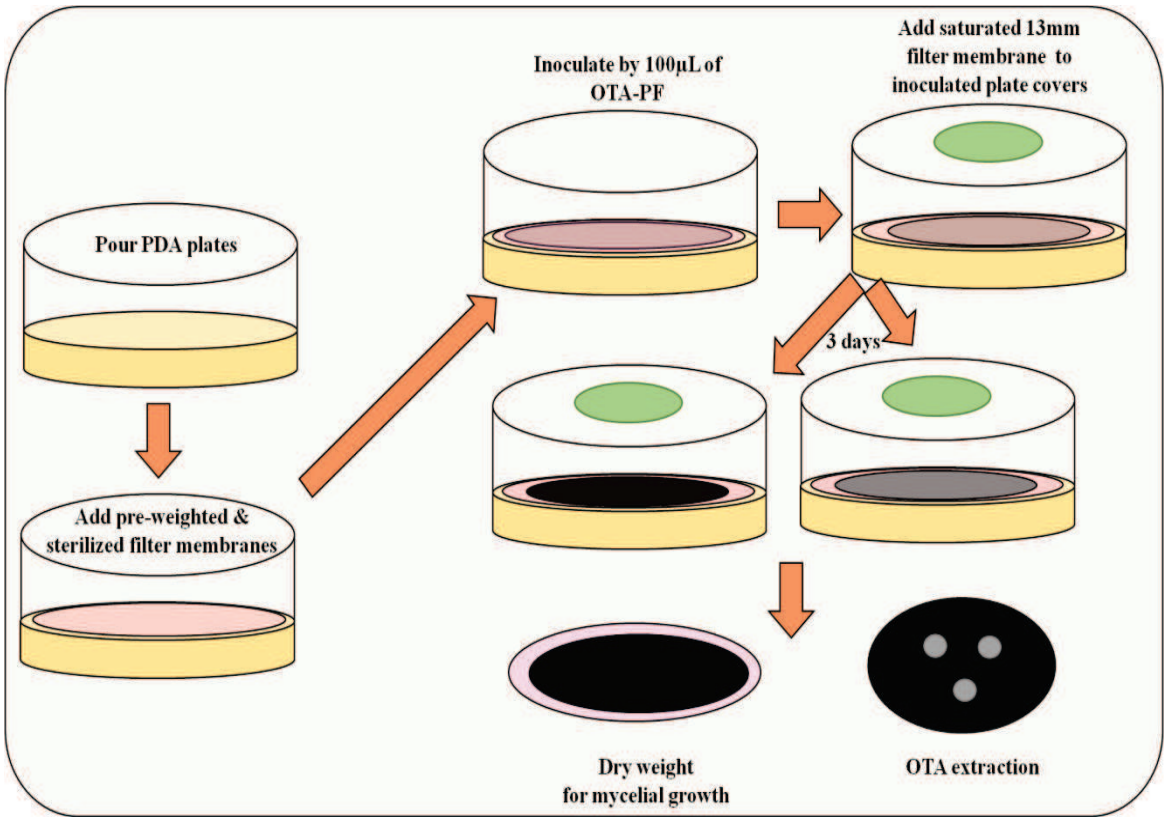



**Figure 2.10: Vapor diffusion method description**

## 2.10. Tests of antifungal activities of isolated strains

### 2.10.1. Antifungal tests

The antagonistic ability of all non-Aspergillus species isolated from Stifénia treatment was tested against *A. carbonarius* [6 strains isolated from GJ and 4 strains from GS]. Three different methods (Fig. 2.11) were applied on PDA petri plates differencing in the distance in between the two fungi and the time of inoculation [at 5 cm distance inoculated at the same time (Bendahmane *et al.*, 2012); at 3 cm distance inoculated at the same time; the antagonist inoculated and incubated for 3 days then inoculated *Aspergillus carbonarius* at 3 cm distance] with three replicates. Then plates were incubated at 25°C and growth reduction and OTA production of *A. carbonarius* was determined (Suarez-Quiroz *et al.,* 2004) at 3, 7, and 10 days. The results were expressed in µg of OTA/g of dry weight. The calibration curve was prepared with OTA dilutions (Sigma) from 0.25 ng/mL to 100 ng/mL for extracted culture samples.

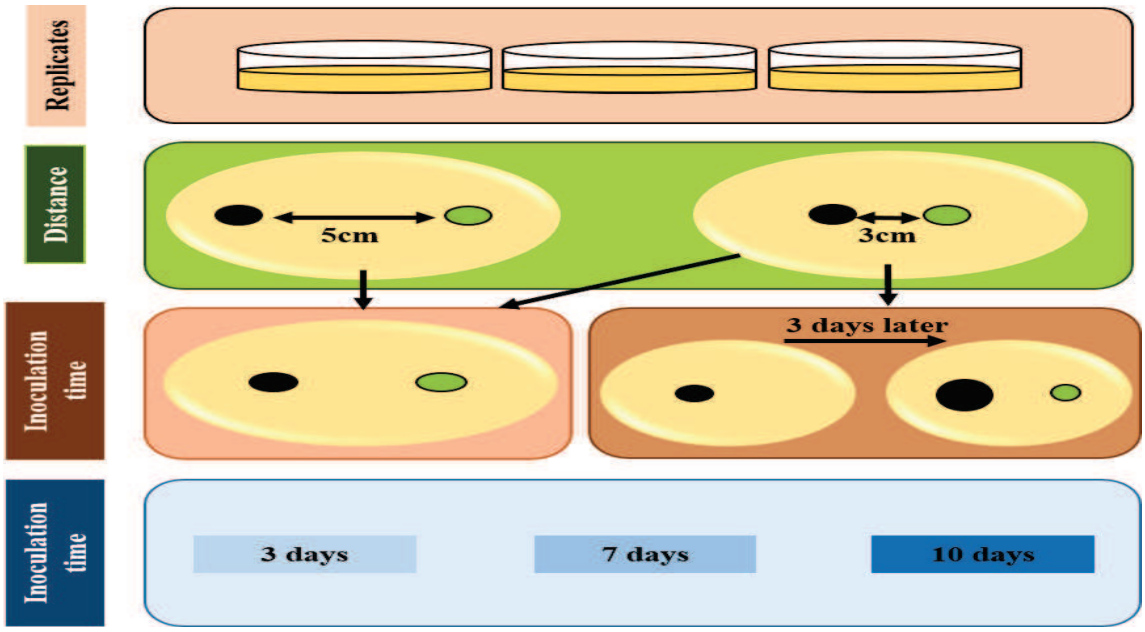



**Figure 2.11: Different tested antagonism modalities**

### 2.10.2. Growth reduction determination

Radical diameters were measured (Fig. 2.12) using a precision measuring tool (Fisher Darex, France). The % of mycelia growth reduction was calculated according the equation of Pandey *et al.,* (1982) and Berber *et al.,* 2009;

$$\% \text{ of Mycelia growth reduction} = \frac{d_c - d_t}{d_t} \times 100$$

Where;

$d_c$ =growth diameter of *A. carbonarius* control colony;

$d_t$ =growth diameter of *A. carbonarius* colony in the presence of different isolated strains under study.

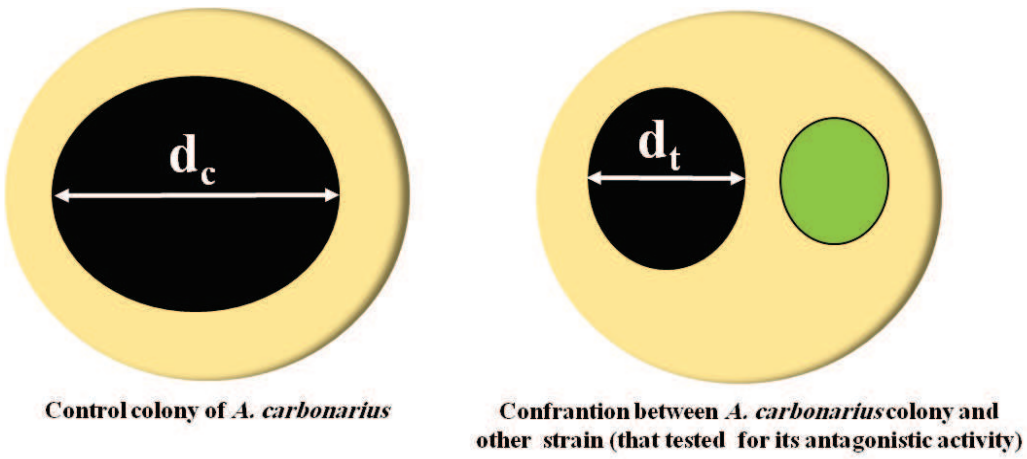



**Figure 2.12.: Radical growth determination method**

## 2.11. Indirect antifungal activity of *Penicillium adametzioides* against OTA-PF

### 2.11.1 Using liquid media culture filtrates

The antifungal activity was tested usind 1, 5, 10, 25, 50, 75, 100% of the cultrures filtrates using four different methods:

**2.11.1.1.** Side Solid diffusion method

By adding saturated sterilized filter with 30 µL of each concentration and inoculating 5 µL of $10^6$ spore suspension of OTA-PF and the same techniques used fot the section 2.9.2.1. were used fot the impact of mycelial growth and OTA production.

**2.11.1.2.** Center Solid diffusion method

The same described protocol with slight modifications, placing the saturated filters at the middle of plates, and inoculating 100 µL of the $10^6$ spore suspension of OTA-PF on all the surface of Petri plates

**2.11.1.3.** Side liquid diffusion method

The same technique described in 1.1.1. but with adding the solution in a whole and the other steps were the same.

**2.11.1.4.** Center liquid diffusion method

The same technique described in 1.1.2. with adding the solution into a whole in the center of plates.

### 2.11.2. Combactin antifungal activity test against OTA-PF:

Four different concentrations (10, 5, 1, and 0 µM) of compactin (Sigma Aldrich, France) mixed with PDA plates in triple replicates were inoculated by 5µL of $10^6$ of *A. carbonarius* spore.ml$^{-1}$ for 3, 7, 13 days. Then their ability of OTA production was measured using HPLC analysis using the method described by Suarez-Quiroz *et al.* (2004).

# Chapter 3

# Results

# Chapter 3

# Results

# Part 1

# PART 1

# IMPACT OF PREHARVEST TREATMENTS (CHEMICAL, BIOLOGICAL, ELICITOR) ON THE BIODIVERSITY AND TOXICIGENICTY OF BLACK ASPERGILLI AND ON THE FUNGAL ECOSYSTEMS

## (MICROBIOLOGICAL, CHROMATOGRAPHICAL AND MOLECULAR BIOLOGY APPROACHES)

### 1. Introduction

Ochratoxin A is a fungal secondary metabolite, mainly produced by the *Aspergillus* and *Penicillium* species. (van der Merwe *et al.*, 1965; Frisvad *et al.,* 1991). This mycotoxin is a common contaminant in food such as coffee cocoa, beer, grains, spices and especially grape juice and wine (van Egmond and Speijers, 1994). OTA has been shown to have a number of toxic effects to animals where the most prominent one is nephrotoxicity (Krogh, 1992). It has been proved to be carcinogenic in the kidney and liver in mice (Kanizawa and Suzuki, 1978) and rats (Bendele *et al*., 1985) in addition to numerous specific toxic effects, such as hepatotoxicity (Chopra *et al*., 2010), teratogenicity (Mayura *et al.,* 1976), carcinogenicity (Bendele *et al*., 1985), and immunosuppressivity (Haubeck *et al.,* 1981). Thus, OTA is suspected of being the main etiological agent responsible for Balkan Endemic Nephropathy (BEN) and associated urinary tract tumor in humans (Vukelic *et al.,* 1992). OTA has been classified as a group 2B carcinogen (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 1993). Thus the European Commission has fixed the maximum limit for OTA in different food and in the case of wine and grape juice this limit wax fixed at 2.0 µg/Kg (Regulation (CE) N° 123/2005, 2006).

Field treatment with fungicides is the traditional prevention technique against fungal contamination of crops as it was mainly done in vineyard (Vagar *et al.*, 2010) and the vine culture is the most fungicide-consuming culture in France. It is in this context that the plan "Ecophyto 2018" was designedin 2008 in order to progressively half the use of pesticides (*i.e.,* fungicides) in France by 2018, while maintaining the economic performance of French agriculture ((MFAP, 2008).

This highlights the importance of finding alternative control methods safer for the environment while ensuring the safety of producers and consumers. This was the main motif for conducting this research in general, by testing the field application of three natural control methods. Two bio-control agents were used (1) *Trichoderma atroviride* which showed efficacy in apple scab control (Kowalska *et al.,* 2011 and (2) *Saccharomyces cerevisiae* which reduced the growth and OTA production of *Penicillium verrucosum* (Petersson *et al.,* 1998) and detoxified the grape juice (Bejaoui *et al.,* 2004). One plant extract (Stifénia® which is a natural product extracted from fenugreek (*Trigonella foenum-greacum*) and its stimulating effect is now recognized on the *Vitis vinifera* L. against *Uncinula necator* pathosystem the causal agent of grape powdery mildew disease. And a comparision between these preharvest treatments and the chemical traditional OTA control method was done.

In this chapter, the impact of these preharvest treatments proposed to control the OTA accumulation in grapes and their products was evaluated by conducting different studies that are illustrated in Fig. R-C1.1 and present in two scientific articles.

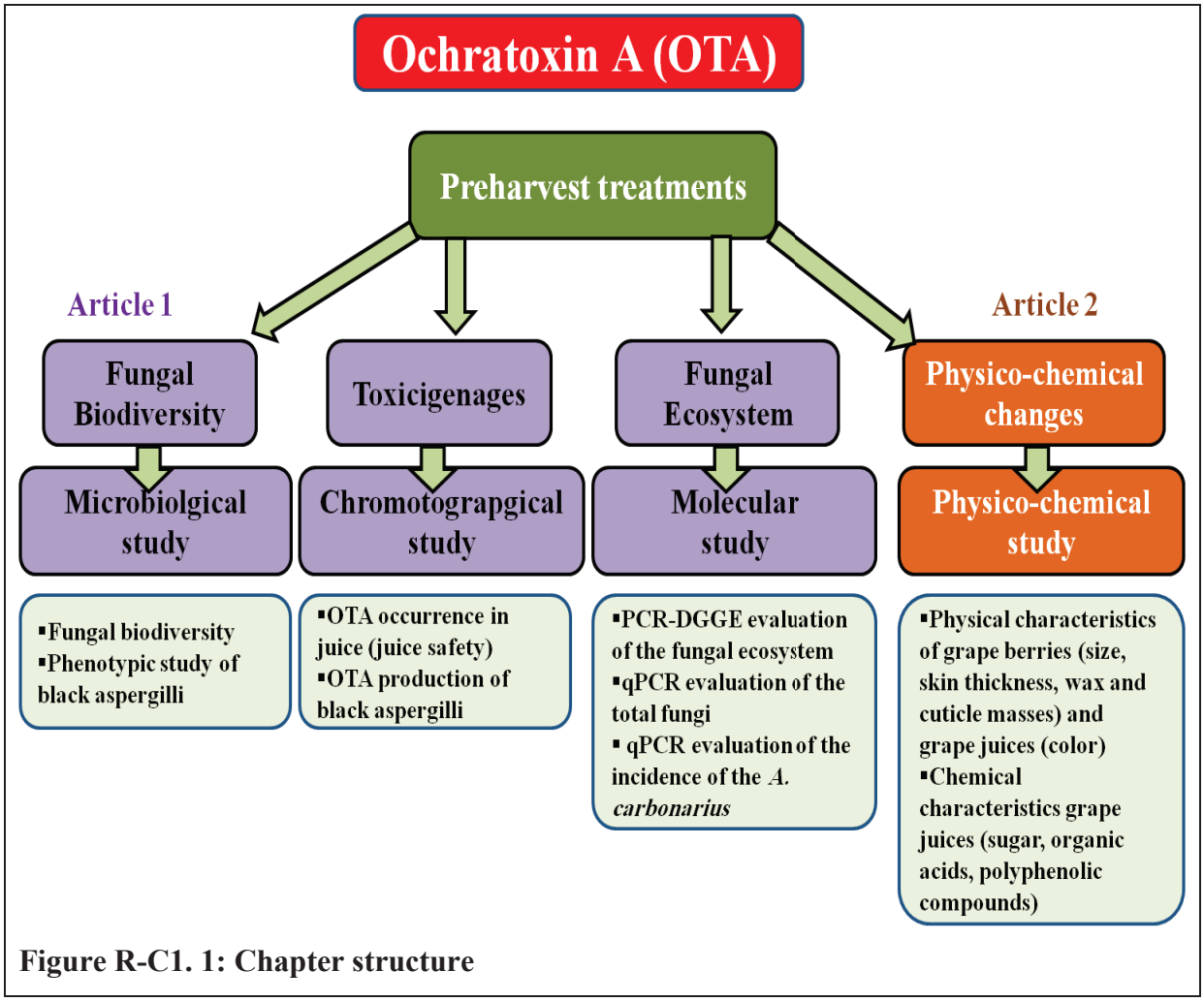



**Figure R-C1. 1: Chapter structure**

# Study of vine preharvest treatments to control *Aspergillus carbonarius* and their effects on the fungal ecosystem and OTA contamination

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Submitted to **Journal of Crop Protection**

**Abstract**

The grape and wine industry is affected by the presence of Ochratoxin A (OTA) in its products because of contamination of grapes by strains of Aspergillus section Nigri. Wine is considered as the second contributor in Europe to the ingestion of this mycotoxin with nephrotoxic, neurotoxic and teratogenic effects, and classified as possibly carcinogenic to humans by the International Agency for Research on Cancer. The European Commission has set the maximum limit for OTA in wine and grape juice to 2 mg/Kg. The objective of this work is to provide non-chemical alternative methods to control OTA contamination in grapes and in processed products as juice and wine, in respect with environment and stakeholder health (producers and consumers). Different treatments were compared in experimental vineyard on near parcels after artificial contamination by *Aspergillus carbonarius*: a chemical fungicide; *Saccharomyces cerevisiae* and *Trichoderma atroviride* as antagonists; and a plant extract as elicitor. Two untreated modalities served as controls, one was artificially contaminated. Q-PCR using universal and specific primers for *Aspergillus carbonarius* had estimated the effect of the different treatments on the presence of *A. carbonarius* .The lowest occurrence of black aspergilli strains was obtained for treatment using elicitation. While, the DGGE gave an overview on effect of treatments on the fungal ecosystem, which showed higher similarity between the non-contaminated and elicitor treatment (76%) followed by the yeast one and the most different was the contaminated one. In addition the microbiological traditional methods of isolation showed that the elicitor treatment had a higher proportion of fungal species not isolated in the other treatments. These results could rapidly evaluate the effect of different treatments on the fungal ecosystem of grapes. Furthermore, the elicitor and yeast bioagent could replace the chemical fungicide because these two treatments had reduced the OTA content at the same level than the fungicide.

**Key words:** OTA, *Aspergillus carbonarius*, *Trichoderma atroviride*, *Saccharomyces cerevisiae,* Stifénia, q-PCR, DGGE, alternative control method, fungicide, fungal ecosystem.

## 1. Introduction

Rousseaux *et al.* (2014) has demonstrated the complex of grape microflora including filamentous fungi, yeasts and bacteria with different physiological characteristics and effects on wine production. Most studies have focused on the wine microbiota, but a few studies have reported the ecology of grape microorganisms. Some of these organisms such as non-Botrytis bunch rotting fungi, which greatly influence the safety or sensory quality of wine, due to the production of mycotoxins [*i. e.* Ochratoxin A (OTA)] and off-flavors, respectively are considered to be spoilage agents.

OTA is a secondary metabolite produced by moulds of the genera *Aspergillus* and *Penicillium*, which has nephrotoxic effect (Vrabcheva *et al*., 2000), and also exhibits immunosuppressive (Petzinger and Weidenbach, 2002), teratogenic (Castegnaro and Pfohl-Leszkowicz, 2002) and carcinogenic (2B group) (IARC, 1993) effects. OTA is one of the most common naturally occurring mycotoxins, contaminating a wide range of different plant products, including cereals, coffee beans, cocoa, nuts, spices, dried fruits, beer and wine (SCOOP, 2002). In Europe, wine and especially red wine has been determined as the second major source of human exposure to OTA following cereals and preceding coffee and beer (SCOOP, 2002; Walker, 1999). Thus the European Commission has fixed the maximum limit for OTA in wine and grape juice at 2.0 µg/Kg (Regulation (CE) N° 123/2005, 2006).

Member of black *Aspergillus* species section *Nigri* are mainly responsible for OTA accumulation in grape juice in particular *Aspergillus carbonarius* and *Aspergillus niger* (Abarca *et al*., 1994; Samson *et al*., 2004; Téren *et al*., 1996; 1997; Varga *et al*., 2004; Suàrez-Quiroz *et al*., 2004b). In the Mediterranean zone where OTA wine and grape juice contamination was mostly found, grapevine are the most frequently contaminated by the fungi of *Aspergillus carbonarius* and other members of the section *Aspergillus niger* (Dachoupakan *et al.,* 2009).

All the studies that were done since 1996 showed that Mediterranean grape juice and wine were the most contaminated by OTA, but not all the Mediterranean viticulture orchards were at the same OTA contamination level (Rousseau, 2004). Regions that were near from sea were highly contaminated by OTA.

The estimation of fungal growth or biomass is not easy, because no primary standard exists. Although, quantifying techniques of food fungi have been improved in recent years, the dilution plating technique is the most relied technique in viable counting (Pitt and

Hocking, 2009) and the PCR-DGGE which is well known as fingerprinting technique for the food bacterial and yeasts ecology (Fleet, 2007; El-Sheikha *et al.,* 2009; 2012*)*

Several studies have been conducted to evaluate the ecology of soil fungi. Oros-Sichlera *et al.* (2006) have developed a semi-nested PCR system coupled with DGGE fingerprinting that offers a robust, reliable and sensitive tool for the analysis of soil fungal community structure. van Elsasa *et al.* (2000) have used 18S rDNA based fungal-specific primers in PCR-DGGE profiling of soil fungal communities, which has allowed the resolution of separation of mixtures of PCR products of several different fungi, as well as products resulting from mixed-template amplifications, into distinct banding patterns. Several authors simply used CFU counting or the global respiratory activity in inoculated soils (Thion *et al.,* 2013).

Molecular methods targeting specific regions of DNA have been developed for rapid, highly specific identification of the grape fungal ecosystem (Rousseaux *et al.*, 2014), but very few studies have focused on the characterization of fungal communities on grapes. Only Doaré-Lebrun *et al.* (2006) and Laforgue *et al*. (2009) have reported the use of PCR-TTGE or PCR-DGGE methods for describing the communities of fungi growing on grapes. Diguta *et al.* (2011) recently reported a rapid, easy and reliable culture-dependent method, PCR ITS-RFLP, for the identification of fungi isolated from grapes to species level without the need for sequencing. However, there is still a need to develop techniques for improving the characterization of fungal diversity on grape berries without the need of culture step, particularly for the minority of genera and species present. For other microorganisms, various culture independent methods, such as *in situ* hybridization flow cytometer with fluorescent antibodies and qPCR, have been used, to analyze the yeast ecosystem in grape must, for example ( Zott *et al*., 2010, Rousseaux *et al.*, 2014).

The main aim of this study is assessing alternative suitable and save control method to the conventional fungicide treatments, in order to minimize the OTA accumulation in the grape juice and avoiding their negative effect of their residues on the environmental natural balance and on human health. A field experiment with different proposed OTA-control treatments was conducted and the impact of these treatments was evaluated on the fungal ecosystem in general and particularly, on the black aspergilli biodiversity and toxigenicity using traditional microbiological method and the new settled molecular methods (qPCR and PCR-DGGE).

## 2. Material and methods

### 2.1. Strains origin and *In vitro* experiment

The fungal strains [*Trichoderma atroviride* and *Aspergillus carbonarius*] and the yeast strain *Saccharomyces cerevisiae* were obtained from IFV, Narbonne, FRANCE.

Six different modalities of treatments were applied on the experimental viticulture orchard PECH-ROUGE of INRA (*Institut Nationale de Recherché Agronomique*) and IFV (*Institut Français de la vigne et du vin*), Narbonne (Languedoc-Roussillon region), France, as shown in Tab. 1, near parcels of *Mourvèdre,* which is a cultivar that was recognized as being sensitive to OTA contamination.

**Table 1. The different treatments applied on the cultivar *Mourvèdre* at the experimental field of IFV, Narbonne, France; during the year 2010.**

| | Characteristics of treatments | | | | |
|---|---|---|---|---|---|
| | Contamination* | Control | | Treatment Time | |
| | | *Type* | *Name* | *Frequency* | *Time* |
| 1 | - | - | - | - | - |
| 2 | + | - | - | - | - |
| 3 | + | Chemical fungicide | ** Scala® | Twice | At the green tip-stage; at leaves output-stage |
| 4 | + | Fungal bio-agent | *Trichoderma atroviride* | Twice | at the green tip-stage, at leaves output-stage |
| 5 | + | Yeast bio-agent | *Saccharomyces cerevisiae* | Once | at leaves output-stage |
| 6 | + | Plant extract (elicitor) | *** Stifénia® | Multiple | Starts at the green-tip stage till harvesting stage with 15days intervals |

* Artificially contaminated by OTA-PF [OTA producing fungi (*Aspergillus carbonarius*)] at the veraison stage.

** Scala®: systemic fungicide used against gray mould (Pyriméthanil 400g/L, BAYER, France)

*** Stifénia®: homogenized fenugreek seed powder, SOFT, France.

### 2.2. Samples

Grapes were harvested in late September 2010. Grape bunches (5 kg/ treatments) were randomly collected for each treatment and placed in previously two sterilized bags, which

were kept at about 4°C until analysis. Mycological analysis was immediately done and the remaining samples were kept frozen at -20°C.

### 2.3. Mycological analysis of grapes

#### 2.3.1. Media cultures

PDA (Potato Dextrose Agar: 4 g potato extract; 20 g glucose; 15 g agar) (Biokar Diagnostics, Beauvais, France), PDA with low pH 3.2 (about 10 mL of sterilized tartaric acid after autoclaving at 121°C for 15 min), and YM (Yeast Malt liquid medium: 3 g yeast extract; 3 g malt extract; 5 g peptone; 5 g glucose; 20 g agar /L distilled water, autoclaved at 121°C for 15 min) (Samson et al., 1995; Pitt and Hocking, 2009).

#### 2.3.2. Enumeration and isolation

##### 2.3.2.1. from grape juice

Decimal dilutions of grape juices [about 150 g of randomly grape clusters parts were homogenized using VWR Star Blender™ LB400 (France) without external water and filtered under pressure] using physiological water containing 0.01% Tween 80 (Merck Germany) were done. 100 µL of each dilution were spread on PDA (3.5 pH) in Petri plates and incubated at 25°C for 5 to 7 days in the dark. After incubation, the number of CFU (colony forming units) of filamentous fungi per milliliter of juice homogenate was evaluated (AFNOR, 2002).

##### 2.3.2.2. from grape stalks

To estimate the microbial population in the grape stalks (GS), the same protocol described above was used using 25-35 g of GS mixed with 100 mL of sterile physiological water and homogenized by stomacher for 2 min and decimal dilutions with sterile physiological water (NaCl; 8.5 g/L, 0.01% Tween 80 (v/v)). The same practices and conditions that were used for grape juices enumeration were applied to evaluate the CFU of filamentous fungi number per milliliter of homogenate grape stalks (AFNOR, 2002).

### 2.4. OTA content

#### 2.4.1. OTA extraction from grape juice

Fifty grams of each sample was homogenized with 200 mL of 70% methanol using a blender at high speed for 1 min and filtered. The filtered extracts were diluted (1: 5) by PBS/0.01% Tween 20 (washing buffer) (Sigma-Aldrich, France). Twenty milliliters of each filtered diluted extracts were passed through OchraTest™ affinity columns at a rate of

approximately 1 drop/sec until air passed through the column. The columns were then washed with washing buffer (1-2 drops/sec) followed by 10 mL of purified water (1-2 drops/sec). The OTA content were collected in 1.5 mL of methanol:acetic acid (98:2, v:v) (1 drop/sec) (Skarkova *et al.*, 2013).

### 2.4.2. OTA extraction from *Aspergillus* cultural media

Colony diameters were measured and three agar plugs of five millimeters of diameter were removed from the *A. carbonarius* colonies at three, seven, ten, fifteen and twenty days of incubation. The plugs were weighed, introduced into 3 mL vials and extracted with 2.5 milliliters of solvent (methanol:formic acid, 25:1) under sonication for 15 min. The solvent was then evaporated under a nitrogen stream at 40 °C. The dried extracts were re-suspended in the mobile phase of the subsequent HPLC (49.5% deionized water, 49.5% acetonitrile, 1% acetic acid) then filtered with a syringe (MiniSart SRP 4 of diameter 0.45 µm, Sartorius, Germany) (Suarez-Quiroz et al., 2004a; Barberis *et al,* 2009; Dachoupakan *et al*., 2009).

### 2.4.3. Analysis OTA by HPLC

OTA was analyzed by HPLC (Pump, Shimadzu LC/9A, Japan) with fluorescence detection ($\lambda_{exc}$333 nm; $\lambda_{em}$460 nm, Shimadzu RF-10AXL, Japan), using a C18 column (25 x 4.6 mm, 5 µm, Phenomenex®, USA). The mobile phase [49% acetonitrile (Carlo Erba Reagent, France, P00637G21), 49% Milli-Q water (Quantum® Ex, MILLIPORE), and 1% acetic acid] was pumped at 1 mL/min. The injection volume was 20 µL, and the retention time was approximately 16.3 min. The calibration curve was prepared with OTA solution (Sigma Aldrich, France, 34037-2ML-R) from 0.05 to 5 ng/ mL. The limit of detection (LOD) and limit of quantification (LOQ) of this method which was applied to grape juice samples, were established at 0.023 µg/L and 0.076 µg /L, respectively (Dachoupakan *et al.*, 2009). OTA amounts were presented in µg for g of the fungal dry weight.

## 2.5. DNA extraction from grape berries

The method described by El-Sheikha *et al*. (2009; 2012) was used for the DNA extraction from grape berries as shown in Fig. 2.4. About 5-8 berries was grounded by hand in 5-8 mL oh peptone water then 2 mL were added to sterile eppendorf tubes with 0.3g of 0.5 mm diameter acid wash glass beads 425 - 600 µm (Sigma, France). The mixture was vortexed vigorously for 30 min in bead beater instrument (Vortex Genie 2 SI-A256, USA) then centrifuged at 12000 rpm/15 min and the suspension discarded. The cell pellet was re-suspended in 300 µL of breaking buffer [2% Triton X-100 (Prolabo, France), 1% SDS(

sodium dodecyl sulphate; Sigma, France), 100 mM NaCl (Sigma), 10mM Tris, pH 8.0, 1mM EDTA, pH 8.0 (Promega, France)]. 100 µL TE [10 mM Tris – HCl, 1mM EDTA, pH 8.0 (Promega), 100 µL lysozyme solution (25 mg/mL, Eurobio, France) and 100 µL proteinase K solution (20 mg/mL, Eurobio, France) were added and the mixture was incubated at 42°C for 20 min. then 50 µL 20% SDS were added to each tube, then incubated at 42°C for 10 min. The tubes were vortexed vigorously for 5 min.400 µL MATAB (mixed alkyltrimethyl ammonium bromide; Sigma) were added to each tube, then incubated at 65°C for 10 min. The lysates were then purified twice by repeated extraction with 700µL phenol: chloroform: Isoamyl alcohol (25:24:1; Carlo Erba, France) and the tubes were handily vortexed for 5 min and then centrifuged at 12000 rpm/15 min. The aqueous layer was transferred to an eppendorf vial and the residual phenol was removed by extraction with 600 µL chloroform: Isoamyl alcohol (24:1) and centrifuged for 15 min at 12000 rpm. The aqueous phase was collected and the DNA was stabilized with 30 µL sodium acetate (3M, pH 5), followed by precipitation by adding equal volume of ice cold Isopropanol, and stored at -20°C for 12 h (overnight). After centrifugation at 12000xg for 15 min, the supernatant was eliminated, DNA pellets were washed with 500 µL 70% ethanol and the tubes were centrifuged at 12000 g for 15 min. The ethanol was then discarded and the pellets were air-dried at room temperature for 45-60 min. finally, the DNA was re-suspended in 50 µL ultrapure water and stored at -20°C until analysis.

### 2.5. Real-time PCR amplification

Two quantitative PCR were conducted using two sets of primers (Table 2). Real time PCR (q-PCR) amplifications and detections were carried out using The LightCycler® 480 Real-Time PCR instrument (Roche, USA). Each sample was prepared in duplicates of 6 µL reaction mixtures (Roche diagnostics, Germany) in Micro-Amplify optical 384-well reaction plates and sealed with optical adhesive covers. Each reaction well contains 2 µl of template DNA, 3 µl of 2× Quantitect SYBRR® Green I Mix (Qiagen, Courtaboeuf, France), and 0.6 pmol/µL (final concentrations) of each forward and reverse primers. Real-time qPCR was conducted the conditions shown in table 3.

**Table 2: A description of the primers group used for the real time PCR assays**

| | Primer | | | Amplified length | Reference |
|---|---|---|---|---|---|
| | Name | Sequence (5'-3') | Region | | |
| Specific primers for *A. carbonarius* | Ac12RL-OTAf | aatatatcgactatctggacgagcg | Non-conserved regions in the AT domain of PKS gene (Ac12RL3) | 141 bp | Atoui *et al.*, 2007 |
| | Ac12RL-OTAr | ccctctagcgtctcccgaag | | | |
| Universal primers for all fungi | ITS1f | tccgtaggtgaacctgcgg | 5.8 S | 300 bp | Fierer *et al.*, 2005 |
| | 5.8s | cgctgcgttcttcatcg | | | |

To generate the standard curve, a: for the specific primers 10-fold dilutions (ranging from 1 µg to 1 pg) of *A. carbonarius* (CBS 120167) DNA whose concentration was previously determined were subjected to real-time PCR. The standard curve is a plot of the threshold cycle (Ct) versus log DNA concentration. Then, DNA grape samples were subjected to real-time PCR and the amount of *A. carbonarius* DNA was calculated according to the generated standard curve. Control sample without DNA template was included in the experiment runs. B: for the universal primers a decimal dilution was made for the two control treatments.

**Table 3: Real time PCR conditions**

| | Pre-cycles steps | | 40 cycle | | |
|---|---|---|---|---|---|
| | 1 | 2 | Denaturation | Annealing | Extension |
| ITS1/5.8s | 50°C/ 2 min | 95°C/ 15 min | 95°C/15s | 58°C/30s | 72°C/30s |
| Ac12RL-OTAf/r | --- | | 95°C/ 1 min | 53°C/ 30 s | 72°C / 1 min |

### 2.6. PCR-denaturing gradient gel electrophoresis (DGGE) analysis

Fragments of 260 bp in the 28S region was amplified using eukaryotic universal primers U1f-GC (50-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA-30, Sigma, France) and the reverse primer U2r (50-GAC TCC TTC GTC CGT GTT-30 , Sigma, France) (El Sheikha *et al.,* 2012). A 30-bp GC-clamp (Sigma, France) was added to the forward primer (the GC-clamp is underlined). PCR was performed in a final volume of 25 mL containing 2 µL of each primers, 0.5 µL dNTPs mixture, 2 µL $MgCl_2$, 5 µL of 10x of reaction Taq buffer $MgCl_2$ free (Promega, France),1.25 U of Taq DNA polymerase (Promega, France), and 2 mL of the extracted DNA (30 ng). PCR was run for 30 cycles with

annealing at 50 °C for 50s, extension at 72 °C for 90s, and denaturation at 95 °C for 45 s (El Sheikha *et al.*, 2012).

Two µL PCR products were analyzed first by conventional electrophoresis in 0.8% (w/v) agarose gel with TAE 1x buffer (40 mM Trise-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM $Na_2$-EDTA), stained with ethidium bromide 0.5 mg $mL^{-1}$ in TAE 1x and quantified by using a standard (DNA mass ladder 100 bp, Promega, France). The PCR products were analyzed by DGGE by using a Bio-Rad Dcode™ universal mutation detection system (Bio-Rad Laboratories, USA) using the procedure first described by El Sheikha *et al.* (2009). Samples containing approximately equal amounts of PCR amplicons (30 µL) were loaded into 8% (w/v) polyacrylamide gels (acrylamide/N,$N^1$-methylene bisacrylamide, 37.5/1, Promega, France) in 1x TAE buffer (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM $Na_2$-EDTA). All electrophoresis experiments were performed at 60 °C using a denaturing gradient ranging from 40% to 70% (100% corresponded to 7 M urea and 40% [v/v] formamide, Promega, France). The gels were electrophoresed at 20 V for 10 min and then at 80 V for 16 h. After electrophoresis, the gels were stained for 30 min with ethidium bromide and rinsed for 20 min in distilled water and then photographed on a UV transilluminator with the Gel Smart 7.3 system (Clara Vision, Les Ulis, France).

Banding patterns were standardized with the control-1 modality as reference patterns included in gel. This software permitted to identify the bands relative positions compared with the standard patterns. In DGGE analysis, the generated banding pattern is considered as an image of all of the major fungi in the populations. Gel images were stored by using The Imager system (Ampligene). Quantification of ethidium bromide-stained bands for competitive PCR experiments was performed with the ImageQuant TL software v.2003 (GE Healthcare, USA). A simple resampling method has been used to evaluate it. Each of the 6 samples was doubled. Differences of fungal communities on berries of different treatments were determined.

### 2.7. Statistical analysis

An ANOVA analysis was conducted using Statistica 10 software (Stat Soft. Inc., Tulsa, USA). The Duncan test was used to detect any significant differences between the examined groups, and significance levels were defined using $P \leq 0.05$. DGGE results were statistically analyzed using a factorial correspondence analysis.

# 3. Results

## 3.1. Quantitative characterization for the fungal microflora

### 3.1.1. Microbiological study

Stifénia® treatment had the lowest CFU of total microflora in general (including the black Aspergilli strains) isolated from grape juices, followed by *Saccharomyces* treatment (Fig 1A) comparing with all treatments under study. The situation was the same for CFU of black *Aspergilli* isolated from grape stems with the Stifénia® treatment followed by *Saccharomyces* one (Fig. 1B).

Both Stifénia® and *Saccharomyces* treatments had reduced the contamination of grape juice by the black *Aspergillus* species number by 66.7% comparing with the control treatment (contaminated by OTA-PF not treated) followed by *Trichoderma* and chemical (Scala®) ones (58.3%). The effect of the *Saccharomyces* treatment was limited, which reduced the number of black Aspergilli species in grape stem by 14%. Stifénia® occupied the highest level of reduction in the number of *Aspergillus* species isolated from grape stems (71.5%) followed by *Trichoderma* and Scala® (43%) each.

The treatment Stifénia® had mainly reduced spore potential of total microflora with references to the black *Aspergillus* strains comparing with other treatments (99.8%) followed by *Saccharomyces* treatment (73.7%) in case of the isolation from grape juice; While, in the case of grape stems isolation, the *Saccharomyces* treatment highly reduced the spore potential of black *Aspergillus* (86%) followed by Stifénia® treatment (83.4%). In grape juice, the treatments *Trichoderma* (- 42%) and Scala® (-10%) had a negative effect on the potential spores of the black *Aspergillus* strains (where, the spore incidence had increased comparing with the control treatment (contaminated not treated)). But the opposite was true using grape stem as a source of isolation, where a positive reduction was observed in *Trichoderma* treatment (42%) followed by Scala® (21%).

The OTA production ability of isolated black aspergilli strains showed high variations. Hence, certain isolates of black Aspergilli species had the same behavior of OTA production with high OTA amounts produced by OTA-PF used for *in-vivo* experiment contamination. Approximately, one isolate from the GJ with the treatment Stifénia and control 1 modality or more than one isolate were isolated from yeast and fungal bioagents, and chemical fungicide) (Fig 2 A & B). Whereas, other strains had produced on cultural media variable quantities of OTA during the aging of the fungi (Fig 2C).

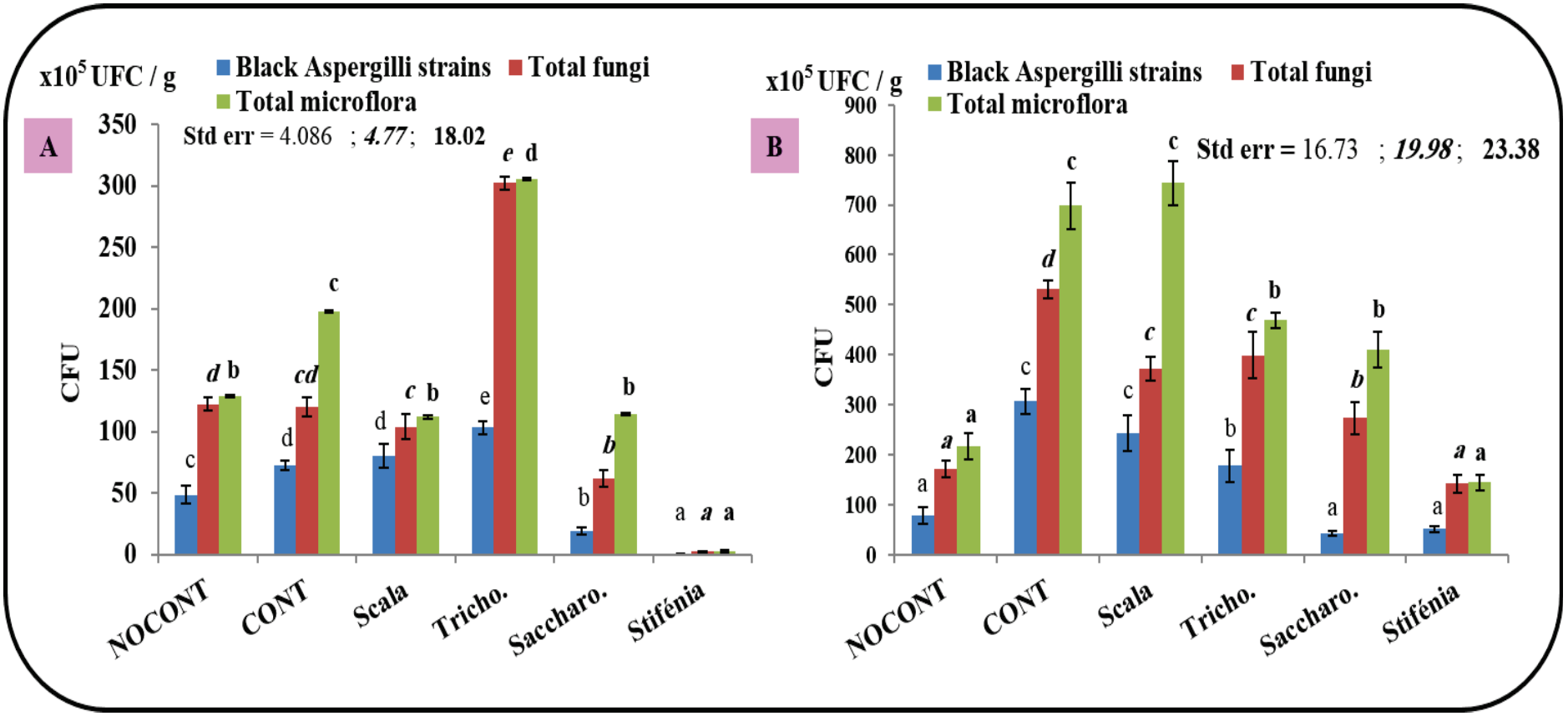


**Figure 1: CFU of total microflora (black Aspergillus, total fungi, and total yeasts) for A: Grape Juice (GJ), B: Grape Stems (GS).**

*Statistically analyzed using ANOVA (one factor analysis test, Duncan, P≤0.05)*

***NOCONT**: OTA-PF-uncontaminated & untreated modality; **CONT**: OTA-PF-contaminated-but-untreated; **Scala**: OTA-PF-contaminated & Scala® treated; **Tricho**: OTA-PF-contaminated & Trichoderma atroviride treated; **Saccharo**: OTA-PF-contaminated & Saccharomyces cerevisiae treated; **Stifénia**: OTA-PF-contaminated & Stifénia®.*

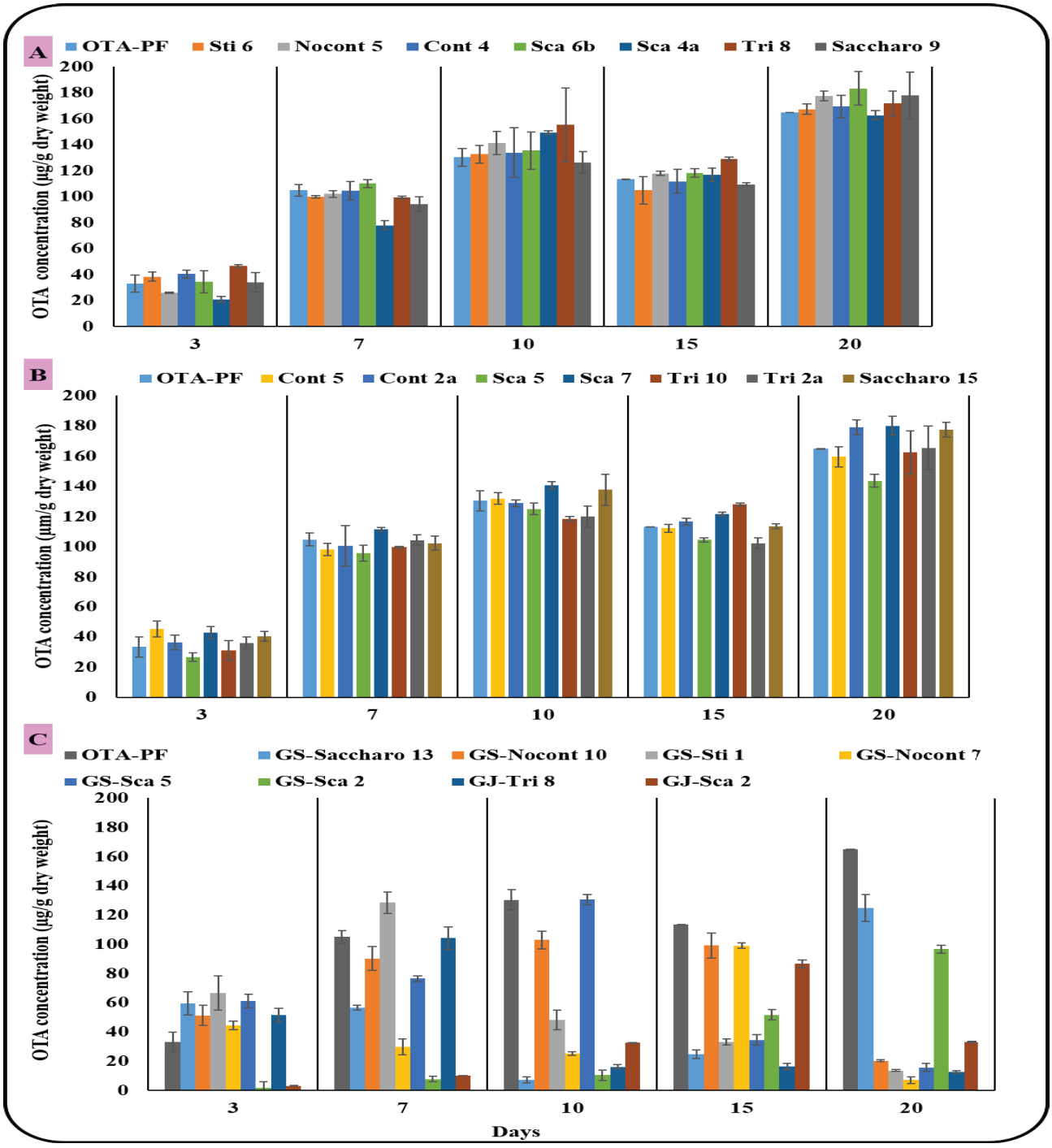


**Figure 2: OTA amounts (µg/g dry weight) produced by major black aspergilli isolates from different treatments isolated from A:** GJ with the same OTA-PF profile of the OTA production; **B** GS with the same OTA-PF profile of the OTA production; **C:** GJ and GS with the different OTA-PF profile of the OTA production.

***Nocont**: OTA-PF-uncontaminated & untreated modality; **Cont**: OTA-PF-contaminated-but-untreated; **Sca**: OTA-PF-contaminated & Scala® treated; **Tri**: OTA-PF-contaminated & Trichoderma atroviride treated; **Saccharo**: OTA-PF-contaminated & Saccharomyces cerevisiae treated; **Sti**: OTA-PF-contaminated & Stiénia®.*

The contamination by OTA-PF permitted the encroachment of the OTA content to be higher than the authorized OTA content (2 µg/L) to be 2.6 ± 0.098 µg/L (*contaminated-and-untreated* control). Whereas, the *uncontaminated-untreated* control grape juice- OTA- content was present at extremely low quantities (1.1 ± 0.014 µg/L). The plant natural elicitor treatment, (Stifénia®) and yeast bioagent treatments allowed OTA reduction at the same efficacy as chemical treatment with no significant difference (1.5, 1.5 and 1.55 µg/L, respectively), which significantly reduced the juice OTA contamination rate by 42.3 to 38%. OTA (Fig. 3). The fungal bioagent treatment had the lowest effectiveness compared with other treatments for the reduction of OTA because the juice contains 2.1 ± 0.14 µg/L.

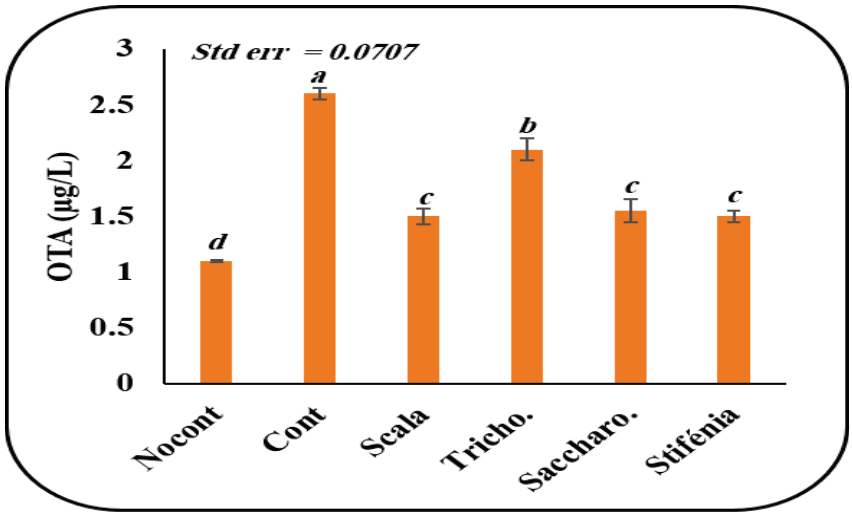


**Figure 3: OTA concentrations of grape juices (µg/L).**

***Nocont**: not contaminated by OTA-PF or by treated modality; **Cont**: artificially contaminated by OTA-PF and untreated modality; **Scala:** artificially contaminated by OTA-PF and treated by the Scala® fungicide; **Tricho:** artificially contaminated by OTA-PF and treated by the fungus Trichoderma atroviride; **Saccharo:** artificially contaminated by OTA-PF and treated by the yeast Saccharomyces cerevisiae; **Stifénia:** artificially contaminated by OTA-PF and treated by the Stifénia® treatment.*

*Statistically analyzed using an ANOVA (Duncan at P≤0.05%), STD err = 0.07.*

### 3.1.2. Molecular quantification by real time PCR (qPCR)

The amplified DNA of different treatment's grape juices using qPCR with the specific OTA-PF primers (efficiency: 1.909) had relatively estimated regarding the OTA-PF incidence in Control-1 (Fig. 4A) and Control-2 (Fig. 4B). The contamination of grapes had significantly increased the potential content of *A. carbonarius* two times more its natural incidence in the open field (Control 1) (Fig. 4A). All the tested OTA-control treatments had significantly reduced the incidence of the OTA-PF in the grape juice (Fig. 4B). The chemical fungicide had

equal OTA-PF to that found in uncontaminated modality (Fig. 4A) and approximately 3.02 % of that found in the contaminated-untreated modality (Fig. 4B).

The elicitor treatment was the most effective treatment in relative reduction of the OTA-PF regarding both of the control modalities, Control-1 and 2 (94% and 99.8%, respectively) (Fig. 4), followed by the fungal and yeast bioagents.

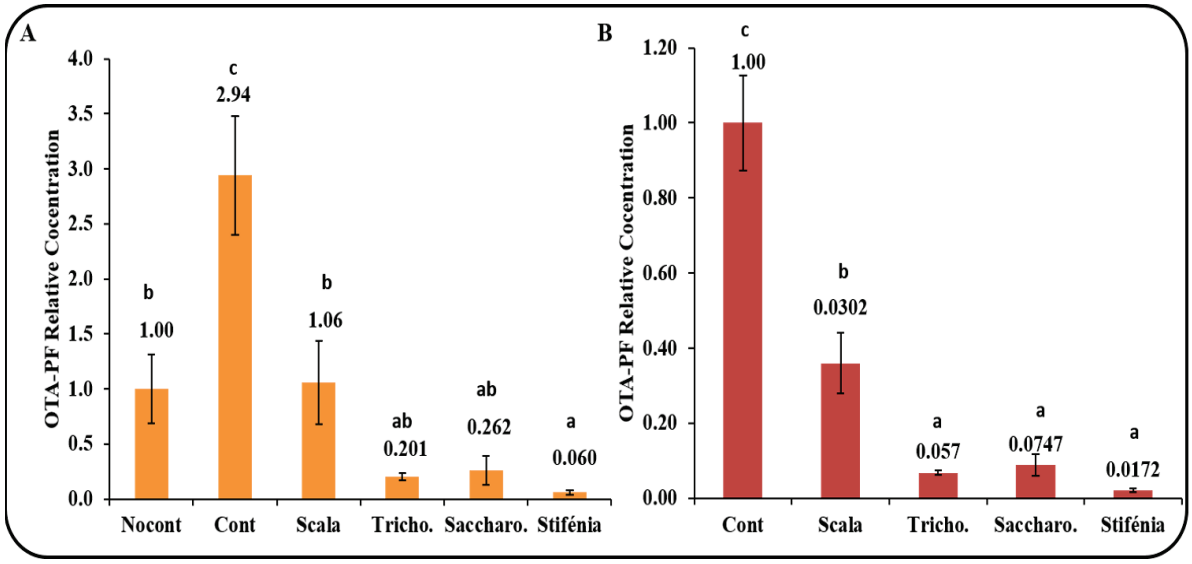


**Figure 4: OTA-PF relative concentration of real time PCR using *A. carbonarius* specific primer set Ac12RL-OTAf/r regarding to A: Control-1 (Nocont), B: Control-2 (Cont).**

***Nocont**: not contaminated by OTA-PF or by treated modality; **Cont**: artificially contaminated by OTA-PF and untreated modality; **Scala:** artificially contaminated by OTA-PF and treated by the Scala® fungicide; **Tricho:** artificially contaminated by OTA-PF and treated by the fungus Trichoderma atroviride; **Saccharo:** artificially contaminated by OTA-PF and treated by the yeast Saccharomyces cerevisiae; **Stifénia:** artificially contaminated by OTA-PF and treated by the Stifénia® treatment. Statistically analyzed using ANOVA (one factor analysis, Duncan test, P≤0.05) std err= 0.174.*

The absolute concentrations were estimated for different amplified total fungal microflora (TFM) – qPCR using the universal primer set [efficiency: 1.728, regarding the OTA-PF standard curve (1 (703.4 µg/µL) to $10^8$ dilutions)] (Fig. 5). The highest significant fungal incidence was observed in the fungal biocontrol treatments (1230µg/µL ± 195.8), followed by that present in the two control modalities (Control-2 and -1; 104.4 ± 81.4 and 923.8 ± 44.2 µg/µL, respectively). The other OTA control treatments had significantly decreased the TFM regarding the two control modalities (Fig. 5). The fungal ecosystem in the Stifénia® treatment had the most significant reduction (109.1 ± 7.1).

Whereas, the absolute concentrations of different amplified OTA-PF DNA upon the specific-qPCR had also calculated and showed in Fig. (5), which has the same effect that described in the relative estimation showed in Fig. (4A).

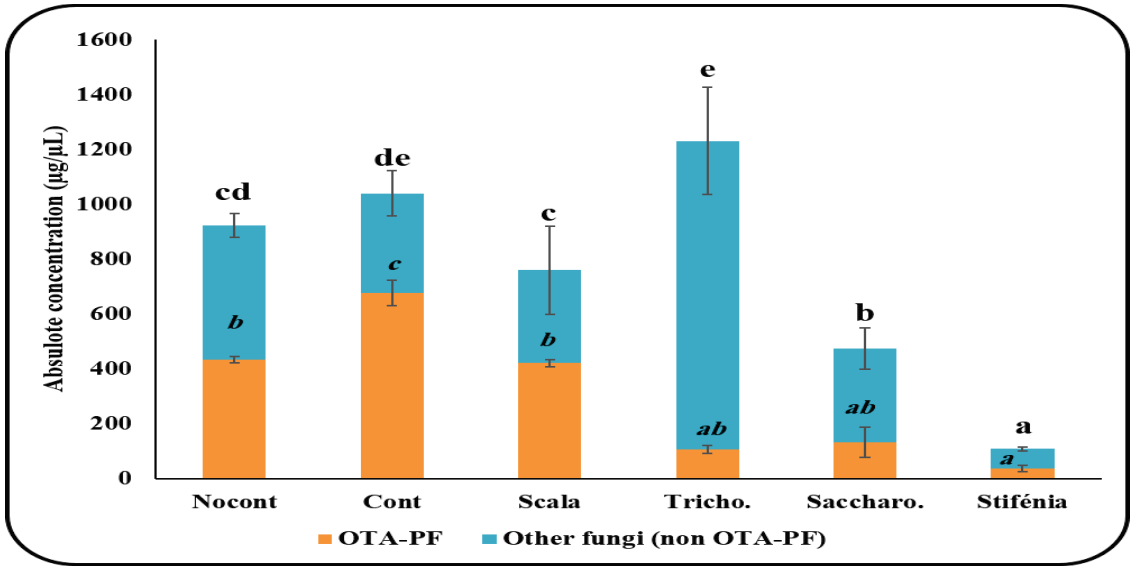


**Figure 5: Real time PCR Absolute concentration of the *Aspergillus carbonarius* (OTA-PF) and other fungi (non OTA-PF, including other black aspergilli strains) using (Ac12RL-OTAf/r and ITS1f/5.8S primer sets, respectively).**

***Nocont:*** *OTA-PF-uncontaminated-untreated modality;* ***Cont:*** *OTA-PF-contaminated-untreated modality;* ***Scala:*** *OTA-PF-contaminated & Scala® treated treatment;* ***Tricho:*** *OTA-PF-contaminated & fungal bioagent treatment;* ***Saccharo:*** *OTA-PF-contaminated & yeast treated treatment;* ***Stifénia:*** *OTA-PF-contaminated & Stifénia® treated treatment.*

*Statistically analyzed using ANOVA (one factor analysis, Dancun test, P≤0.05) std err (OTA-PF) = 73.3, Std err (total fungi) = 66.16.*

## 3.2. Qualitative study

### 3.2.1. Microbiological study

According to the morphological characteristics of different fungal isolates on several culture media (PDA, MEA and CYA) and microscopic observations (data was not included), different isolates were divided in two major types: black aspergilli strains (BA) and non-black aspergilli strains (NBA). The highest number of black aspergilli strains (BA) were isolated from the GS of the control-2 modality (n =12) and from the GJ of the control-1 modality (Fig. 6). The fungal bioagent treatment had a variation in NBA species number isolated from different grape source (GS and GJ, n = 4 and 9 isolates respectively).

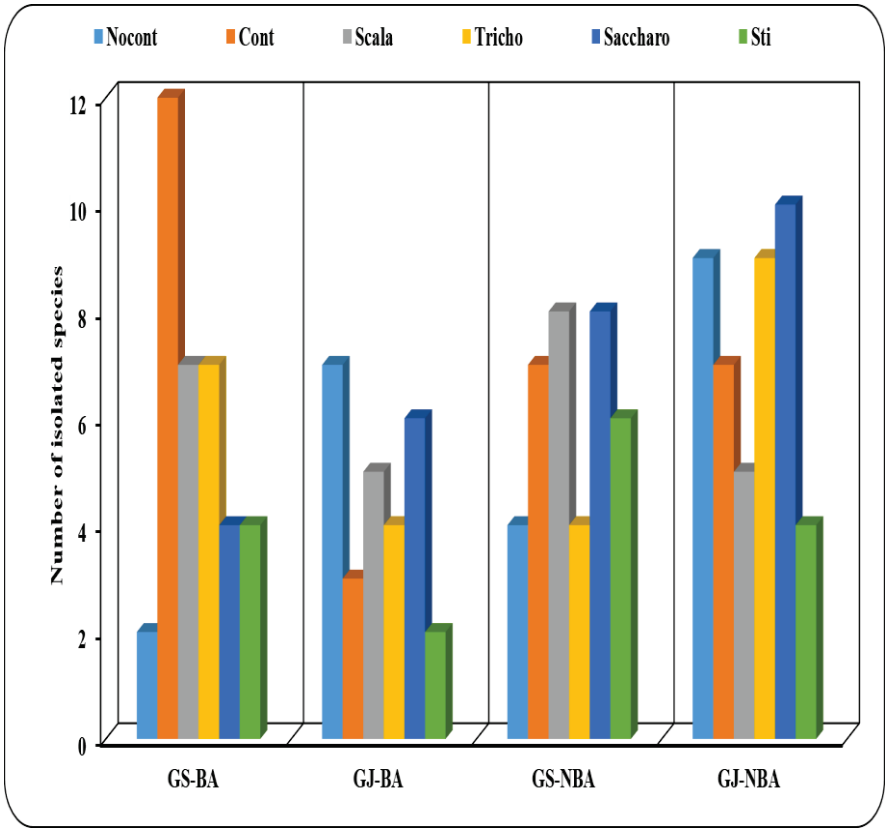


**Figure 6: Number of fungal strains isolated from GJ and GS of different treatments**

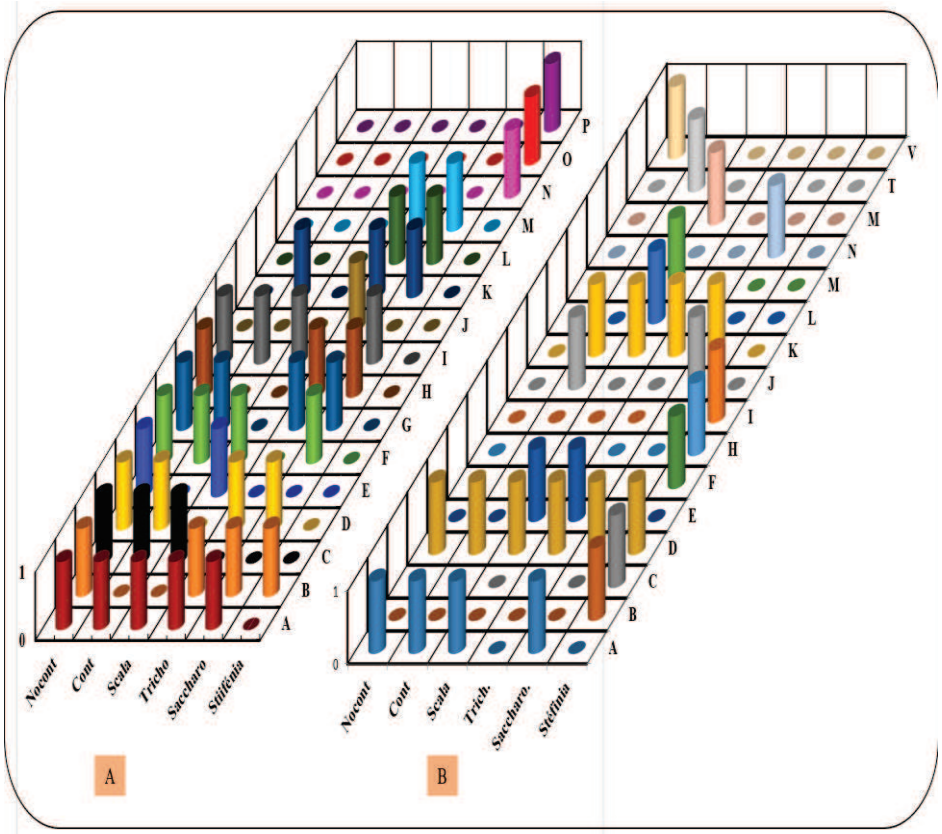


**Figure 7. Non-black aspergilli strains isolated from: A:** GS, **B:** GJ.

***GS:*** *Grape stem,* ***GJ:*** *Grape juice,* ***BA:*** *Black aspergilli species, NBA: Non-black aspergilli species,* ***Nocont:*** *OTA-PF-uncontaminated-untreated modality;* ***Cont:*** *OTA-PF-contaminated-untreated modality;* ***Scala:*** *OTA-PF-contaminated & Scala® treated treatment;* ***Tricho:*** *OTA-PF-contaminated & fungal bioagent treatment;* ***Saccharo:*** *OTA-PF-contaminated & yeast treated treatment;* ***Sti:*** *OTA-PF-contaminated & Stiférnia® treated treatment.*

Whereas, the GJ of the Stifénia treatment had the lowest number of the BA and NBA strains (n = 2 and 6, respectively) (Fig. 6), and also had the highest number of emerged new NBA strains which was not isolated from other treatments and modalities (Fig. 7), which highlights its effect on the grape fungal ecosystem. Only two or three fungal strains could be isolated from all or most of the treatments (GJ and G) (Fig. 7), while all other NBA strains might be isolated only once or twice in all the treatments (Fig. 7).

### 3.2.2. Impact of different treatments on the fungal ecosystem using PCR-DGGE

The partial denaturation of the ds-DNA, which amplified from the total DNA extracted of different preharvest treated grape berries, using the fungal universal primer set in the DGGE migration had permitted the separation the different fungal species found in the GJs (corresponding to bands which varied from 13 to 26 bands with different density) (Fig. 8).

The statistical analysis of different treatment's pattern lines (Fig. 8) showed the impact of the preharvest treatments regarding their fungal profiles. Hence, the different treatments and modalities were well separated in the factorial chart which refereeing to different fungal ecosystem (FE) (Fig. 9A).

For the similarity between different treatments, the FE of Stifénia treatment was the closest treatment to the control-1 modality with 76.6 %. The other OTA-control treatments had a similarity of 55%. The chemical fungicide treatment was more similar to the control-2 modality (65.5 %) followed by the fungal bioagent treatment (61.5). And for the yeast bioagent treatment was at the same similarity of the two groups (55% similarity) (Fig.9B)

## 4. Discussion

The grape juice's OTA content was extremely increased by the *A. carbonarius* field contamination (Control-2), which is significantly reduced by the chemical, yeast bioagent and Stifénia treatments to be within the authorized uptake limit. While, the fungal bioagent treatment failed to reduce this OTA content to the max authorized OTA level.

According to the results obtained from GJ and GS microbiological isolations, all of the preharvest treatments had affected the fungal ecosystem (FE), which was naturally isolated from the uncontaminated modality (Control-1). The elicitor treatment was the one that affected most the FE and the fungal biodiversity (FB) (regarding to both the BA and NBA isolated strains). The highest significant fungal increase was observed in the fungal biocontrol treatments due to the introduction of *Trichoderma* specie in the ecosystem.

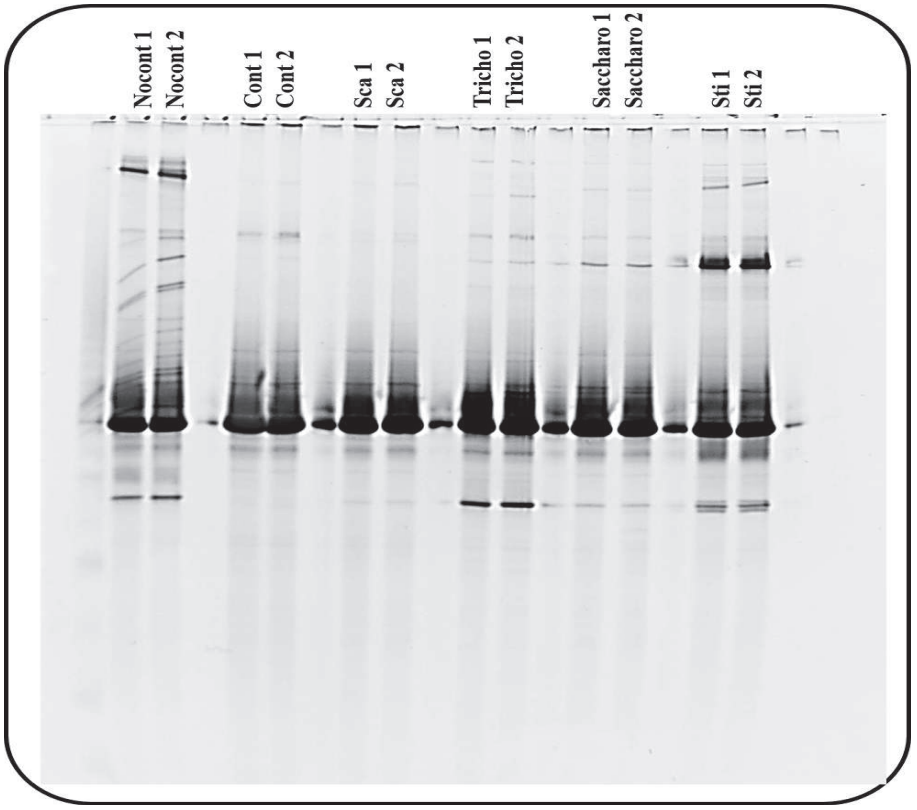


**Figure 8: PCR-DGGE for 28S DNA band profile of different treatments**.

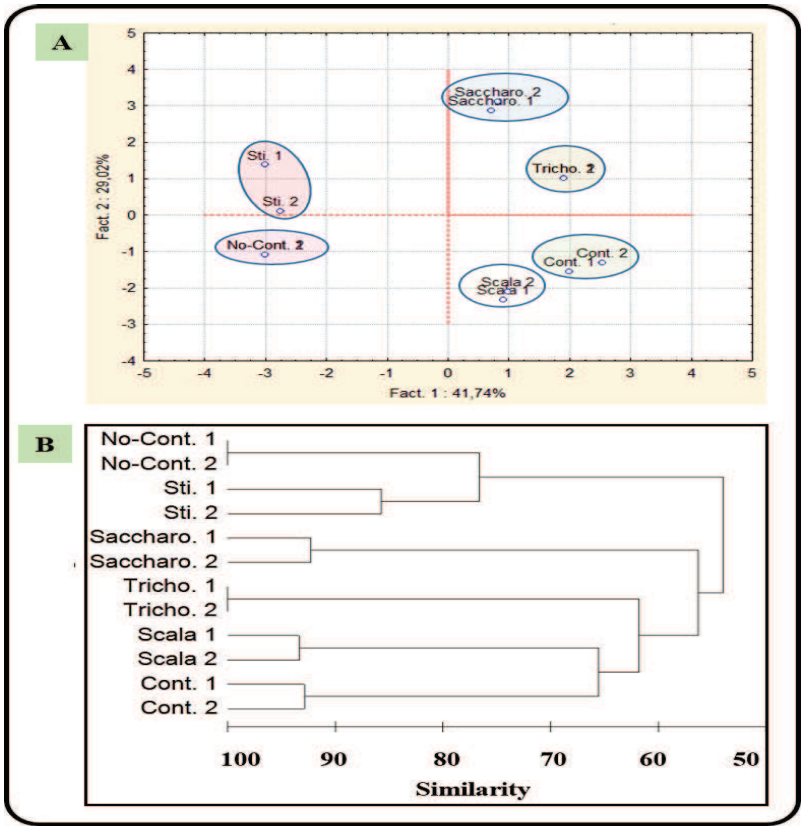


**Figure 9 :A:** Factorial variance analysis of 28S DNA banding profiles of different treatments; **B:** Dendrogram of 28S DNA banding profiles of different treatments.

***Nocont:*** *OTA-PF uncontaminated-untreated modality;* ***Cont:*** *OTA-PF-contaminated-untreated modality;* ***Sca:*** *OTA-PF-contaminated-Scala®-treated treatment;* ***Tricho.:*** *OTA-PF-contaminated- Trichoderma atroviridie-treated treatment by;* ***Saccharo.:*** *OTA-PF-contaminated-Saccharomyces cervisea-treated treatment;* ***Sti.****: OTA-PF-contaminated-Stiénia®-treated treatment.*

Zoetendal *et al*. (2004) have demonstrated that the choice of a particular molecular-based approach is depending on the questions being addressed. PCR-DGGE fingerprinting technique is a monitoring of community/population shifts and rapid comparative analysis usually performed. While this technique has certain limitations as it is a subject to PCR biases, it is semi-quantitative and the specie identification requires clone library.

However, the PCR-DGGE technique had indicated the different impacts of the preharvest treatments on the FE. The DGGE should be accompanied with caution in the qualitative assessment and comparison analysis of different samples (Although DGGE electrophoresis does provide a broad overview of a microbial community, interpretation of DGGE data (Ercolini, 2004; Prakitchaiwattana *et al.*, 2004)). That is agreed with our observation of incorrect evaluation of certain treatments, especially for the elicitor treatment. The DGGE results, which demonstrated the elicitor FE profile as the closest one to that of the uncontaminated treatment, were not agreed with the results obtained from the microbiological isolation,. In the other hand, the FE profile of this treatment was the highest treatment affecting the FE (reduction) and FB (new isolates) regarding all treatments and modalities (especially, the uncontaminated modality). Hence, the DGGE technique cannot differentiate dead and living cells (Ercolini, 2004; Prakitchaiwattana *et al.*, 2004). Which could clarify the partial disagreements with the microbiological approaches.

Although, the high density of some bands in the pattern lines of different treatments, bands were purified (data not included) from DGGE gels of the separated amplified DNA in order to run the sequencing subsequent process which might demonstrate the fungal species. That agreed with Thanh *et al.* (2008) results, which demonstrated that the band intensity of DGGE is not always correlated with population density, as DNA extraction efficiencies vary between microorganisms, and also could be due to the low concentration of the extracted DNA from GJ of different treatments as observed by Gothwal *et al*. (2007).

The results obtained from the *A. carbonarius* quantification in different preharvest treatment using the primers set (Ac12RL-OTAf/Ac12RL-OTAr) designed by Atoui *et al*. (2007) on the basis of polyketide synthase (pks) sequences had detected low quantities of OTA-PF as it has been observed in the plant extract treatment. That agreed with other studies which were conducted at the same genetic region such as Selma *et al.*, 2008; Spadaro *et al*., (2010), and Castella and Cabañes (2011). Ayoub *et al.* (2010) has reported the use of specific primers binding to sequences encoding the polyketide synthases (PKSs) involved in OTA biosynthesis for specific detection of *A. carbonarius* and *A. niger*, even in symptomless grape

samples. Also the absolute quantitative fungal estimations corresponding to different treatments according to the microbiological process had the same incidence level that demonstrated by specific OTA-PF qPCR, while the qPCR was more sensitive, for the very low quantities.

However, Gonzalez-Salgado *et al.* (2009) has developed a highly efficient quantitative PCR assay based on the multi-copy internal transcribed region 2 (ITS 2 of the rRNA gene) for the specific detection of *A. carbonarius* on grapes. In this research, the ITS1-5.8S region was used for the total fungal quantification. In addition, the results obtained were approximately within the same trend of those found in the microbiological study with slight changes.

From the above we conclude that, the combination of specific and universal qPCR could be used as a fast monitoring technique. Particularly, in the presence of the low target microorganism concentrations even with symptomless samples (i. e. the absence of the fungal signs, or symptoms) (Atoui et al., 2007). That is highlighting the importance of this technique in the favorable climates for the problematic fungi or disease, particularly, as in our case, for the experiments on prevention mycotoxin-producing fungi.

Furthermore, the microbiological approaches are very important in the case of overlapping the disadvantages of certain molecular techniques, by avoiding the dead spores or fungi units counting that could affect the final result giving incorrect evaluations (as found, by DGGE). Also in detecting the new fungal emerging species as it was the case for the Stifenia treatment, which could act by antagonism on the target pathogens (OTA-PF in our case).

Last not least, the yeast bioagent and elicitor treatments could be alternative or complementary treatments for the chemical fungicide traditional OTA control method. However, further studies should be performed to setup the other impacts of these OTA reducing preharvest treatments.

**Acknowledgements**

Financial support by the Egyptian government is gratefully acknowledged. We also thank to Ratomahenina R. and Baccou JC for their contentious intellectual input and help. We also appreciate Montet D., Meile J. C. and El-Sheikha A. F. for their intellectual input and help in the DGGE technique.

**References**

Abarca, M.L., Bragulat, M.R., Castellá, G., Cabañes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. App Environ Microbio. 60, 2650-2652.

AFNOR, 2002. Microbiologie Alimentaire. Saint-Denis La Plaine Cedex, 8ème éd. Tome, vol. 1, p. 64.

Atoui, A., Mathieu, F., Lebrihi, A., 2007. Targeting a polyketide synthase gene for *Aspergillus carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. Inter J Food Microbio. 115, 313-318. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.11.006

Ayoub, F., Reverberi, M., Ricelli, A., D'Onghia, A.M., Yaseen, T., 2010. Early detection of *Aspergillus carbonarius* and *A. niger* on table grapes: a tool for quality improvement. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 27, 1285-1293. DOI: 10.1080/19440049.2010.489576.

Barberis, C., Astoreca, A., Fernandez-Juri, M.G., Dalcero, A.M., Magnoli, C., 2010. Effect of Antioxidant Mixtures on Growth and Ochratoxin A Production of Aspergillus Section Nigri Species under Different Water Activity Conditions on Peanut Meal Extract Agar. Toxins 2(6), 1399-1413. DOI: 10.3390/toxins2061399

Castegnaro, M., Pfohl-Leszkowicz, A., 2002. Les mycotoxines: Contaminations omniprésentes dans l'alimentation animales et humaines. La sécurité alimentaire du consommateur, Lavoisier, Tec & doc., Moll &Moll Eds., Paris.

Castella, G., Cabañes, F.J., 2011. Development of a Real Time PCR system for detection of ochratoxin A-producing strains of the *Aspergillus niger aggregate*. Food Cont. 22, 1367-1372. http://dx.doi.org/10.1016/j.foodcont.2011.02.014

Dachoupakan, C., Ratomahenina, R., Martinez, V., Guiraud, J.P., Baccou, J.P., Schorr-Galindo, S. (2009). Study of the phenotypic and genotypic biodiversity of potentially ochratoxinogenic black aspergilli isolated from grapes. Inter J Food Microbio. 132:14-23. DOI: 10.1016/j.ijfoodmicro.2009.03.011.

Diguta, C.F., Vincent, B., Guilloux-Bénatier, M., Alexandre, H., Rousseaux, S., 2011. PCR ITS-RFLP: a useful method for identifying filamentous fungi isolates on grapes. Food Microbio. 28, 1145-1154. DOI: 10.1016/j.fm.2011.03.006

Doare´-Lebrun, E., El Arbi, A., Charlet, M., Guerin, L., Pernelle, J.J., Ogier, J.C., Bouix, M., 2006. Analysis of fungal diversity of grapes by application of temporal temperature gradient gel electrophoresis – potentialities and limits of the method. J Appl Microbiol 101, 1340–1350. DOI: 10.1111/j.1365-2672.2006.03030.x

El Sheikha, A.F., Condur, A., Métayer, I., Le Nguyen, D.D., Loiseau, G., Montet, D., 2009. Determination of fruit origin by using 26S rDNA fingerprinting of yeast communities by PCR-DGGE: Preliminary application to *Physalis* fruits from Egypt. Yeast 26(10), 567-573. DOI: 10.1002/yea.1707.

El Sheikha, A.F., Durand, N., Sarter, S., Okullo, J.B.L., Montet, D., 2012. Study of the microbial discrimination of fruits by PCR-DGGE: Application to the determination of the geographical origin of *Physalis* fruits from Colombia, Egypt, Uganda and Madagascar. Food Cont. 24(1-2), 57-63. http://dx.doi.org/10.1016/j.foodcont.2011.09.003

Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J Microbio Meth. 56(3), 297-314.

European Commission, European Commission Regulation No. 1881/2005 (2006). Amending Regulation (EC) No. 1881/2006 setting maximum levels for certain in foodstuffs as regards ochratoxin A. Off.J EurUnion 364, 5-24.

Fierer, N., Jackson, J.A., Vilgalys, R., Jackson, R.B., 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbio. 71(7), 4117-4120. DOI: 10.1128/AEM.71.7.4117-4120.2005

Fleet, G.H., 2007. Yeasts in foods and beverages: impact on product quality and safety. Curr Opin Biotechno. 18(2), 170-175. http://dx.doi.org/10.1016/j.copbio.2007.01.010

González-Salgado, A, Patiño, B, Gil-Serna, J, Vázquez, C, González-Jaén, M.T., 2009. Specific detection of Aspergillus carbonarius by SYBR Green and TaqMan quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. FEMS Microbiol Lett. 295(1), 57-66. DOI: 10.1111/j.1574-6968.2009.01578.x.

Gothwal, R.K., Nigam, V.K., Mohan, M.K., Sasmal, D., Ghosh, P., 2007. Extraction of bulk DNA from Thar Desert soils for optimization of PCR-DGGE based microbial community analysis. Electr J Biotechno. [online]. 15 July 2007, 10(3), [10/11/2013]. http://www.ejbiotechnology.info/content/vol10/issue3/full/6/index.html. ISSN 0717-3458. DOI: 10.2225/vol10-issue3-fulltext-6.

IARC (1993). Ochratoxin A. in some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monographs on the evaluation of carcinogenic risk of chemicals to humans, Lyon, France: International Agency for Research on Cancer, 56:489-521.

Laforgue, R., Guérin, L., Pernelle, J.J., Monet, C., Dupont, J., Bouix, M., 2009. Evaluation of PCR-DGGE methodology to monitor fungal communities on grapes. J Appl Microbio. 107:1208-1218. DOI: 10.1111/j.1365-2672.2009.04309.x

Oros-Sichlera, M., Gomesa, N.C.M., Neuberb, G., Smallaa, K., 2006. A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. J Microbio Meth. 65(1):63-75. http://dx.doi.org/10.1016/j.mimet.2005.06.014

Petzinger, E., Weidenbach, A., 2002. Mycotoxin in the food chain: the role of ochratoxins. Livestock Produc Sci. 76:245-250.

Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage. 3rd edition. Springer Dordtecht Heidelberg London New York. DOI: 10.1007/978-0-387-92207-2.

Prakitchaiwattana, C.J., Fleet, G.H., Heard, G.M., 2004. Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. FEMS Yeast Res. 4, 865-877. DOI: 10.1016/j.femsyr.2004.05.004

Rousseau, J., 2004. Ochratoxine A in wines: current knowledge, second part: mycotoxins and wines. Vinidea.net Wine Internet Technical Journal, N.5. ) http://www.infowine.com/default.asp?scheda=1038

Rousseaux, S., Diguta, C. F., Radoï-Matei, F., Alexandre, H., Guilloux-Bénatier, M., 2014. Non-*Botrytis* grape-rotting fungi responsible for earthy and moldy off-flavors and mycotoxins. Food Microbio. 38, 104-121. http://dx.doi.org/10.1016/j.fm.2013.08.013

Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O., 1995. Introduction to Food Borne fungi. 4th ed. Centraalnureau voor Schimmelcucutures, Netherlands.

Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F.A., Frank, J.M., Frisvad, J.C., 2004. New ochratoxin A or sclerotium producing species in *Aspergillus* section Nigri. Stud Myco. 50, 45-61.

SCOOP, 2002. European commission, 2002. Task 3.2.7. Assessment of dietary intake of ochratoxin A by the population of EU member states. Rome, Italy.

Selma, M.V., Martínez-Culebras, P.V., Aznar, R., 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. Inter J Food Microbio. 122(1-2), 126-134. DOI:10.1016/j.ijfoodmicro.2007.11.049

Selma, V.M., Martinez-Culebras, P.V., Elizaquivel, P., Aznar, R., 2009. Simultaneous detection of the main black aspergilli responsible for ochratoxin (OTA) contamination in grapes by multiplex real-time polymerase chain reaction. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 26, 180-188. DOI: 10.1080/02652030802345623.

Skarkova, J., Ostry, V., Malir, F., Roubal, T., 2013. Determination of ochratoxin A in food by high performance liquid chromatography. Analy Lett. 46(10), 1495-1504. DOI: 10.1080/00032719.2013.771266

Spadaro, D., Patharajan, S., Kartikeyan, M., Lorè, A., Garibaldi, A., Gullino, M.L., 2010. Specific PCR primers for the detection of isolates of *Aspergillus carbonarius* producing ochratoxin A on grapevine. *Ann Microbio.* 61, 267-272. DOI: 10.1007/s13213-010-0131-3

Suarez-Quiroz, M.L., Gonzalez-Rios, O., Barel, M., Guyot, B., Schorr-Galindo S., Guiraud J.P., 2004a. Study of ochratoxinA-producing strains in coffee processing. Inter J Food Sci Techno. 39, 501-507. DOI: 10.1111/j.1365-2621.2004.00810.x

Suarez-Quiroz, M.L., Gonzalez-Rios, O., Barel, M., Guyot, B., Schorr-Galindo, S., Guiraud J.P., 2004b. Effect of chemical and environmental factors on *Aspergillus ochraceus* growth and toxigenesis in green coffee. Food Microbio. 21, 629-634. http://dx.doi.org/10.1016/j.fm.2004.03.005

Téren, J., Palágyi, A., Varga, J., 1997. Isolation of ochratoxin A producing aspergilli from green coffee beans of different origin. Cereal Res Communica. 25, 303-304.

Téren, J., Varga, J., Hamari, Z., Rinyu, E., Kevei, F., 1996. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathologia*, 134, 171-176. DOI: 10.1007/BF00436726

Thanh, V.N., Mai, T., Tuan, D.A., 2008. Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. *Inter J Food Microbio.* 128(2), 268-73. DOI: 10.1016/j.ijfoodmicro.2008.08.020.

Thion, C., Cébron, A., Beguiristain, T., Leyval, C., 2013. Inoculation of PAH-degrading strains of *Fusarium solani* and *Arthrobacter oxydans* in rhizospheric sand and soil

microcosms: microbial interactions and PAH dissipation. Biodegradation 24, 569-581. DOI: 10.1007/s10532-013-9628-3.

van Elsas, J.D., Duarte, G.F., Keijzer-Wolters, A., Smit, E., 2000. Analysis of the dynamics of fungal communities in soil via fungal-specific PCR of soil DNA followed by denaturing gradient gel electrophoresis. J Microbio Meth. 43(2), 133-151. http://dx.doi.org/10.1016/S0167-7012(00)00212-8

Varga, J., Juhász, A., Kevei, F., Kozakiewicz, Z., (2004). Molecular diversity of agriculturally important *Aspergillus* species. Eur J Plant Patho. 110, 627-640. DOI: 10.1023/B:EJPP.0000032402.36050.df

Vrabcheva, T., Usleber, E., Dietrich, R., Märtlbauer, E., 2000. Co-occurrence of Ochratoxin A and citrinin in cereals from Bulgrian villages with a history of Balkan endemic nephropathy. J Agri Food Chem. 48, 2483-2488. DOI: 10.1021/jf990891y

Walker, R., 1999. Mycotoxins of growing interest, ochratoxin. Presented at the 3rd joint FAQ/WHO/UNEP International conference on mycotoxins, Tunis, Tunisia.

Zoetendal, E.G., Collier, C.T., Koike, S., Mackie, R.I., Gaskins, H.R., 2004. Molecular Ecological Analysis of the Gastrointestinal Microbiota: A Review. *J Nutr.* 134, 465-472.

Zott, K, Claisse, O, Lucas, P, Coulon, J, Lonvaud-Funel, A, Masneuf-Pomarede, I., 2010. Characterization of the yeast ecosystem in grape must and wine using real-time PCR. Food Microbiol. 27(5), 559-67. DOI: 10.1016/j.fm.2010.01.006

# Influence of preharvest treatments (chemical, fungal and yeast bio agents, and elicitor) on the OTA juice content and the physical and chemical characteristics of the *Mourvèdre* grape cultivar

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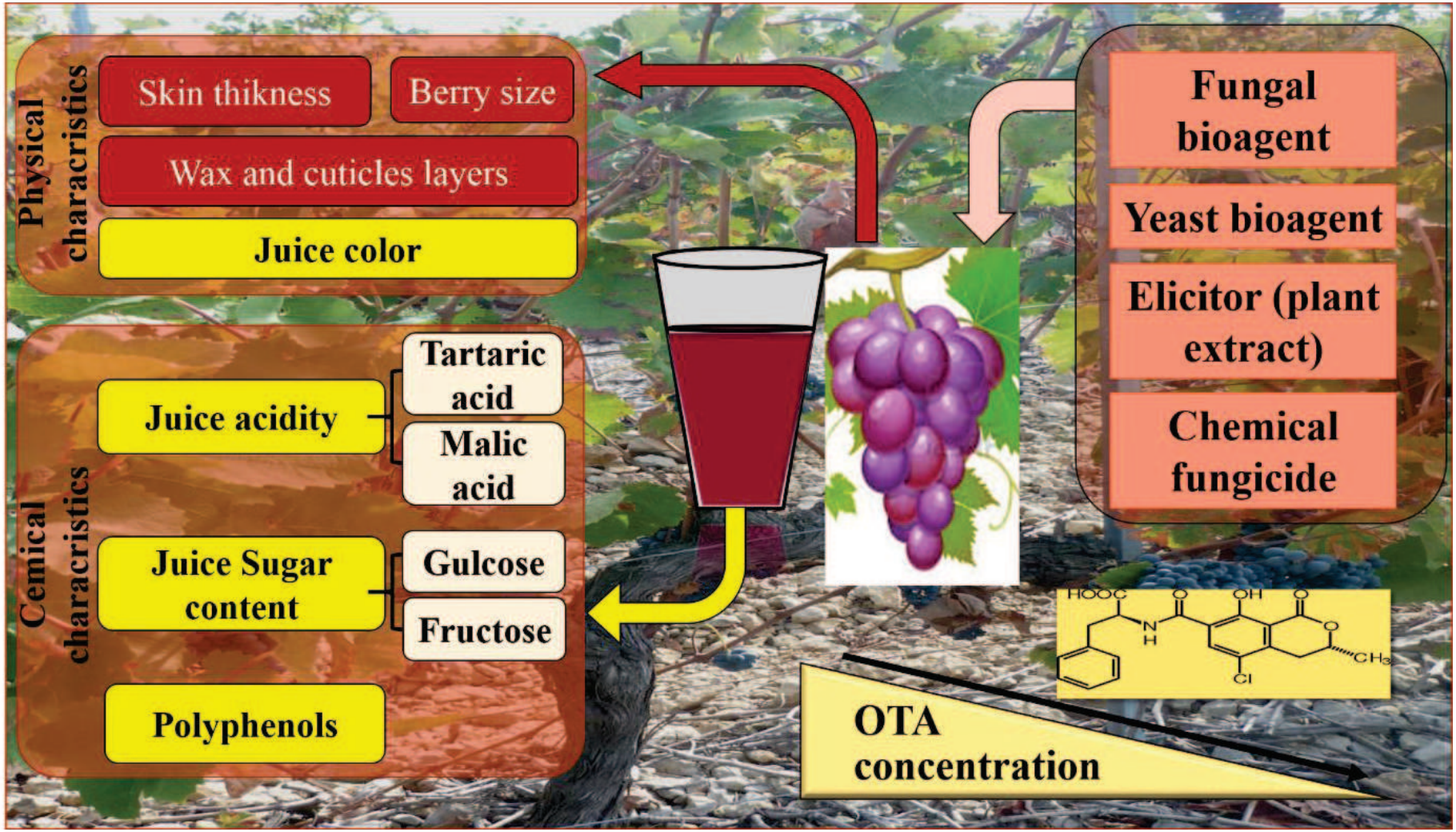
Submitted to **the Agricultural and Food Chemistry**

**TOC Graphic**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Juice Sugar content
Gulcose
Fructose
Polyphenols
Fungal bioagent
Yeast bioagent
Elicitor (plant extract)
Chemical fungicide
OTA concentration

**Abstract**

The Ochratoxin A (OTA) content and quality of grape juice in grape berries of the cultivar *Mourvèdre* was characterized by studying their morphology (diameter and skin thickness), measuring the color and main soluble chemical compound contents in grape juices (sugars, acids and total polyphenols), and comparing the effects of six preharvest treatments [chemical fungicide (Scala®), two different biocontrol-agents (*Trichoderma atroviride* and *Saccharomyces cerevisiae*), and elicitor (Stifénia®)]. A significant reduction (38 - 42%) was observed in the OTA juice content by the chemical, yeast bioagent and elicitor treatments with juice safety improvement. The two biological treatments and one elicitor treatment significantly increased the thickness of the berry skins in general (regarding the wax, cuticle layers and skin thickness), which could be related to the enhancement of the disease resistance of the grape berries to certain pathogens and could also simultaneously explain the OTA reduction and the grape juice quality improvement.

**Key words**

*Aspergillus carbonarius*, food safety, juice quality, polyphenols, acidity, sugars, OTA.

**Introduction**

According to an estimate of the United Nations Food and Agriculture Organization (FAO), which was conducted in 1985, 25% of the foods in the world are infected with mycotoxins.[1] Ochratoxin A (OTA) is one of the most common mycotoxins. This mycotoxin was discovered in 1965 by a group of researchers in South Africa and was first detected in cereals, as well as in coffee, beer, dried fruits, and spices. Mycotoxin's occurrence was demonstrated in samples of table wine in 1996. Three years later, it was discovered that the OTA contamination of grapes took place at the vineyard, which was in contrast to cereals, where OTA is produced during storage. [2]

*Aspergillus carbonarius*, followed by *A. niger aggregate*, plays a major role in the OTA contamination of French grapes. Harvest time is the critical time when almost all ochratoxigenic fungi were detected and when the highest amounts of OTA were measured in grapes. Southern Mediterranean regions, particularly Languedoc-Roussillon had the greatest amount of OTA contamination.[3] Those fungi grow on grapes at veraison and proliferate until harvest, settling on the berries after injury, explosions or wilting of the film, and are extremely adaptable to tropical conditions.[4] EU legislation permits a maximum of 2 µg/L OTA as a tolerable limit, and a maximum of 120 ng OTA /kg body weights for the tolerable weekly intake (TWI) in grape juice and wine. [5,6] OTA contamination in grape juice is of great concern because children are its main consumers and because the consumption of juice is greater than that of wine.[7] Ochratoxin A ($C_{20}H_{18}ClNO_6$) is a molecule that combines an amino acid (phenylalanine) in a chlorinated di-hydrocoumarin with a molar mass of 403.8 g/mol.[8] The following strategies have been proposed to prevent the toxic effects of mycotoxins on consumers in general, and of ochratoxins, particularly in foods and feeds: (i) the prevention of mycotoxin contamination; (ii) the decontamination or detoxification of foods that are contaminated with mycotoxins; and (iii) the inhibition of the absorption of consumed mycotoxins in the gastrointestinal tract.[9] The prevention of mycotoxin contamination in the field is the main goal of the agricultural and food industries. Because mycotoxin-producing molds can usually only colonize damaged parts of plants, crops must be protected against damage that is caused by either mechanical processes or insects. Field treatment with fungicides is the traditional prevention technique.[10] Because, the vine culture is the most fungicide-consuming culture in France, it is in this context that the plan "Ecophyto 2018" was designed which aims to progressively half the use of pesticides (*i.e.,* fungicides) in France by 2018, while maintaining the economic performance of French agriculture.

Chemical composition is one of the most important quality criteria for fruit products. Organic acids have important effects on characteristic fruit flavors and play a major role in quality criteria such as stability, color and flavor. High acidity not only influences the palatability of table grapes but also affects the suitability of wine grapes.[11] Tartaric and malic acids, which are dominant grape organic acids, account for 90% or more of the total acidity in grapes.[12] Although possessing the same genotype, grapes, that are harvested from different climates and soils have different organic acid contents. During grape ripening, continuous warm conditions result in a lower acid content at maturity primarily due to the increasing degradation of malic acid, which affects the juice and wine flavor.[11]

Polyphenols are the most important phytochemicals in grapes because these phytochemicals possess many biological activities and health-promoting benefits.[13] The phenolic compounds primarily include anthocyanins, flavanols, flavonols, stilbenes (resveratrol) and phenolic acids. Anthocyanins are pigments that primarily exist in grape skins. Flavonoids are widely distributed in grapes, particularly in seeds and stems, and principally contain (+)-catechins, (-)-epicatechin and procyanidin polymers. Anthocyanins are the main polyphenolics in red grapes, whereas flavan-3-ols are more abundant in white varieties.[14] The strong correlation that was observed between the antioxidant activity and phenolic content in the commercial grape juice suggests a role for grape juice total polyphenol in quality control studies.[15]

The aim of this study is to compare the effect of six preharvest treatment modalities (two controls, one chemical fungicide, two different biocontrol-agents, and one elicitor) on the content of OTA in grape juices. A grape characterization was also made by studying the morphology of grape berries (diameter and skin thickness) and by measuring the color and content of the main soluble chemical compounds of grape juices (sugars, acids, polyphenols) to control the impact of the treatment on fruit quality as well as to explain the action of the biological and elicitor treatments on the plant production and protection against pathogens including *Aspergillus carbonarius*

## Materials and Methods

### 1. Grape and grape juices

#### 1.1. Treatments

Six different modalities of treatments were applied on the experimental viticulture orchard PECH-ROUGE of INRA (*Institut Nationale de Recherché Agronomique*) and IFV (*Institut Français de la vigne et du vin*), Narbonne (Languedoc-Roussillon region), France, as shown in Table 1, near parcels of *Mourvèdre,*which is a cultivar that was recognized as being sensitive to OTA contamination.

#### 1.2. Sampling

Samples were harvested in late September 2010 during the grape harvest season from the experimental field in Narbonne, France. Grape bunches (5 kg/ treatments) were taken and placed in two previously sterilized bags, which were kept at approximately 4°C until analysis. Mycological analysis was immediately performed and the remaining samples were kept frozen at -20°C. Grape juices were made by crushing berries as described in laboratory publication by Fritz and Athmann, [16] and were stored at -20°C.

### 2. OTA content in grape juice

#### 2.1. OTA extraction

Specific grape juice was prepared from each treatment in a blender using 50 g of grape berries with 200 mL of 70% methanol (VWR, PROLABO, 20837.320) that was diluted with deionized water by two steps (30 mL water, then 140 mL methanol and 320 mL water),and then filtered (by a cellulose acetate filter 0.2 µL, Sartorius®, Germany). In total, 10 mL of the juice was diluted in 40 mL of PBS (8 g NaCl, 1.2 g $Na_2HPO_4$, 0.2 g KCl, 0.2 g $KH_2PO_4$, pH 7.0) (Sigma Aldrich, France, P4417) + 0.01% Tween 20 (Sigma Aldrich, France, P-1379). Then 20 mL of the diluted juice was passed through an *OCHRAPREP*® affinity column (R-Biopharm Rhone, France) (1 drop/sec). Next the column was washed by 10 mL of PBS/0.01% Tween-20, followed with 10 mL purified water. The OTA were collected by 1.5 mL methanol:acetic acid (VWR, PROLABO, 84-528.290) (98:2, v:v) twice (at the flow rate of 1-2 drop/sec).[17] Then the extracts were stored at -20°C until measured.

**Table 1: The different treatments applied on the cultivar *Mourvèdre* at the experimental field of IFV, Narbonne, France during the year 2010.**

| | Name | Contamination by OTA-PF | | Treatment | | |
|---|---|---|---|---|---|---|
| | | Case | Time | Type | Frequency | Time |
| 1 | **Nocont** | - | - | - | - | |
| 2 | **Cont** | + | At the veraison stage | - | - | |
| 3 | **Scala** | + | At the veraison stage | Chemical fungicide: Commercial name; SCALA®, active ingredient; Pyriméthani 400g/L | Twice | One at the green tip stage, and the other at leaves output stage |
| 4 | **Tricho.** | + | At the veraison stage | Fungal bioagent using the fungi *Trichoderma atroviride*. | Twice | One at the green tip stage, and the other at leaves output stage |
| 5 | **Saccharo.** | + | At the veraison stage | Yeast bioagent using the yeast *Saccharomyces cerevisiae*. | Once | At the leaves output stage |
| 6 | **Stifénia** | + | At the veraison stage | Plant extracts FEN 560 (fenugreek seed powder) (Stifénia®). | Multiple | Starts at the green tip stage and followed by 15 days intervals ones |

3. + Artificially contaminated by OTA-PF *[OTA producing fungi (Aspergillus carbonarius)]*

### 3.1. Analysis OTA by HPLC

OTA was analyzed by HPLC (Pump, Shimadzu LC/9A, Japan) with fluorescence detection ($\lambda_{exc}$333 nm; $\lambda_{em}$460 nm, Shimadzu RF-10AXL, Japan), using a C18 column (25 x 4.6 mm, 5 µm, Phenomenex®, USA). The mobile phase [49% acetonitrile (Carlo Erba Reagent, France, P00637G21), 49% Milli-Q water (Quantum® Ex, MILLIPORE), and 1% acetic acid] was pumped at 1 mL/min. The injection volume was 20 µL, and the retention time was approximately 16.3 min. The calibration curve was prepared with OTA solution (Sigma Aldrich, France, 34037-2ML-R) from 0.05 to 5 ng/ mL. The limit of detection (LOD) and limit of quantification (LOQ) of this method which was applied to grape juice samples, were established at 0.023 µg/L and 0.076 µg /L, respectively.[18]

## 4. Grape berry physical characters analysis

### 4.1. Skin thickness

Thin sections of berry skin (approximately 0.5 x 1.0 cm) were taken with a scalpel, air dried at room temperature [19] and measured under a scanning electron microscope (Quanta FEG, Type 200, D8399, FEI Company™, USA). In total, 100 measurements were completed at various zones of skin from 50 berries/treatment (5 berries /bunch).

### 4.2. Size

One hundred randomly chosen berries from each treatment were analyzed using a precision measuring tool (Fisher Darex, France).[20] The average of each treatment was compared with the average of other treatments.

### 4.3. Cuticle and wax quantification.

The method for cuticle and wax quantification, as described by Gabler, [21] was used. Fifty berries were selected from the middle portion of the clusters and kept frozen at –20°C until analyzed. Several epidermal disks, which were 5 mm in diameter, were cut with a cork borer from different parts of each berry. The cuticle and wax contents of three replicates, each containing 50 disks, were determined. Cuticle and wax layers were separated from underlying tissues by soaking the disks for 24 h at 30°C in 2.5 ml of a digestion solution containing 0.5% cellulase (from *Aspergillus niger*, Sigma Chemical C1184-5KU) and 0.1% pectinase (from *Aspergillus niger*, Sigma Chemical P4716-5KU), which was dissolved in 0.1 M sodium acetate buffer (pH 4) (Sigma Aldrich, France, S2889-250G). The disks of the digestion resistant material (cuticle plus epicuticular wax) were collected on a metal screen and rinsed with deionized water until clean. The disks were air-dried and the epicuticular wax was

dissolved by immersing the disks in 2 ml of 100% chloroform (VWR, PROLABO, 22711.290) for 24 h to separate the wax from the cuticle. The wax which was dissolved in chloroform, was transferred to pre-weighed beakers. Separate beakers containing either cuticle or wax were placed in an oven at 100°C for 24 h, and their weights were recorded.

### 4.4. Evaluation of the color of juice by colorimeter L*a*b*

This method determines 3 parameters for the juice color: *L* (lightness) with values ranging from 0 (black) to100 (white), point *a* on the green-red axis, and point *b* on the blue-yellow axis. Two milliliters of juice were centrifuged at 8,000 g and filtered (0.45 µm, MiniSart, Sartorius®, Germany). The analyses were performed using the Tri-Stimulus program (Konica-Minolta), and the three color parameters ($L^*a^*b^*$) were recorded. Each sample was measured three times and the average value were calculated.[22]

## 5. Grape juice biochemical analysis

### 5.1. Grape Juice Acidity

#### 5.1.1. pH

The pH of each juice was measured using a Series pH720 pH meter; (WTW® InoLab, Germany). All analyses were performed in duplicate at 20°C.

#### 5.1.2. Organic Acid analysis

The grape organic acids were separated according to the method that was demonstrated by the International Organization of Vine and Wine, [23] which uses HPLC after filtration with a filter syringe (0.45 µm, MiniSart, Sartorius®, Germany). The HPLC system was composed of a pump (Shimadzu LC-10A, Japan), which was connected to a UV detector (Shimadzu SPD-6A, Japan) that was set at 210 nm[20], with the following conditions: loop injection of 20 µL; C610H column (9 µm, 300 mm x 7.8 mm) (SUPELCO®, USA). The column was kept at a constant temperature of 40°C. The mobile phase was composed of ultra-pure water containing 0.5% of sulfuric acid (VWR, PROLABO, 220685.330) with a flow rate of 0.6 mL/min. Concentrations were determined according to standard curves for malic acid (Sigma Aldrich, France, M-0875) (0.1 to 0.6 g/ L) and tartaric acid (MERK Eurolab, 20-716.293) (0.1 to 0.4 g/ L).

### 5.2. Sugar analysis

A grape juice from each treatment was diluted 1000 times to match the calibration curve. Sugars (glucose and fructose) were determined by HPLC. [24] The same HPLC system was used for the organic acid determination, with the same column and conditions, coupled to a

refractometer (Shimadzu RID-6A, Japan). Concentrations were determined according to standard curves for glucose (Sigma Aldrich, France, G0350500-1EA) and fructose (Sigma Aldrich, France, F-0127) (0.1 to 1 g of each sugar/ L).

### 5.3. Total polyphenol analysis

The total concentration of polyphenols in grape juice was estimated with reagent Folin-Ciocalteau (MERCK, Germany, 1.09001.0100).[25,26,13] A sodium carbonate (Sigma Aldrich, France, S7795) solution (0.25 g/mL) was filtered through a nitro-cellulose membrane (0.2 µm, Sartorius®, Germany) and added to 200 mL of water. For the analysis 20 µL of each juice sample, which was pre-diluted 10-fold, was used following the Folin-Ciocalteau method. Concentrations were determined according to the standard curve of gallic acid (50 to 500 mg / L) (Sigma Aldrich, France, G-7384).

## 6. Statistical analysis

An ANOVA analysis was conducted using the Statistia 10 software (Stat Soft. Inc., Tulsa, USA). Duncan and Tukey's tests were used to detect any significant differences between the samples that were examined, Significance levels were defined using $P \leq 0.05$.

## Results

### 1. OTA juice content

OTA juice content was present at extremely low quantities, which varied from 1.1 ± 0.014 µg/L (*uncontaminated-untreated* control) to 2.6 ± 0.098 µg/L (*contaminated-and-untreated* control). The contamination by OTA-PF permitted the encroachment of the OTA content to be higher than the authorized OTA content (2 µg/L).

The *Trichoderma* treatment had the lowest effectiveness compared with other treatments for the reduction of OTA because the juice contains 2.1 ± 0.14 µg/L. No significant differences were found between Scala®, Stifénia® and *Saccharomyces* treatments (1.5, 1.5 and 1.55 µg/L, respectively), which significantly reduced the juice OTA contamination rate by 42.3 to 38%. The natural treatment, Stifénia®, and *Saccharomyces* treatments allowed OTA reduction at the same efficacy as chemical treatment (Fig. 1).

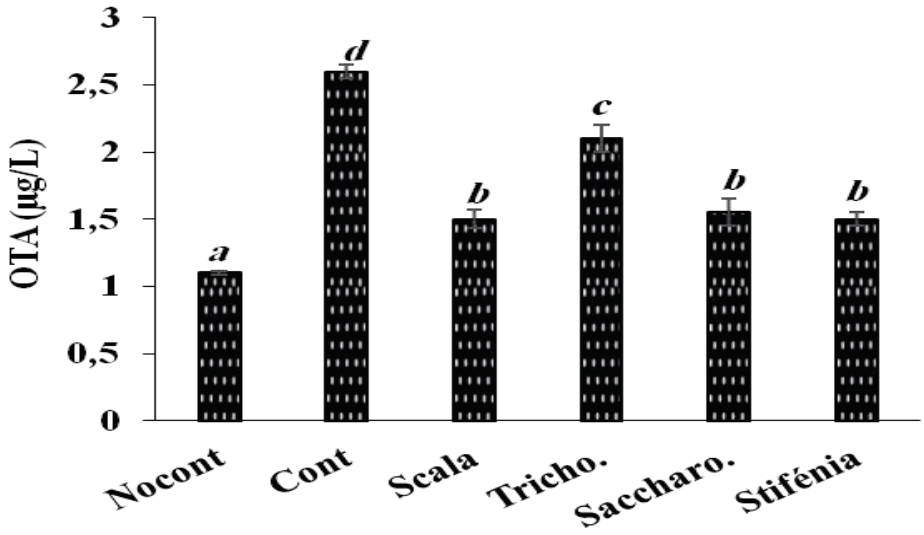


**Figure 1: OTA concentrations of grape juices (µg/L).**

***Nocont**: not contaminated by OTA-PF or by treated modality; **Cont**: artificially contaminated by OTA-PF and untreated modality; **Scala:** artificially contaminated by OTA-PF and treated by the Scala® fungicide; **Tricho.:** artificially contaminated by OTA-PF and treated by the fungus Trichoderma atroviride; **Saccharo.:** artificially contaminated by OTA-PF and treated by the yeast Saccharomyces cerevisiae; **Stifénia:** artificially contaminated by OTA-PF and treated by the Stifénia® treatment*

*Statistically analyzed using an ANOVA (Duncan at P≤0.05%), STD err = 0.07.*

## 2. Grape berry physical characteristic analysis

### 2.1. Skin thickness

The results of the skin thickness of grape berries, which were analyzed using a scanning electron microscope, are shown in Fig.2 A and Fig. 3. Samples of grapes that were treated by Stifénia® (132 ± 8.87 µm) had 57% thicker berry skin than those berries that were treated with the *contaminated-and-untreated* modality (84.3 ± 5.04 µm), followed by *Saccharomyces* (125.4 ± 3.44 µm) at 49%, *Trichoderma* (114.7 ± 7.04 µm) at 36%, and last, by Scala® (109.9 ± 5.23 µm) at 25.6%. Moreover, the order of the effectiveness of the previous treatments was the same as that of the grape skin thickness regarding the *uncontaminated-and-untreated* modality (97.8 ± 9.63 µm) (35%, 28%, 17%, and 12.4%, respectively).

### 2.2. Size

Berry size varied from 0.34 to 1.99 µm. The OTA-PF contamination greatly affected the average size of the berries (1.08 ± 0.05 cm) compared with the other control treatment (1.22 ± 0.04 cm). All four treatments increased the berry size average, with significant increases using the two biological and the elicitor treatments compared with the contaminated control

modality. Stifénia® and *Saccharomyces* treatments had the highest increase in berry size average (1.27 ± 0.04, and 1.25 ± 0.05 cm, respectively), whereas the Scala® treatment had the lowest increase by 1.13 ± 0.01 cm (Fig. 2B).

### 2.3. Waxes and Cuticle

The OTA-PF contamination significantly reduced the cuticle around the grape berries (64.9 ± 8.75 mg) compared with the uncontaminated treatment (75.03 ± 2.63 mg) (Fig. 2C). Whereas Scala® and *Saccharomyces* treatments had a significant increase in of the berry cuticle layer (93.27 ± 17.35 and 93.27 ± 12.16 mg, respectively) compared with the uncontaminated control. Stifénia® (89.70 ± 16.11) and *Trichoderma* (74.27 ± 21.11) treatments had no significant effect on the cuticle layer (whereas, Stifénia® had an increasing trend) (Fig. 2C).

Wax layers varied from 1.3 to 2.67 mg. The wax mass was significantly reduced by the OTA-PF contamination, with a mass of 1.7 ± 0.40 mg compared with the mass of the uncontaminated control (1.96 ± 0.06 mg). In contrast, the *Saccharomyces* treatment had a positively significant wax layer mass of 2.50 ± 0.17 mg. Other treatments (Stifénia®, *Trichoderma* and Scala®) (2.23 ± 0.17, 2.07 ± 0.10 and 2.00 ± 0.10, respectively) had an increasing wax mass compared with the uncontaminated control; however, the increased wax masses were not significantly different (Fig. 2D).

### 2.4. Evaluation of the color of juice by colorimeter $L^*a^*b^*$

The grape juice brightness ($L^*$) varied from 29.42 ± 0.013 (*contaminated-and-untreated* control) to 34.56 ± 0.011 (Scala® treatment). Whereas the blue-yellow ($b^*$) grape juice color varied from 2.58 ± 0.0044 (*contaminated-and-untreated* control) to 3.77 ± 0.0033 (*uncontaminated-and-untreated* control), and the green-red ($a^*$) grape juice color was varied from 9.91 ± 0.029 (Scala® treatment) to 14.29 ± 0.24 (Stifénia® treatment).

The contamination by OTA-PF had the highest significant reduction in the grape juice brightness and was nearest treatment to the blue rather than to the yellow.

Stifénia® treatment was significantly the highest treatment in the red among all treatments, and closer to the yellow rather than to the blue, with no significant difference compared with *Saccharomyces* and *Trichoderma* treatments, and darker than the Scala®, *uncontaminated-and-untreated*, and *Trichoderma* treatments (Fig. 2E).

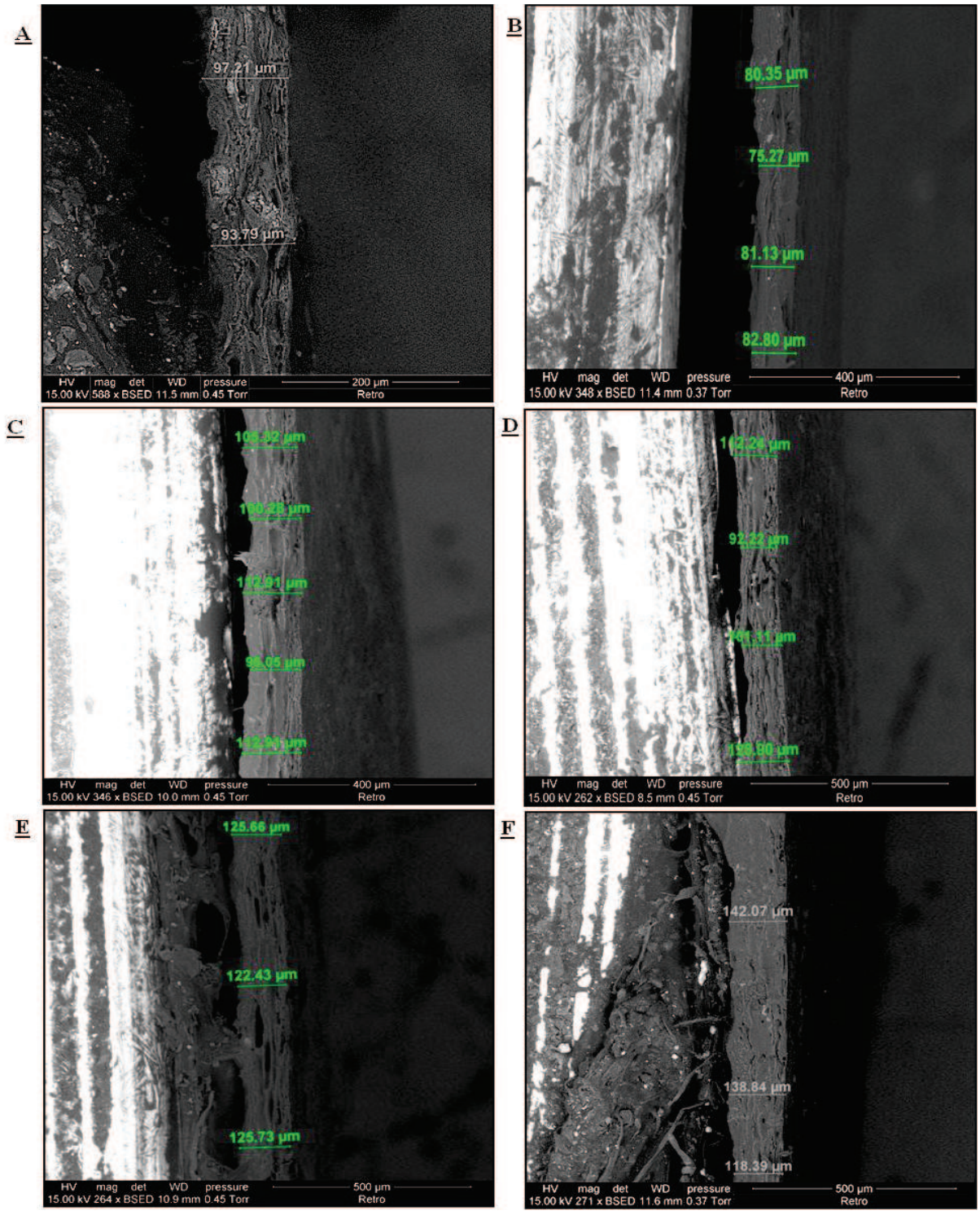


**Figure 3: Scanning electron microscopy of skin thickness of different preharvest treatments.** ***A:*** Uncontaminated and untreated modality**,** ***B:*** Contaminated untreated modality, ***C:*** Contaminated and treated by Scala®, ***D:*** Contaminated and treated by *Trichoderma atroviride*, ***E:*** Contaminated and treated by *Saccharomyces cerevisiae*, ***F:*** Contaminated and treated by Stifénia®.

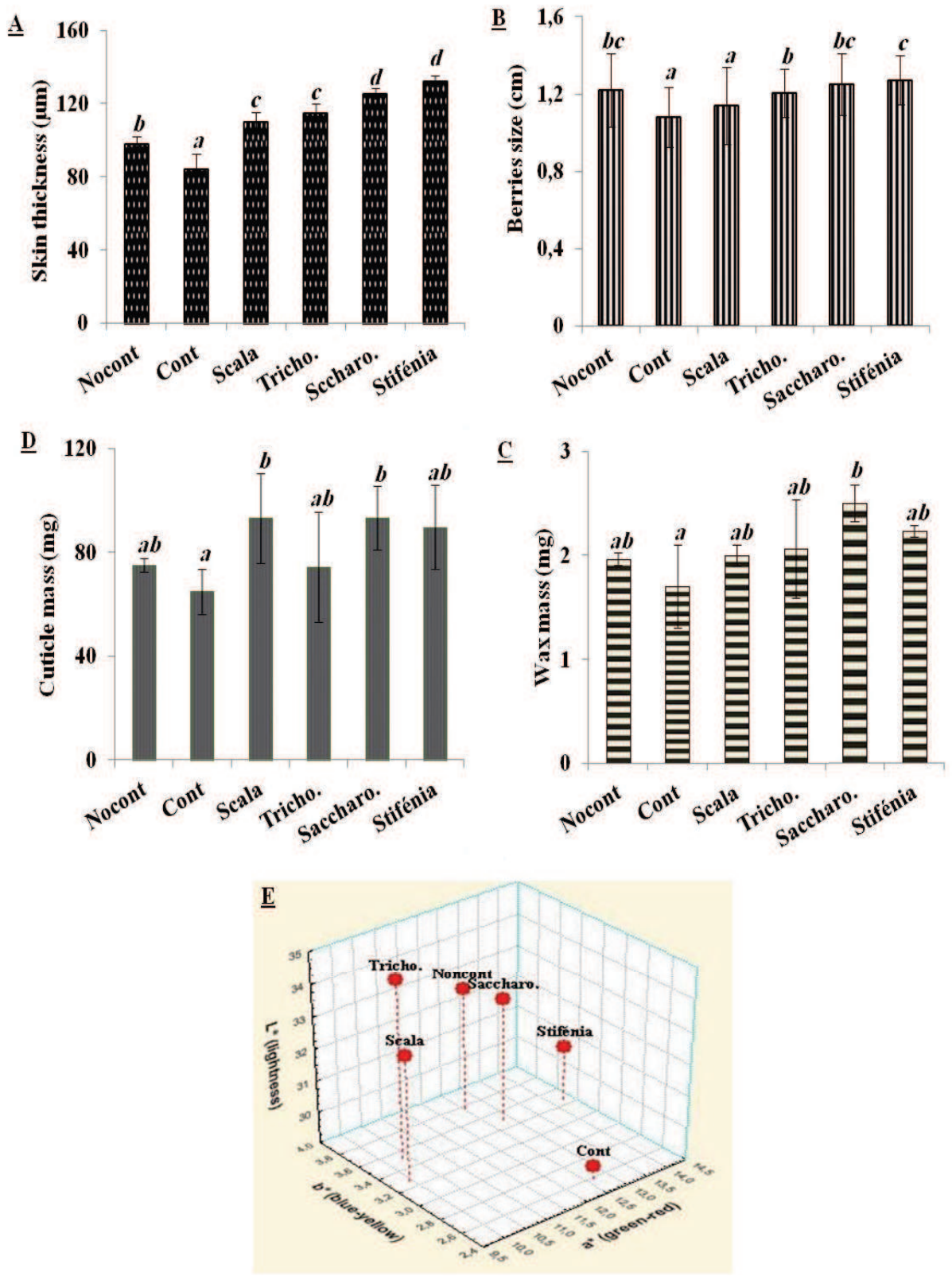


**Figure 2: Physical characteristics of different treatments. A:** Skin thickness (µm) of grape berries; **B:** Berry size with different treatments; **C:** Cuticle mass (mg) of grape berries; **D:** Wax mass (mg) of grape berries; **E:** 3D Scatter plot of juice color (Statistica 10).

***Nocont**: not contaminated by OTA-PF or by treated modality; **Cont**: artificially contaminated by OTA-PF and unreated modality; **Scala:** artificially contaminated by OTA-PF and treated by the Scala® fungicide; **Tricho.:** artificially contaminated by OTA-PF and treated by the fungus Trichoderma atroviride; **Saccharo.:** artificially contaminated by OTA-PF and treated by the yeast Saccharomyces cerevisiae; **Stifénia:** artificially contaminated by OTA-PF and treated by the Stifénia® treatment*

*Statistically analyzed using an ANOVA (Duncan at P≤0.05%), STD err for skin thickness: 2.93; berry size: 0.2; cuticle mass: 8.3. and for wax mass (Tukey at P≤0.05), STD err: 0.15.*

## 3. Chemical characteristics

### 3.1. pH and Acids content by HPLC

All grape juice samples presented pH values ranging between 3.45 and 3.7. The chemical and elicitor treatments were significantly more acidic than the biological treatments (fungus and yeast bioagents) and the two control treatments (Fig. 4A).

In all tested grape juices, malic acid was the most prevalent with concentrations varying from 5.3 ± 1.1 g/L (Stifénia® treatment) to 13.4 ± 1.1 g/L (*Trichoderma* treatment), unlike tartaric acid, which varied from 2.6 ± 1.2 g/L (Scala® treatment) to 8.8 ± 1 g/L (*uncontaminated-and-untreated* modality) (Fig. 4B).

In contrast, malic and tartaric acid juice contents decreased significantly in all of the treatments that were contaminated by OTA-PF. Excluding the *Trichoderma* treatment, which had the highest levels of these two organic acids among all contaminated treatments, the malic acid content was at the same level for the *uncontaminated-and-untreated* modality, and the tartaric acid content was significantly lower than the two control treatments and higher than other treatments (Fig. 4B). The pH and acid contents for Scala® and Stifénia® treatments had the same significantly reduced levels (Fig. 4A and 4B).

### 3.2. Sugar analysis by HPLC

In general, glucose was found in a higher quantity than fructose in all the treatments (1.086 ± 0.026 glucose: 1 fructose). Significant differences between different treatments were observed with ranges from 98.5 ± 0.6 g/L (Stifénia®) to 225.7 ± 18.1 g/L (*Trichoderma*) for glucose and from 90.8 ± 0.7 g/L (Stifénia®) to 217.9 ± 11.9 g/L (*Trichoderma*) for fructose. Fructose content decreased significantly (12%) by the contamination of *A. carbonarius* compared with the *uncontaminated-and-untreated* modality, whereas the reduction in glucose content was insignificant. The grape sugar content (glucose and fructose) of *Saccharomyces*, Scala® and Stifénia® treatments decreased significantly. The *Trichoderma* treatment was the

only treatment that had no significant difference in the sugar content compared with the *uncontaminated-and-untreated* modality (Fig. 4C).

### 3.3. Total polyphenol analysis

There was an approximately 50% reduction in the polyphenol content was observed in chemically treated juice (77.78 ± 2.36 mg gallic acid/L) compared with the *uncontaminated-and-untreated* control (146.1 ± 4.32). No significant differences were observed between the *contaminated-and-untreated* modality (166.11 ± 13.75) and the alternative treatments; the fungal biological treatment (168.89 ± 8.64) and the Stifénia® treatment (194.4 ± 13.75) had highly partial significance in the polyphenol content compared with the uncontaminated control and low partial significance compared with the other biocontrol treatment (*Saccharomyces*; 202.2 ± 1.57) (Fig. 4D).

**Discussion**

This work was performed to demonstrate the impact of alternative treatments on the OTA content of grapes and on the total quality of berries. The effect on OTA was demonstrated with all the alternative control treatments except for the fungal biocontrol treatment, which reduced the grape juice OTA concentration to within the EU permitted limit at the same OTA content as the chemical treatment. In addition, many consequences were highlighted regarding the measured total quality of the grape.

Certain tested characteristics gave an indication of the quality of grape berries and its juice (*i.e.*, berry size, sugar content, juice color, polyphenol content, etc.), whereas other characteristics indicated the safety of these plant products (*i.e.,* OTA contamination). Additional characteristics were measured to determine the induced plant defense barriers [passive defense (wax and cuticle layers and skin thickness) and chemical barriers (pH, acids and sugar contents)].[27]

The presence of wounds on grape skin strongly favors the infection of berries by *A. carbonarius*, [28] which is an OTA producer and a saprophytic organism that is not thought to penetrate the intact grape berry epidermis.[29] Thus, the thickness of the wax and cuticle layers as well as the berry skin thickness in general (which is significantly enhanced by the two biological treatments and the elicitor treatment) appeared to reduce the damage probability during the post-harvest handling of grape products (which could provide an entry path for OTA-PF infection) and reduce the infection by certain other important pathogens such as *Botrytis cirerea*.[21]

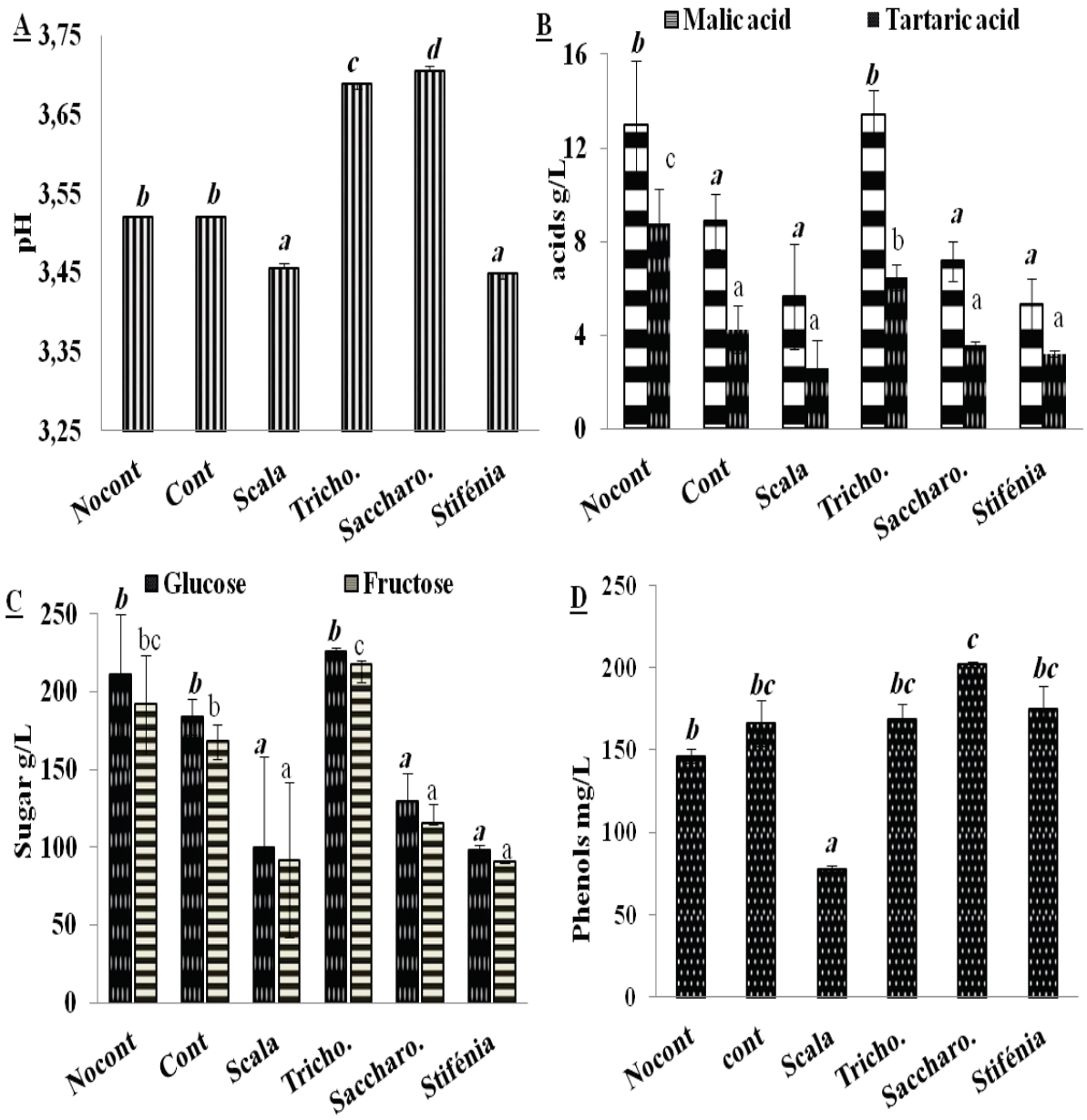


**Figure 4: Biochemical characteristics of grape juices. A:** pH; **B:** Concentrations of acids (malic and tartaric acids; g/L); **C:** Concentrations of sugars (glucose and fructose; g/L); **D:** Concentrations of polyphenols (mg/L).

***Nocont**: not contaminated by OTA-PF or by treated control; **Cont**: artificially contaminated by OTA-PF and not treated modality; **Scala:** artificially contaminated by OTA-PF and treated by the Scala® fungicide; **Tricho.:** artificially contaminated by OTA-PF and treated by the fungus Trichoderma atroviride; **Saccharo.:** artificially contaminated by OTA-PF and treated by the yeast Saccharomyces cerevisiae; **Stifénia:** artificially contaminated by OTA-PF and treated by the Stifénia® treatment.*

*Statistically analyzed using an ANOVA (Duncan at P≤0.05%), STD err for pH: 0.003; Malic acid: 1.15; Tartaric acid: 0.63; Glucose: 12.9; Fructose: 10.3; Polyphenols: 12.6.*

The grape juice sugar content had a strong correlation with the OTA content, where the significantly reduced grape juice OTA contamination was parried by the reduced sugar content as found with the chemical fungicide, yeast bioagent and plant extract elicitor treatments. Moreover, the opposite was true in the case of the fungal bioagent treatment. This result is consistent with Battilani's [30] result that, at veraison, the berry skin softens, and the sugar content increases; from this stage until harvest, berries are most susceptible to infection by *A. carbonarius* and are capable of supporting OTA production, particularly when damaged.

A positive correlation was observed between the higher acidity of the grape juice for Scala® and Stifénia® treatments and the OTA juice contamination, which agreed with findings by Serra. [31] Whereas this situation was not true with the *Saccharomyces* treatment, which had low total acidity with the same reduced level of OTA content compared with the previous two treatments that had assumed another mechanism of OTA removal action after its formation.[32]

The color of the anthocyanins in foods is influenced by several factors, including heat, pH, ascorbic acid, sugars, metal ions, and co-pigments, such as tannins. Small structural differences in anthocyanins result in differences in color under physiological conditions. The presence of blue anthocyanins is marked by high pH, whereas red anthocyanins are found in more acidic juices.[13] This observation could explain the Stifénia treatment, with the lowest acidity among all treatments, which led to transformation of colorless anthocyanins to red anthocyanins with the highest red color concentration apparent.[33] However, the situation was different in the Scala® treatment, which had the same total acidity as the Stifénia® treatment, with the lowest red color concentration. This result could be due to the low polyphenol concentrations (-50% of the total polyphenols in the Stifénia® treatment) which had a positive strong correlation with anthocyanin concentrations.

Košmerl *et al.*[34] observations that the ability of phenols to act as antioxidants depended on the degree of conjugation, number and distribution of the functional group and molecular weight. Mono-hydroxy-benzoic acids are weak antioxidants, whereas tri-hydroxy-benzoic acid (gallic acid) is the best antioxidant of all hydroxy-benzoic acids. This observation indicated the importance of the two biological treatments and the elicitor treatment that were tested this study (that was tested to OTA contamination control) because of their high concentration of total polyphenols compared with the loss of total polyphenols that is caused by the chemical treatment. Additionally, the effects of grape and red wine polyphenols on

vascular human health have been extremely controversial, although certain studies have shown improved endothelial function in healthy individuals as well as smokers.[13]

The elicitor (Stifénia®) and yeast (*Saccharomyces cerevisiae*) treatments appeared to be the most effective treatments for juice OTA contamination reduction (food safety), which was in keeping with the enhancement of the disease resistance of grape berries against certain pathogens with the improvement of the grape juice quality observed at the same time. For this global benefit, the Stifénia® or yeast treatments could potentially substitute for the chemical treatment.

**Acknowledgements**

Dr. R. Ratomahenina and Dr. J. C. Baccou are gratefully acknowledged for their contentious intellectual input and help. Our deep thanks to S.O.F.T., France for providing the Stifénia product and to I.F.V. for field experimentation. We also thank Dr. C. Gril for his help with the electron microscopic analysis.

**References**

(1) Brochard, G.; Le Bâcle, C. Mycotoxines en milieu de travail : Origine et propriétés toxiques des principales mycotoxines. Documents pour le Médecin du travail, **1999,** *119*, 299-310.

(2) Walker, R. Mycotoxins of growing interest, Ochratoxins. Tunis, Tunisia; **1999**. 3rd joint FAO/WHO/UNEP inter. Confer.on mycotoxins. ftp://ftp.fao.org/es/esn/food/myco5b.pdf.

(3) Bejaoui, H.; Mathieu, F.; Taillandier, P.; Lebrihi, A. Black aspergilli and ochratoxin A production in French vineyards. *Inter. J. Food Microbiol.* **2006,** *1*(111, 1), 46-52.

(4) Battilani, P.; Pietri, A. Ochratoxin A in grapes and wine. *Eur. J. Plant Pathol*. **2002**, *108*, 639-643.

(5) European Commission, European Commission Regulation No. 1881/2005. Amending Regulation (EC) No. 1881/2006 setting maximum levels for certain in foodstuffs as regards ochratoxin A. *OJEU* **2006**, 364, 5-24.

(6) European Commission, European Commission Regulation No. 105/2010. Amending Regulation (EC) No. 1881/2006 setting maximum levels for certain in foodstuffs as regards ochratoxin A. *OJEU* **2010**, *35*, 7-8.

(7) Varga, J.; Kozakiewicz, Z. Ochratoxin in grapes and grape-derived products. *Trends Food Sci. Technol.* **2006***, 17*, 72-81.

(8) Pohland, A., E.; Nesheim, S.; Friedman, L. Ochratoxin A: A review. *Pure App. Chem.,* **1992,** *64*(7)*,* 1029-1046.

(9) Kabak, B.; Dobson, A. D. W.; Var, I. Strategies to prevent mycotoxin contamination of food and animal feed. *Crit. Rev. Food Sci. Nutr.* **2006,** *46*, 593-619.

(10) Varga, J.; Kocsubé, S.; Péteri, Z.; Vágvölgyi, C.; Tóth, B. Chemical, physical and biological approaches to prevent ochratoxin induced toxicoses in humans and animals. *Toxins*, **2010,** *2*, 1718-1750.

(11) Soyer, Y.; Koca, N.; Karadeniz, F. Organic acid profile of Turkish white grapes and grape juices. *J. Food Compos. Anal.* **2003,** *16*, 629-636.

(12) Patil, V. K.; Chakrawar, V. R.; Narwadkar, P. R.; Shinde, G. S. Grape. In *Handbook of Fruit Science and Technology*; Salunkhe, D. K.; Kadam, S. S., Eds.; Marcel Dekker: New York, USA, 1995, 7-38.

(13) Soto-Vaca, A.; Gutierrez, A.; Losso, J. N.; Xu, Z.; Finley, J. W. Evolution of phenolic compounds from color and flavor problems to health benefits. *J. Agri. Food Chem.* **2012,** *60*, 6658-6677.

(14) En-Qin, X.; Gui-Fang, D.; Ya-Jun, G.; Hua-Bin, L. Biological Activities of Polyphenols from Grapes. *Inter. J. Mol. Sci.* **2010,** *11*, 622-646.

(15) Burin, V. M.; Falcão, L. D.; Gonzaga, L. V.; Fett, R.; Rosier, J. P.; Bordignon-Luiz, M. T. Colour, phenolic content and antioxidant activity of grape juice. Ciência e Tecnologia de Alimentos, **2010,** *30* (4), 1027-1032.

(16) Fritz, D. J.; Athmann M. Vignes en biodynamie: le jus de raisin est-il différent? Expérimentation sur du jus de raisin à l'aide de trois méthodes d'étude des forces formatrices. (http://www.bio-dynamie.org/wp-content/uploads/2012/08/12_Vignes-en-biodynamie-Jus-de-raisin-2006.pdf) (18/09/2013)

(17) Skarkova, J.; Ostry, V.; Malir, F.; Roubal, T. Determination of ochratoxin A in food by high performance liquid chromatography. *Anal. Lett.* **2013,** *46*(10)*,* 1495-1504.

(18 Dachoupakan, C.; Ratomahenina, R. M.; Martinez, V.; Guiraud, J. P.; Baccou, J. C.; Schorr-Galindo, S. Study of the phenotypic and genotypic biodiversity of potentially ochratoxigenic black aspergilli isolated from grapes. *Inter. J. Food Microbiol.* **2009**, *132*(1), 14-23.

(19) Pathan, A. K.; Bond, J.; Gaskin, R. E. Sample preparation for scanning electron microscopy of plant surfaces—Horses for courses. *Micron* **2008,** *39*, 1049-1061.

(20) Ojeda, H.; Deloire, A.; Carbonneau, A. Influence of water deficits on grape berry growth. *Vitis*, **2001,** *40*(3), 141-145.

(21) Gabler, F. M.; Smilanick, J. L.; Mansour, M.; Ramming, D. W.; Mackey, B. E. Correlations of morphological, anatomical, and chemical features of grape berries with resistance to *Botrytis cinerea. Phytopathology* **2003,** *93*(10), 1263-1273.

(22) León, K.; Mery, D.; Pedreschi, F.; León, J. Color measurement in *L*a*b** units from RGB digital images. *Food Res. Inter.* **2006,** *39*(10), 1084-1091.

(23) OIV. Recueil International des Méthodes d'Analyses. O. I. V.: Paris, France, **2009;** 2, 476.

(24) Castellari, M.; Versari, A.; Spinabelli, U.; Galassi S.; Amati, A. An improved HPLC method for the analysis of organic acids, carbohydrates and alcohols in grape. *J. Liq. Chromatogr. Relat. Technol.* **2000,** *23* (13)*,* 2047-2056.

(25) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Meth. Enzymol.* **1999,** *299,* 152-178.

(26) Tawaha, K.; Alali, F. Q.; Gharaibeh, M.; Mohammad, M; El-Elimat, T. Antioxidant activity and total phenolic content of selected Jordanian plant species. *Food Chem.* **2007,** *104*(4), 1372-1378.

(27) Guest, D.; Brown, J. Plant defences against pathogens**.** In: *Plant Pathogens and Plant Diseases*; Brown, J., F.; Ogle, H., J. Eds. Rockvale publications: Armidale, Australia, **1997,** 263-286.

(28) De Curtis, F.; De Felice, D. V.; Ianiri, G.; De Cicco, V.; Castoria, R. Environmental factors affect the activity of biocontrol agents against ochratoxigenic *Aspergillus carbonarius* on wine grape. *Inter. J. Food Microbiol*. **2012,** *159*, 17-24.

(29) Leong, S. L.; Hocking, A. D.; Pitt, J. I. Occurrence of fruit rot fungi (*Aspergillus* section *Nigri*) on some drying varieties of irrigated grapes. *Aust. J. Grape Wine Res.* **2004,** *10*, 83-88.

(30) Battilani, P.; Magan, N.; Logrieco, A. European research on ochratoxin A in grapes and wine. *Inter. J. Food Microbiol.* **2006,** *111*(Suppl.), S2-S4.

(31) Serra, R.; Mendonça, C.; Venâncio, A. Ochratoxin A occurrence and formation in Portuguese wine grapes at various stages of maturation. *Inter. J. Food Microbiol.* **2006,** *111*(Suppl. 1), S35-S39.

(32) Petruzzi, L.; Bevilacqua, A.; Baiano, A.; Beneduce, L.; Corbo, M. R.; Sinigaglia, M. Study of *Saccharomyces cerevisiae* W13 as a functional starter for the removal of ochratoxin A. *Food Cont.* **2014,** *35,* 373- 377.

(33) Heredia, F. J.; Francia-Aricha, E. M.; Rivas-Gonzalo, J. C.; Vicario, I. M.; Santos-Buelga, C. Chromatic characterization of anthocyanins from red grapes-I. pH effect. *Food Chem.* **1998,** *63*(4), 491-498.

(34) Košmerl, T.; Bertalanič, L.; Maraš, V.; Kodžulović, V.; Šućur, S.; Abramovič, H. Impact of yield on total polyphenols, anthocyanins, reducing sugars and antioxidant potential in white and red wines produced from Montenegrin autochthonous grape Varieties. *Food Sci. Technol.* **2013,** *1*(1), 7-15.

# COMPLEMENTARY RESULTS- PART 1

This part of results give supplementary data to differentiate fungal ecosystem of vine.

## 1. Sporulation Index

About thirty-five black *Aspergillus* strains were isolated from grape stem (GS) and twenty-four strains from grape juice (GJ). These black Aspergilli strains were grown on PDA medium in order to evaluate their sporulation and determine another criterion for their differenciation. A scale had been designed to differenciate these strains and establish a sporulation index (Fig. S1.1). The results were shown in Tab. (S1.1).

| $x10^6$ Spore/mL | Low | | | Medium | | | High | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | 1-2,5 | 2.6-5 | 5.1-7.5 | 7.6-10 | 10.1-12.5 | 12.6-15 | 15.1-17.5 | 17.6-20 | > 20 |

**Figure S1.1: Scale for Black *Aspergillus* Sporulation.**

**Table S1.1: Spore production for some of black *Aspergillus* isolates**

| Strain source | Black *Aspergillus* strains | Number of spores (x $10^6$ conidia/mL) | Sporulation Scale | |
|---|---|---|---|---|
| GS | CONT 6 | 1.125 | 1 | Low |
| | TRI 1 | 1.125 | | |
| | SACCHARO 4b | 1.125 | | |
| | STI 1 | 1.125 | | |
| | TRI 10 | 1.125 | | |
| GJ | TRI 8 | 1.4375 | | |
| GS | SACCHARO 10 | 1.8125 | | |
| | NOCONT 10 | 2.375 | | |
| | CONT 5 | 2.375 | 2 | |
| | STI 2 | 2.75 | | |
| | NOCONT 9a | 2.9375 | | |
| | SACCHARO 13 | 2.9375 | | |
| | SCA 2 | 3 | | |
| | SCA 7 | 3.4375 | | |
| | TRI 2a | 3.4975 | | |
| | TRI 11 | 3.5 | | |
| GJ | SACCHARO. 9 | 3.5 | | |
| | SCA 6a | 3.625 | | |
| | NOCONT 1 | 3.75 | | |
| | SCA 6b | 4 | | |
| | CONT 4 | 4.1875 | | |
| | NOCONT 1 | 4.1875 | | |
| GS | STI 6 | 4.1875 | | |
| | SCA 6b | 4.4375 | | |
| | SCA 9a | 4.525 | | |
| | SACCHARO 15 | 4.725 | | |
| | CONT 9c | 4.725 | | |
| | SCA 5 | 4.8125 | | |

| | | | | |
|---|---|---|---|---|
| GJ | NOCONT 5 | 5.125 | 3 | |
| | SCA 2 | 5.25 | | |
| | TRI 7 | 5.25 | | |
| | TRI 14 | 6.375 | | |
| | SCA 11 | 7.5 | | |
| | SACCHARO 8 | 7.575 | 4 | Medium |
| | CONT 14 | 7.575 | | |
| | SCA 4a | 7.6875 | | |
| GS | TRI 8b | 7.75 | 5 | |
| | SACCHARO 4a | 7.75 | | |
| | CONT 2a | 7.925 | | |
| | SACCHARO 12 | 8.375 | | |
| GJ | TRI 7 | 10.75 | | |
| | SACCHARO 7 | 11.125 | | |
| | TRI 5 | 11.375 | | |
| | SCA 4b | 12.625 | | |
| | SCA 3 | 13.125 | 6 | |
| GS | CONT 9a | 13.375 | | |
| GJ | SCA 1a | 13.625 | | |
| | TRI 9 | 14 | | |
| | TRI 5b | 16.1875 | | |
| | SCA 1b | 18.9375 | 7 | High |
| | SCA 9 | 20 | 8 | |
| | STI 5 | 21.375 | | |
| | CONT 2b | 22 | 9 | |
| GS | TRI 16 | 22.5 | | |
| | TRI 2b | 25.125 | | |
| | NOCONT 12b | 25.125 | | |
| | SACCHARO 16 | 25.125 | | |
| | CONT 3 | 26 | | |
| | CONT 9b | 26.125 | | |

The isolated black aspergilli straind had a wide variation of their sporulation that varied from 1.125 to 26.125 x $10^6$ conidia/mL. And a positive corrloation between the low sporulation and the high OTA produced amounts by the fungus was observed. These results combined with the morphologic (*Appendix 6*) and microscopic characterization of these fungi on different cultural media had permitted the species differenciation.

## 2. Separation of ds-DNA fragments of more than 500 bp using PCR-DGGE

Given to the followings:

- The separation principle of DGGE is based on the melting behavior of DNA molecules;
- These double-stranded DNA (ds-DNA) of same length but different base composition are subjected to a denaturant environment and will melt in decrete segments called melting domains. The melting temperature ($T_m$) of these domains is sequence-specific. When the

$T_m$ of the lowest domain is reached, the DNA will become partially melted, creating branched molecules;

- This partial melting reduces the DNA's mobility in a polyacrylamide gel;
- The denaturing environment is created by a combination of uniform temperature and a linear denaturant gradient formed with urea and formamide;
- The limited size of the ds-DNA that could be separated using the DGGE technique.

So, the selection of the used primers are a key factor in the efficacy of the DGGE particularly in the subsequent step: the sequencing one which enables the identification of the separated species. A further study was conducted to modify the DGGE conditions in order to permit the separation of longer fragments of dsDNA (more than 500 bp) which could improve the identification of the separated fungal strains.

PCR-DGGE using the specifically designed primers P65F-GC/P580R (515 bp) was performed with certain modifications in the two denaturation chemicals concentrations, which was setted by El-Sheikha *et al*. (2011) for the separation of different type of fungi, within the two gradient gel solutions used during the preparation of the DGGE gel.

### 2.1. DGGE gel with 50 and 40% concentration

A control migration was done using the setted denaturing chemicals concentrations by El-Sheikha *et al*. (2011) for the separation of different type of fungi (40% and 70%) using the ds-DNA fragments of 515 bp. The partial denaturation of the ds-DNA fragments had blocked the early during the migration process which affected the separation and evaluation processes for the different tested treatments (Figure not included).

### 2.2. DGGE gel with 50 and 40% concentration

As shown in Fig. (S1.A), the denaturation process was early blocked also. However, it was blocked after the control migration (70 % and 40 %). The statistical analysis of this DGGE was not performed due to the different denaturized ds-DNA fragments that were very close each other's

### 2.3. DGGE gel with 40 and 30% concentration

The blocking of migration of the partially denaturized ds-DNA equal to the control migration (70 % and 40 %) (Fig. S1.2B), which had also prevent the statistical analysis of this DGGE.

### 2.4. DGGE gel with 50 and 30% concentration

As shown in Fig. (S1.2C), a successful separation was achieved with comparison to this one achieved using the control denaturation with chemical concentrations (70 % and 40 %) combined with the ds-DNA 260 bp fragments (in the first article). These modified concentrations of the denaturing chemicals had showed approximately the same similarity of the: control 1 modality and Stifénia treatment, which have the highest similarity regarding what was already found in article 1, followed by yeast treatment. Whereas, the only difference was found in the similarity order between fungal bioagent treatment and the chemical fungicide one. However, Control 2 had always the highest dissimilarity regarding to the control 1 (Fig. S1.3). The sequencing step was not performed due to the low concentration of the ds-DNA fragments content used for the DGGE migration.

So, this result could permit the differentiation between fungal microflora-ds-DNA with size more than 500bp.

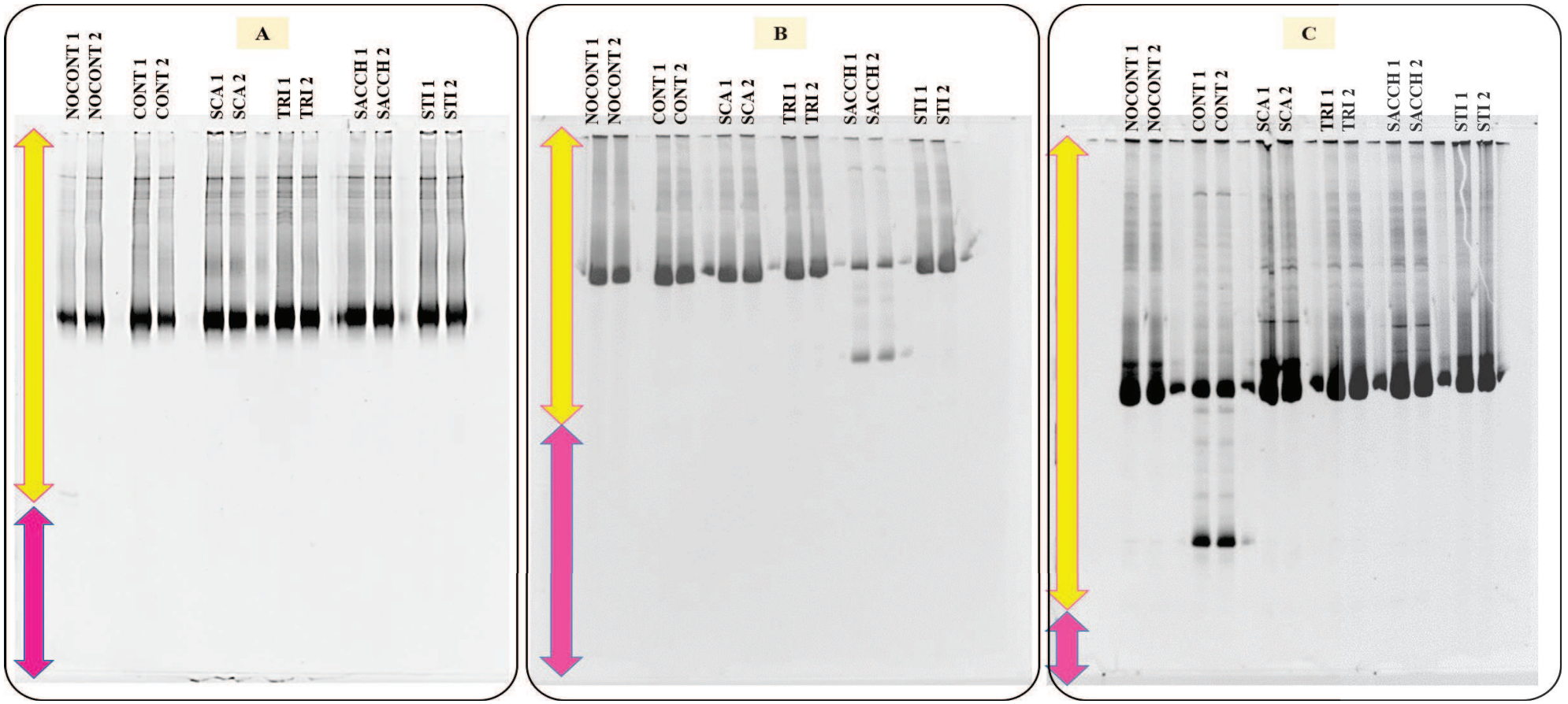


**Figure S1.2: Migrated DGGE gels for different grapevine preharvest treatments-PCR-products using P65F-GC/P580R primers with denaturant concentrations of: A: 50 and 40%, B: 40 and 30%, C: 50 and 30%.**

***NOCONT**: uncontaminated-untreated modality, **CONT**: OTA-PF-contaminated-untreated modality, **SCA**: OTA-PF-contaminated- treated by Scala® treatment, **TRI**: OTA-PF-contaminated- treated by fungal bioagent treatment, **SACCH**: OTA-PF-contaminated- treated by yeast bioagent treatment, **STI**: OTA-PF-contaminated-treated by Stifénia® treatment, **1&2**: replicates.*

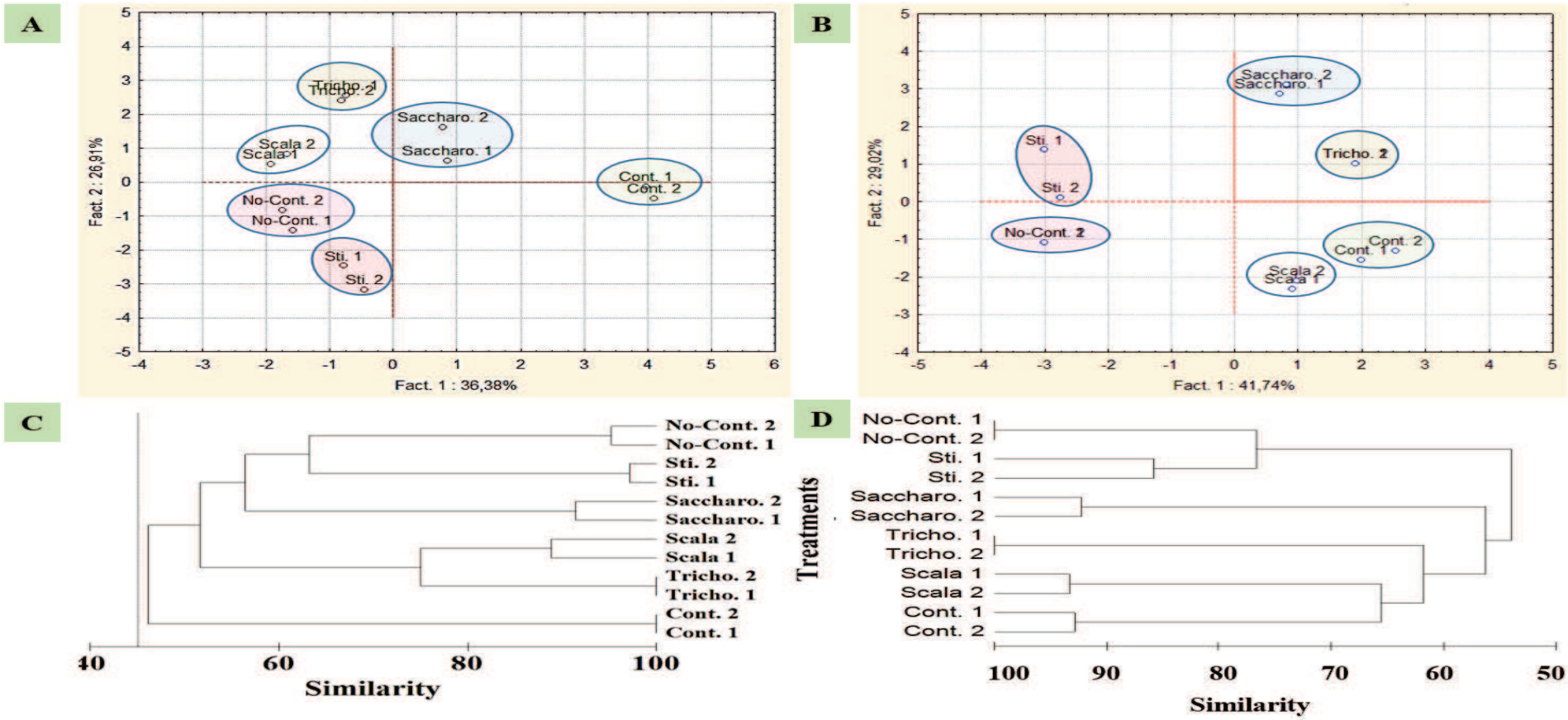


**Figure S1.3: Factorial analysis of different grapevine preharvest treatments-DGGE profiles and their similarity using: A & C: P65F-GC/P580R primers with denaturants concentrations of 50 and 30%, B & D: U1F-GC/U2R primers with denaturant concentrations of 70 and 40%.**

*NOCONT: uncontaminated-untreated modality, CONT: OTA-PF-contaminated-untreated modality, SCA: OTA-PF-contaminated- treated by Scala® treatment, TRI: OTA-PF-contaminated- treated by fungal bioagent treatment, SACCH: OTA-PF-contaminated- treated by yeast bioagent treatment, STI: OTA-PF-contaminated-treated by Stiénia® treatment, 1&2: replicates.*

## Chapter 1 Conclusion

Stifénia and yeast bioagent preharvest treatments had significantly reduced the grape juice OTA contamination at the same level of that resulted by the chemical fungicide one. While, the fungal bioagent treatment was not able to reduce the OTA production.

The two biological treatments and the elicitor treatment significantly increased the thickness of the berry skins in general (regarding the wax, cuticle layers and skin thickness), which could be related to the enhancement of the disease resistance of the grape berries to certain pathogens and could also simultaneously explain the OTA reduction and the grape juice quality improvement. In addition, the two biological treatments and the elicitor treatment gave juices with high concentration of total polyphenols compared with the loss of total polyphenols that is caused by the chemical treatment. Polyphenol content (*i. e.* antioxidants) could be another strategy reducing fungal growth and mycotoxin production which affected both, fungal growth and OTA synthesis (Kabak *et al.*, 2009; Ponsone *et al.*, 2012).

However, all the tested OTA control treatments had reduced the OTA-PF incidence in the grape juices with comparison to the two control modalities. The microbiological isolations and quantifications for the fungal biodiversity and the fungal ecosystem changes still one of the common techniques that were used in the evaluation of fungi affecting food, although, they involve time consuming assays.

The qPCR technique using different primer set could be used as a fast monitoring molecular technique. The qPCR is most accurate technique regarding the DGGE technique which is also used as a fast qualitative monitoring technique for the fungal ecosystem. However, molecular methods could be accompanied with some disadvantages, such as, the disability of differentiating between vial and dead fungi. The fungal ecosystem estimations were approximately the same using the microbiological enumeration and quantitative PCR.

A promising result was obtained by the separation of higher ds-DNA size more than the maximum limit, which could reduce the disadvantages of PCR-DGGE (Durate *et al.,* 2012) and improve its efficacy in evaluation and differentiation between different microflora ecosystems by increasing the microflora sequence variance.

Given to the previous findings, the yeast bioagent and elicitor treatments have improved the juice quality and safety. Whereas, the Stifénia® treatment had showed the highest effect on the fungal ecosystem and especially the black aspergilli including the OTA-PF. Moreover this treatment allowed the isolation of new fungal strains, non-black aspergilli

(NBA), which were not isolated from other treatments. That was the motive to study the effect of these NBA strains isolated of the Stifénia on the OTA-PF in order to understand the mechanism of action of the Stifenia in the plant defense stimulation.

## References

**Bendele A. M., W. W. Carlton, P. Krogh and E. B. Lillehoj (1985).** Ochartoxin A carcinogenesis in the C57BL/6JxC3HF1 mouse. *Journal of the National Cancer I*, **75(4)**:733-742.

**Chopra M., P. Link, C. Michels and D. Schrenk (2010).** Characterization of ochratoxin A-induced apoptosis in primary rat hepatocytes. *Cell Biology and Toxicol*ogy, **26**:239-254.

**Duarte S., F. Cássio and C. Pascoal (2012).** Sofia Duarte, Fernanda Cassio and Claudia Pascoal (2012). Denaturing Gradient Gel Electrophoresis (DGGE). *In*: Magdeldin S. (Ed.), Microbial Ecology - Insights from Freshwaters, Gel Electrophoresis - Principles and Basics, ISBN: 978-953-51-0458-2, pp: 173-196.

**El-Sheikha A. F., N. Durand, S. Sarter, J. B. L. Okullo and D. Montet (2012).** Study of the microbial discrimination of fruits by PCR-DGGE: Application to the determination of the geographical origin of Physalis fruits from Colombia, Egypt, Uganda and Madagascar. *Food Control,* **24(1-2)**:57-63.

**Frisvad J. C. and R. A. Samson (1991).** Mycotoxins Produced in Species of *Penicilllium* and *Apergillus* Occurring in Cereals. In: *Cereal Grain. Mycotoxins, Fungi and Quality in Drying and Storage*; J. Chelkowski, (Ed.); Elsevier: Amsterdam, The Netherlands, **1991**; pp. 441-476.

**Haubeck H. D., G. Lorkowski, E. Kolsch and R. Roschenthaler (1981).** Immunosuppression by ochratoxin A and its prevention by phenylalanine. *Applied and Environmental Microbiology*, **41(4)**:1010-1042.

**IARC (1993)**. Some naturally occurring substance: some food items and constituents, heterocyclic aromatic amines and mycotoxins. *In*: Monographs on the Evaluation of Carcinogenic Risks to Humans, Lyon, France. *International Agency for Research on Cancer.* **56***:*489.

**Kabak B. and A. D. W. Dobson (2009).** Biological strategies to counteract the effects of mycotoxins. *Journal of Food Protection*, **72**:2006-2016.

**Kanizawa M. and S. Suzuki (1978).** Induction of renal and hepatic tumors in mice by ochratoxin A, a mycotoxin. *Gann*, **69(4)**:599-600.

**Kowalska J., D. Remlein-Starosta and D.Drozdzyriski (2011).** Efficacy of bioagents against apple scab in organic orchards, preliminary results. http://www.inhort.pl/files/ekotechprodukt/prezentacje_wynikow/publikacje/PWC%20Korea2011%20dla%20EcotachProduct.pdf

**Krogh P. (1992).** Role of chratoxin in disease causation. *Food and Chemical Toxicology,* **30(2)**:213-224.

**Mayura K., R. V. Reddy, A. W. Hayes and W. O. Berndt (1976).** Teratogenic and toxic effects of ochratoxin A in rats. *Toxicology and Applied Pharmacology*, **37(2)**:331-338.

**Ministère Française de l'Agriculture et de la pèche (MFAP) (2008).** http://agriculture.gouv.fr/IMG/pdf/PLAN_ECOPHYTO_2018.pdf (09/10/2013).

**Petersson S., M. W. Hansen, K. Axberg, K. Hult and J. Schnürer (1998).** Ochratoxin A accumulation in cultures of *Penicillium verrucosum* with the antagonistic yeast *Pichia anomala* and *Saccharomyces cerevisiae*. *Mycological Research*, **102(8)**:1003-1008.

**Ponsone M. L., M. L. Chiotta, J. M. Palazzini, M. Combina and S. Chulze (2012).** Control of ochratoxin A production in grapes. *Toxins*, **4**:364-372. DOI: 10.3390/toxins4050364.

**van der Merwe K. J., P. S. Steyn, L. Fouris, De. B. Scott and J. J. Theron (1965).** Ochratoxin A, a toxic metabolite produced by *Aspergillus* Wilk. *Nature*, **205***:*1112-1113.

**van Egmond H. P.and G. J. A. Speijers (1994).** Survey of data on the incidence and levels of ochratoxin A in food and animal feed wordwide. *Journal of Natural Toxins*, **3**:125-143.

**Varga J., S.Kocsubé, Z. Péteri, C. Vágvölgyi and B. Tóth (2010).** Chemical, physical and biological approaches to prevent ochratoxin induced toxicoses in humans and animals. *Toxins*, **2**:1718-1750.

**Vukelic M., B. Sostaric and M. Belicza (1992).** Pathomorphology of Balkan endemic nephropathy. *Food and Chemical Toxicology*, **30**:193-200.

# Chapter 3

# Results

# Part 2

# PART 2

# STIFÉNIA MODE OF ACTION FOR THE TOXICIGENICTY OF OTA-PF (*ASPERGILLUS CARBONARIUS*) REDUCTION REGARDING BIOLOGICAL CONTROL AND VOLATILE COMPOUNDS ENHANCEMENT.

## 1. Introduction

Although chemicals have been commonly used to reduce fungal proliferation and mycotoxin production under field conditions, nowadays a strict legislation about their use has been established in the European Union, due to the increasing number of resistant fungal strains and the impact of fungicides on the environment and human health. Maximum residue levels of pesticides have been regulated in many products, including grapes (EC, 2008). Therefore, alternative methods are necessary to substitute or complement fungicide treatments to control toxigenic fungi at pre- and postharvest stages (Ponsone *et al*., 2012). Even though the main objective of the food and feed chain providers is to avoid the extended contamination of plant-derived foods and animal feeds with OTA, until now, complete OTA removal from foods and feedstuffs is not feasible (Kabak and Dobson, 2009). Many efforts are being made to achieve OTA reduction. Prevention through pre-harvest management is the best method for controlling mycotoxin contamination (Amézqueta *et al*., 2009; Ponsone *et al*., 2012).

The plant extract, Stifénia® (its active ingredient is an extract of fenugreek seeds) is primarily recommended for the fight against powdery mildew (Martinez and Loison, 2006), it seems to be effective against other diseases such as peach leaf curl, *Taphrina deformans*. And downy mildew various plants. Approved in 2005 by the Society Occitane Manufacturing and Technologies (Port-la-Nouvelle, France), the Stifénia® has been marketed since 2006 in France and neighboring countries such as Spain, Italy, Morocco and Lebanon (Benhamou and Rey, 2012).

However the effectiveness of Stifénia® is the subject of controversy (Simon 2007), where Camele *et al.* (2009) has demonstrated that the Stifénia® was significantly less effective against powdery mildew of melon than sulfur or fungicide chemicals in addition to induce significant yield losses as a result of repeated applications.

*Chapter 3: Results-Part 2*

An advantage is noteworthy, the results obtained in the first part of results, had demonstrated the reduction impact of Stifénia preharvest treatment on the OTA-PF incidence with more than 90% regarding the natural incidence in the open field. In addition, Stifénia® had reduced the grape juice's OTA contamination approximately with 40 % regarding the artificially contaminated vines. And the isolating of new emerged NBA strains in this preharvest treatment.

In this part, further studies were conducted assaying to understand the Stifénia® mode of action (Fig. R2.1.) by the following

1- Biological control tests between the NBA-Stifénia isolated strains against the OTA-PF growth and OTA production.
2- Antifungal activity test of the direct effect of Stifénia against the OTA-PF
3- Volatile compound Stifénia leaves profile assessment regarding the VOC profile of chemically treated leaves
4- Antifungal activity of the VOC that have highest concentrations in the Stifénia treated vine leaves

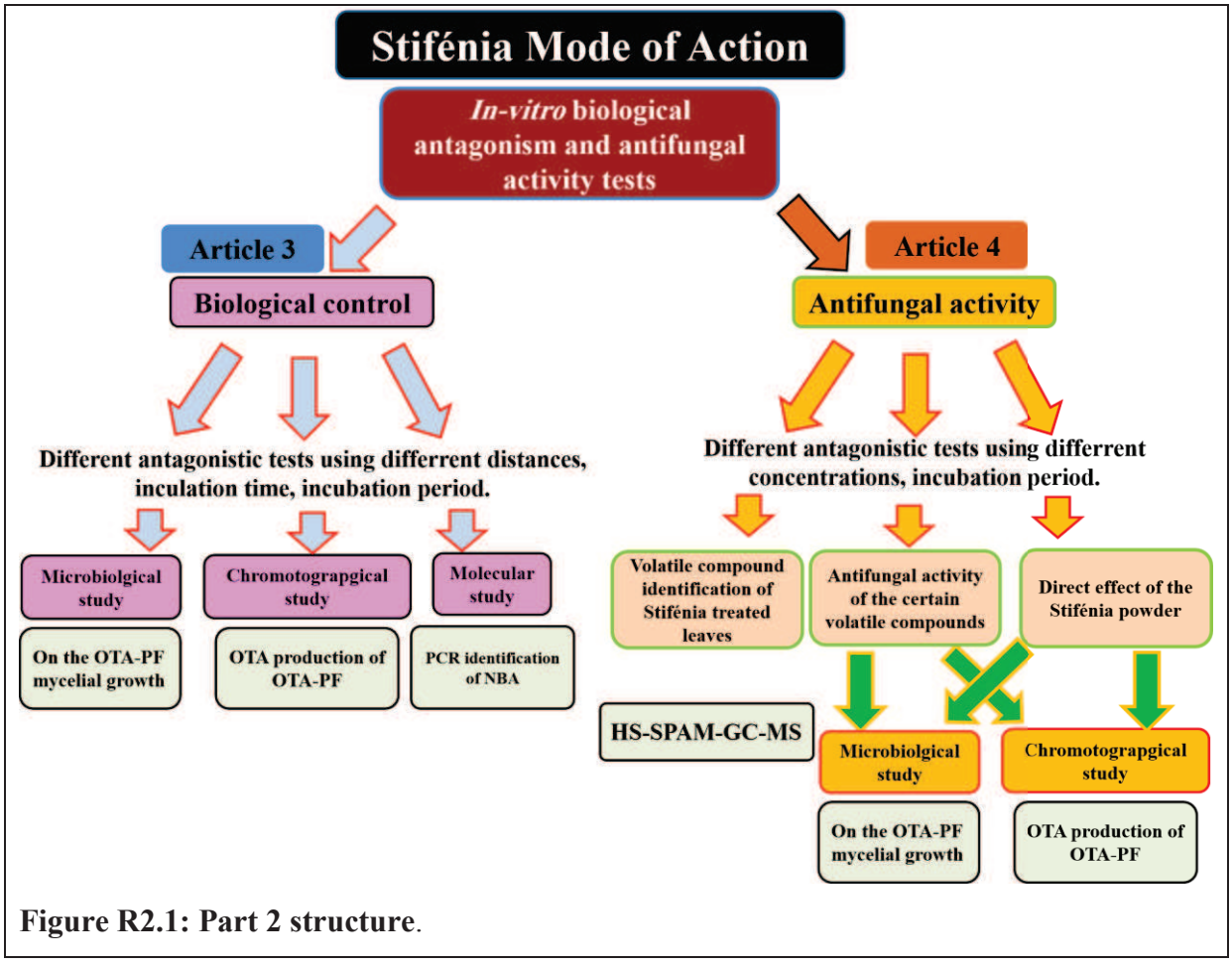


**Figure R2.1: Part 2 structure**.

# First report: *Penicillium adametzioides*, an emerging biocontrol agent for ochratoxin-producing fungus in grapes, resulting from natural product pre-harvest treatment

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Submitted to Food Control

Manuscript Number: FOODCONT-D-13-01559

**Abstract**

Ochratoxin A (OTA) is a secondary metabolite produced mainly by *Aspergillus* section *Nigri* (*A. carbonarius* is the most relevant strain in the Mediterranean region with a group 2B carcinogenic effect in humans. *In vivo* experiments were conducted in southern France involving applying pre-harvest Stifénia® (elicitor), Scala® (chemical fungicide) and two other control treatments [not contaminated by *A. carbonarius* (OTA-PF) and not treated and artificially contaminated by OTA-PF but not treated]. The Stifénia® and Scala® treatments significantly reduced the OTA juice contamination so that it was under the authorised uptake OTA limit. Stifénia® highly affected the grape fungal ecosystem with new non-*Aspergillus* strains isolated from grape stems and juices. *In vitro* antagonistic tests were performed with Stifénia® non-*Aspergillus* isolates (n= 10). Three antagonistic tests were applied using different distances (3 and 5 cm in between the two microorganisms) with two different inoculation times (at the same time and with three day intervals in between). Certain strains had a positive mycelial growth effect on *A. carbonarius* colonies, such as *Penicillium* spp. and *Fusarium* sp. Other strains displayed a reduction effect on OTA production of OTA-PF, such as *Penicillium* spp. (J2, J3). *P. adametzioides* (S3) and *P. expansum* (J1) (at certain stages) reduced the OTA production and mycelial growth. *P. expansum* was excluded as a bio-control agent because of its mycotoxin production ability. The higher challenge distance between certain strains of *P. adametzioides* (S3) and other *Penicillium* strains (as J1, J2, J3 and J4; at three and seven days) reduced the secretion of OTA by OTA-PF. This OTA production reduction could possibly prevent OTA contamination prevention in the case of epidemic favourable conditions by reducing the OTA produced in grape post-harvest products (i.e., juice). This could be accomplished by applying as the elicitor one of the tested fungi with an antagonistic effect on OTA production, such as *P. adametzioides* (at 10 days). Certain strains, such as *P. adametzioides* (S3) and J2 (*P.* spp.) should be further investigated to determine the details of the underlying mechanism of their OTA reduction and their ecosystem effects in cases of *in vivo* application.

**Key words:** *Aspergillus carbonarius, Penicillium adametzioides, OTA, biological-control, Stifénia®.*

**Abbreviations:** ***OTA-PF****; Ochratoxin A producing fungus*

**Introduction**

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, teratogenic, and immunosuppressive properties (Pitt *et al.,* 2001). It has been classified as a possible human carcinogen (group 2 B) by the International Agency for Research on Cancer (IARC) (Reddy and Bhoola, 2010). OTA is suspected to be involved in Balkan Endemic Nephropathy (BEN) (a fatal kidney disease occurring in some areas of south-eastern Europe) and in the high frequency of urinary tract tumours observed in some Balkan areas (Pfohl-Leszkowicz *et al*., 2000). A relationship between kidney disease in association with focal segmental glomerulosclerosis (FSGS) in humans and Ochratoxin A has been demonstrated by Hope and Hope (2012).

Human OTA exposure mainly occurs via the food chain. Thus, EU legislation has established OTA maximum uptake limits for certain plant products, such as grape juice and wine (maximum of 2 µg/L) because the tolerable weekly intake (TWI) of OTA is 120 ng/kg body weight (European Commission Regulation (EC) No. 1881/2005, 2006; EC No. 105/2010, 2010). OTA contamination in grape juice is of great concern, as children are its main consumers, and the consumption of juice is greater than that of wine (Varga and Kozakiewicz, 2006).

OTA is related to grape contamination in the vineyard by several OTA-producing species of fungi, especially the black aspergilli, mainly *A. carbonarius* and the members of the *A. niger* aggregates. *A. carbonarius* is considered the major OTA-producing species in vine grapes (Cabañes *et al*., 2002). Numerous studies have demonstrated that moulds can be found on grapes from the veraison stage, and mould development increases rapidly between veraison and maturation (Bejaoui *et al.*, 2006).

Several strategies have been proposed to prevent the toxic effects of mycotoxins in general, and of ochratoxins in particular, in food and feed: (i) prevention of mycotoxin contamination; (ii) decontamination or detoxification of foods contaminated with mycotoxins; and (iii) inhibition of the absorption of consumed mycotoxin in the gastrointestinal tract (Kabak *et al.,* 2006). The prevention of mycotoxin contamination in the field is a main goal of the agricultural and food industries. As mycotoxin-producing moulds can usually colonise damaged parts of plants, crops must be protected against damage caused by either mechanical processes or insects. Field treatment with fungicides is the traditional prevention technique (Vagar *et al.,* 2010). The public's demand for reduced pesticides in food and the environment have resulted in an energetic debate over the safety of the present

control practices for postharvest diseases. Natural plant extracts and antagonism may provide alternatives to chemical preservatives (Sukorini *et al*., 2013). Biological control using certain microorganisms for postharvest diseases could of the use in the management of the beneficial microflora that already exist on fruit and vegetable surfaces or artificial introduction of antagonists against postharvest pathogens. Our knowledge of methods to manipulate the naturally occurring populations of mixed species of microorganisms in a beneficial manner, however, is meager, and the greatest use of biological control (pre- and postharvest) has been achieved through the artificial introduction of large numbers of a known antagonist (Michael *et* al., 1992). Although it is not possible to entirely prevent the formation of OTA in food products, OTA accumulation can be minimised (Varga *et al.,* 2010). Over the years, much effort has been devoted to the search for new antifungal materials from natural sources for food preservation (Yin and Tsao, 1999; Galvano *et al.,* 2001; Soliman and Badeaa, 2002; Boyraz and Ozcan, 2005; Irkin and Korukluoglu, 2007). Certain natural compounds have antifungal activity that inhibit ochratoxigenic black aspergillii growth, especially *A. carbonarius*, such as fusapyrone produced by *Fusarium semitectum* (Favilla *et al.,* 2008) and natamycin produced by *Streptomyces natalensis* (Medina *et al.,* 2007).

The excessive use of chemical fungicides and the negative effects of their residuals on the microflora natural balance in the environment also motivated this study. This work aimed to find a safe control method for reducing the OTA production by *Aspergillus carbonarius* and its accumulation in the human food, particularly in grape juice by applying an alternative to chemical fungicides, including a natural plant extract (elicitor, Stifénia®) at an experimental station. The study examined the microbial interactions in the most effective treatment for reducing the OTA production as a possible explanation for its mode of action by studying the antagonistic activity of its non-*Aspergillus* isolated strains against *Aspergillus carbonarius*.

## 1. Material and methods

### *1.1.* *In vivo* experiment and fungal strains

Four pre-harvest treatments were applied to the cultivar Mourvèdre, at the IFV (French Institute of Vine and Wine), Pech Rouge, Narbonne, France. The vines were not treated nor contaminated with *Aspergillus carbonarius* [previously isolated from French vineyards; OTA producing fungus (OTA-PF)] for the first modality (Control 1). The other three treatments were contaminated with OTA-PF by inoculation at the veraison stage. The first treatment involved no application (Control 2), whereas the other two treatments consists of treatment

with Stifénia® (homogenised fenugreek seed powder, SOFT, France) from the green-tip stage to harvest with 15-day interval (as recommended by producers to encourage the eliciting effect), and the other treatment involved two treatments with Scala® (chemical fungicide with pyrimethanil 400 g/L as active ingredients, Bayer, France) at green-tip stage and leaves-output stage. The samples were harvested in late September 2010, during the grape harvest period. Grape bunches (5 kg/ treatments) were randomly taken and placed in two previously sterilised bags, which were kept at 4°C until analysis. Mycological analysis was immediately performed and the remaining samples were kept frozen at -20°C.

### *1.2.* Media cultures

PDA (Potato-Dextrose agar; Biokar Diagnostics, Beauvais, France) medium with chloramphenicol was used for fungal isolation at pH 3.5 from GJ and GS. Normal PDA was used for strain purification, conservation and antagonistic tests. CYA and MEA culture media were used for morphological and microscopic identification.

### *1.3.* Isolates

The *Aspergillus carbonarius* strain that was used for the *in vitro* grape contamination was previously isolated from French vineyards and displays high OTA production (Dachoupakan *et al*., 2009). All of the other non-*Aspergillus* tested strains were naturally isolated from grape juices (GJ) and stems [empty clusters; (GS)] that were treated with Stifénia®.

## 2.4. Enumeration

### 2.4.1. From grape juice

Decimal dilutions of grape juice (approximate 150 g of randomly grape clusters parts were homogenised in a stomacher (France) without external water and filtered under pressure). Then100 µL of each dilution were spread on PDA (3.5 pH) in Petri plates and incubated at 25°C for five to seven days in the dark. After incubation, the number of CFU of the filamentous fungi per millilitre of juice homogenate was evaluated (ISO 7218:1996/Amd. 1:2001 (F)) (AFNOR, 2002).

#### 2.4.2. From grape stalks (GS)

To estimate the microbial population in the grape stalks (GS), the same protocol described above was performed using 25-35 g of GS mixed with 100 mL of sterile physiological water and homogenised by stomacher for 2 min and decimal dilutions with sterile physiological water (NaCl; 8.5 g/L). The same spreading dilution quantities that was used for grape juice enumeration were applied on PDA (3.5 pH) with two replicates at the same incubation conditions. The CFU of filamentous fungi number per millilitre of homogenate grape stalks was evaluated (ISO 7218:1996/Amd. 1:2001 (F)) (AFNOR, 2002).

### 2.5. *In vitro*, survey of the antagonistic effect of the non-*Aspergillus* isolated strains from the Stifénia pre-harvest treatment against *A. carbonarius*.

The antagonistic ability of all non-*Aspergillus* species isolated from Stifénia treatment was tested against *A. carbonarius* [six strains isolated from GJ and four strains from GS]. Three different methods were applied to PDA petri plates differing in the distance between the two fungi and the time of inoculation [5-cm distance inoculated at the same time (Bendahmane *et al.*, 2012), at 3-cm distance inoculated at the same time, the antagonist inoculated and incubated for three days and then *Aspergillus carbonarius* inoculated at 3-cm distance] with three replicates. Then, the plates were incubated at 25°C and the growth reduction and OTA production of *A. carbonarius* were determined (Suarez-Quiroz *et al.,* 2004) at three, seven, and 10 days. The results were expressed in µg of OTA/g of dry weight. The calibration curve was prepared with OTA dilutions (Sigma) from 0.25 $ng.mL^{-1}$ to 100 $ng.mL^{-1}$ for extracted culture samples.

The % of mycelial growth reduction was calculated according to the equation of Pandey *et al.* (1982). The % of mycelial growth reduction = $[(d_c - d_t) / d_c] \times 100$, where $d_c$ =the growth diameter of the *A. carbonarius* control colony, $d_t$ =the growth diameter of the *A. carbonarius* colony in the presence of the different strain isolates under study.

### 2.6. OTA determination

#### 2.6.1. Extraction

##### 2.6.1.1. From grape juices

Fifty grams of each sample was homogenised with 200 mL of 70% methanol using a blender at high speed for 1 min and filtered. The filtered extracts were diluted (1: 5) by

PBS/0.01% Tween 20 (washing buffer) (Sigma-Aldrich, France). Twenty millilitres of each filtered diluted extracts were passed through OchraTest™ affinity columns at a rate of approximately 1 drop/sec until air passed through the column. The columns were then washed with washing buffer (1-2 drops/sec) followed by 10 mL of purified water (1-2 drops/sec). The OTA content were collected in 1.5 mL of methanol:acetic acid (98:2, v:v) (1 drop/sec) (Skarkova *et al.*, 2013).

#### 2.6.1.2. From culture media

Colony diameter was measured, and three agar plugs that were 5 mm in diameter were removed from the *A. carbonarius* colonies (in the area where the fungus were challenging the other tested fungus in terms of antagonist activity) at three, seven and 10 days of incubation. The plugs were weighed, introduced into 3-mL vials and extracted with 2.5 mL of solvent (methanol/formic acid, 25:1) under sonication for 15 min. The solvent was then evaporated under a nitrogen stream at 40 °C. The dried extracts were re-suspended in the mobile phase of the subsequent HPLC (49.5% deionised water/49.5% acetonitrile/1% acetic acid) and then filtered with a syringe (Minisart SRP 4 of diameter 0.45 µm, Sartorius, Germany) (Suarez-Quiroz et al., 2004).

### 2.6.2. HPLC analysis

Twenty microlitres of the extracts (juice and culture media ones) were quantified by injection into a C18 column (25 x 4.6 nm, 5 µm, Brownlee, USA) on HPLC system equipped with a fluorescence detector ($\lambda_{exe}$ 333 nm; $\lambda_{em}$ 460 nm, Shimadzu RF-10AXL, Japan). The HPLC mobile phase was pumped at 0.9 mL/ min. (pump, Shimadzu LC/9A, Japan), and the retention time was 15 min. The calibration curve was prepared with OTA dilutions (Sigma) from 0.25 ng/mL to 5 ng/mL for extracted culture samples. The results were expressed in µg of OTA/g of dry weight for the culture media and in µg of OTA/L of grape juices.

## 2.7. Molecular identification

The Dachoupakan *et al.* (2009) protocol for pre-DNA extraction for all of the fungal strain preparations was performed. DNA was extracted using the El-Sheikha *et* al. (2009) protocol with modifications in the first steps using liquid nitrogen (Atoui *et al.,* 2005) for the extraction instead of glass beds.

PCR was conducted with a designed primer pair at the ITS1 region and 5.8S using online software and databases (NCBI database, Multalin, and oligonucleotide properties calculator), *P65f 3'caacctcccacccg '5* and *P580r 3'ctacctgatccgagg 5'* were used to identify

the non-*Aspergillus* isolated strains. The PCR products for important isolated strains were then sequenced (MilleGen, Labège, France) and identified using the online NCBI database. The obtained results were confirmed using the primer pair ITS1 and 5.8S used by Fierer *et al.* (2005).

### 2.8. Statistical analysis

An ANOVA analysis was conducted using Statistica 10 software (Stat Soft. Inc., Tulsa, USA). The Duncan test was used to detect any significant differences between the examined groups, and significance levels were defined using $P \leq 0.05$.

## 3. Results

### 3.1. In vivo experiment

The OTA content was significantly reduced in the grape juice at the same rate (40%) using the two Stifénia and Scala (1.5 µg OTA/L) treatments in comparison to the OTA content of the contaminated and not-treated treatment (2.5 µg/L) (Fig. 1).

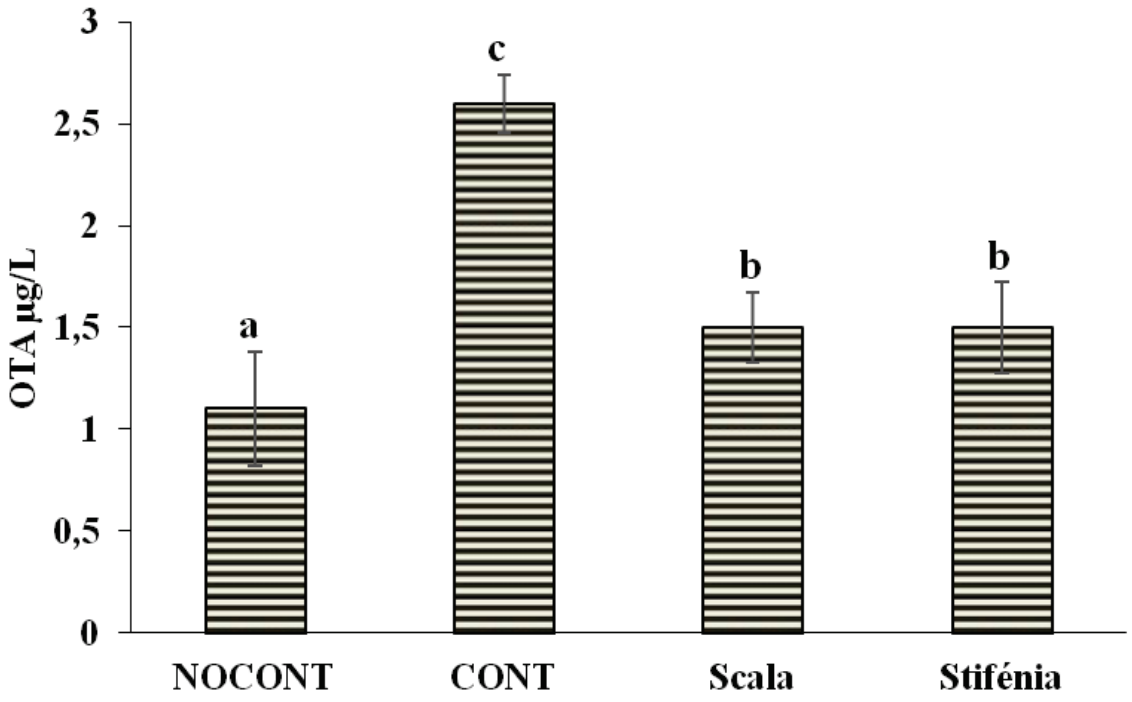


**Figure 1: OTA content in grape juice of different treatments.**

***NOCONT**: not contaminated by OTA-PF and untreated treatment; **CONT**: artificially contaminated by OTA-PF and untreated treatment; **Scala:** artificially contaminated by OTA-PF and treated with Scala® fungicide; **Stifénia:** artificially contaminated by OTA-PF and treated with Stifénia®*

*Statistically analysed using ANOVA, Tukey at 0.05% (std err = 0.05).*

The Stifénia® treatment mainly reduced the spore potential by 99.8% of the total microflora compared with references the black *Aspergillus* strains when the treatment fungi were compared with the contaminated but not treated control treatment (Fig. 2 A) in the case of the isolates from grape juice, and there was an 83.4% reduction (Fig. 2 B) in the case of the grape stalks isolates. The spore potential of black *Aspergillus* strains in the Scala® treatment was increased by 9.9% in grape juice compared with the contaminated not-treated control treatment (Fig. 2 A) and was reduced by 20.8% when examining grape stalk isolates (Fig. 2 B). The microbial ecosystem was highly affected by the Stifénia treatment compared with the other three treatments, as shown in Fig. 2.

In addition, Stifénia® treatment reduced the contamination in terms of species number of black *Aspergillus* to 66.7% in grape juice and to 71.4% in grape stalks compared with the control treatment (contaminated by OTA-PF but not treated) (Fig. 2A and 2B). The number of black aspergilli contaminating the grapes treated by Scala was reduced in comparison with the Stifénia treatment regarding the control treatment (contaminated by OTA-PF but not treated) by 58% and 42.8%, respectively (Fig. 2A and 2B). The Stifénia® treatment reduced the isolated number of *non-Aspergillus* strains compared with the other three treatments. Moreover, emerging new non-*Aspergillus* species were isolated from the Stifénia® treatment compared with the control treatments (Fig. 3).

## 3.2. *In vitro* antagonistic survey

### 3.2.1. *Effect on OTA-PF mycelial growth.*

The new emerging strains of *non-Aspergillus* stains and their limited number of species (Fig. 3) isolated from the Stifénia treatment encouraged the study of the antagonistic activity of these strains against OTA-PF growth and OTA production ability.

Most of the 10 tested isolates (isolated from the Stifénia treatment) significantly affected *A. carbonarius* mycelial growth. The OTA-PF occupied 0.66 µm to 3.93 µm of the region that challenged the tested isolates, corresponding to the mycelial growth reduction (varying from -36.11% to 84.79%) (Tab. 1).

The first method (5 cm) had the lowest effect on the OTA-PF growth. Moreover, certain isolates significantly increased the mycelial growth (J2 and J4) in the early stages of growth. This negative effect reversed with more through colonisation of these fungal colonies.

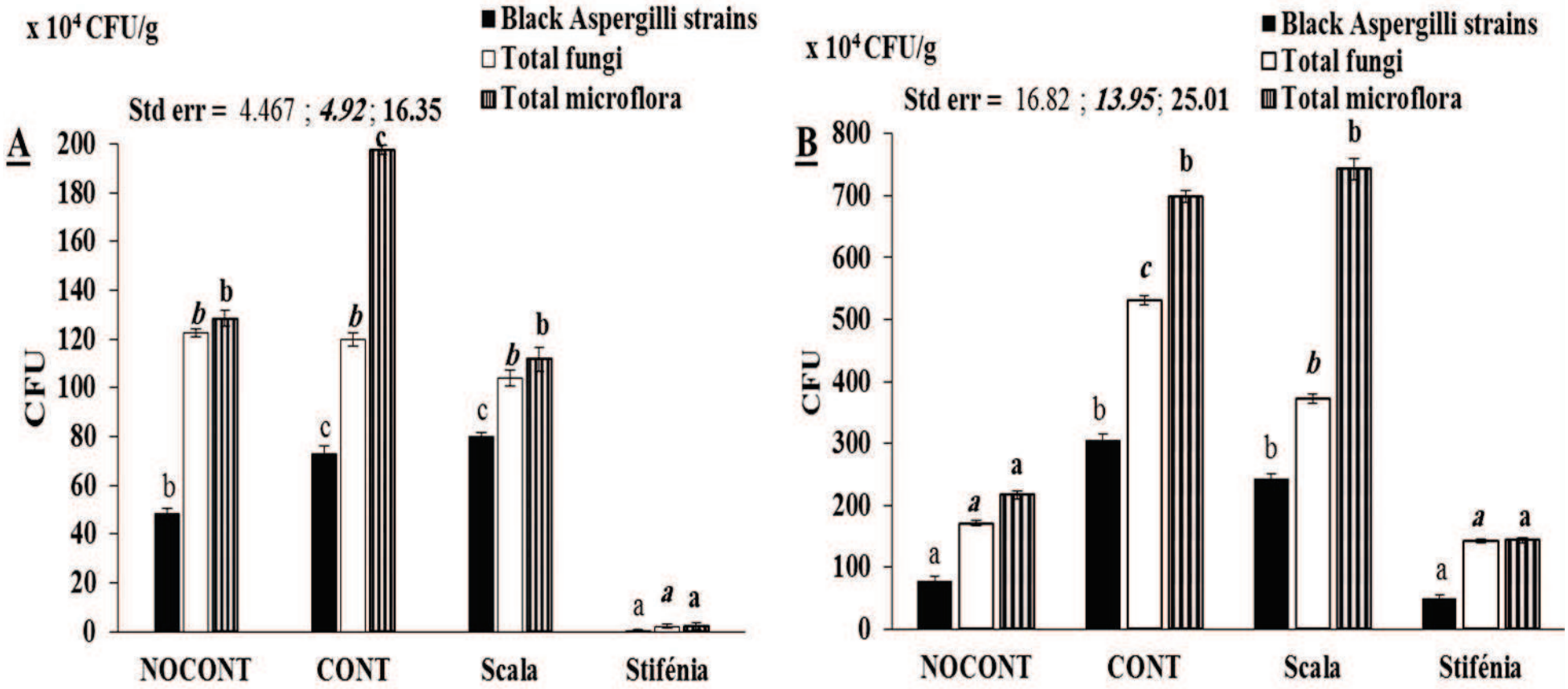


**Figure 2: CFU of total microflora** (black Aspergillus, total fungi, and total yeasts) **for grape; A:** juice, **B:** stems.

***NOCONT****: not contaminated by OTA-PF and untreated treatment;* ***CONT****: artificially contaminated by OTA-PF and untreated treatment;* ***Scala:*** *artificially contaminated by OTA-PF and treated with Scala® fungicide;* ***Stifénia:*** *artificially contaminated by OTA-PF and treated with Stifénia®*

*Statistically analysed using ANOVA (Duncan test at P ≤ 0.05)***.**

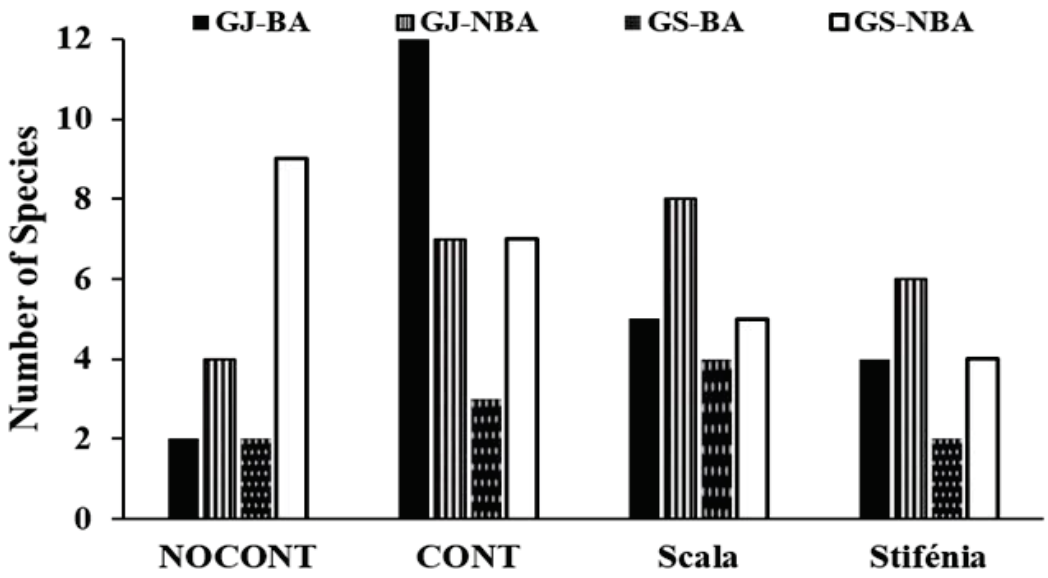


**Figure 3: Number of fungal species isolated from all treatments with different isolation source.**

***NOCONT**: not contaminated by OTA-PF and untreated treatment; **CONT**: artificially contaminated by OTA-PF and untreated treatment; **Scala:** artificially contaminated by OTA-PF and treated with Scala® fungicide; **Stifénia:** artificially contaminated by OTA-PF and treated with Stifénia®; **BA:** black Aspergilli species, **NBA**: non-black Aspergilli species, **GJ:** grape juice, **GS:** grape stalks.*

With the Second and third methods, all of the tested strains reduced the OTA-PF mycelial growth at the same rate, and a positive relationship with the date of incubation and the rate of growth reduction was recorded. J1 (*Penicillium expansum*) (Fig. 4 and 7G), J8 (*Fusarium* sp.) (Fig. 5 and 7H), J2 (*Penicillium* sp.), S6 (*Penicillium* sp.), J4 (*Penicillium* sp.), J7 (*Penicillium* sp) and S3 (*P. adametzioides*) (Fig. 6) had the highest significant rates of growth inhibition for OTA-PF mycelia with the third method at 10 days compared with control treatments (Fig 7: D, E, and F), whereas the strain J4 highly reduced, with no significant difference, the OTA-PF mycelial growth at seven days, as did another *Penicillium* strain (S5) at 10 days of growth, compared with the third method. In addition such reduction was also observed with S3 (*P. adametzioides*), S4, J7 with the third method at 10 days and J1 with the second method at 10 days.

Most of the strains that reduced OTA-PF mycelial growth [J2 (Fig. 7 A, B and C), J7, J8 (*F.* sp) (Fig. 7H), S3 (*P. adametzioides*) (Fig. 7I), and S6] had the ability to produce pigments on PDA culture media (green yellow, light brown orange, purple, dark brown orange, light green pigment, respectively), which may explain the antifungal activity.

All the tested methods at different time of incubation had affected significantly the mycellium growth of OTA-PF.

**Table 1: Mycelial growth inhibition percentage of *A. carbonarius* from direct challenge with different isolated strains from grape juice and stems after three, seven and 10 days of incubation on PDA**

| | Incubation time (days) | Mycelia Growth Reduction (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Non-*Aspergillus* Strains isolated from GJ | | | | | | Non-*Aspergillus* Strains isolated from GS | | | |
| | | J1 | J2 | J3 | J4 | J7 | J8 | S3 | S4 | S5 | S6 |
| **Method 1** | **3** | 00.00 | -36.11 | 10.56 $^{e}$ | -34.44 | 06.11 $^{ab}$ | 07.22 $^{c}$ | 11.67 | 05.56 $^{a}$ | 06.67 $^{bc}$ | 10.56 $^{e}$ |
| | **7** | 15.65 $^{f}$ | -00.68 | -08.16 | -01.36 | -07.48 | 09.86 $^{d}$ | 03.74 | -02.72 | 07.14 $^{c}$ | 04.76 |
| | **10** | 26.73 | 31.57 $^{i}$ | 09.45 $^{d}$ | 58.53 | 15.90 $^{f}$ | 30.88 $^{gh}$ | 28.34 | 12.90 | 30.65 $^{g}$ | 31.34 $^{hi}$ |
| **Method 2** | **3** | 12.22 | 02.22 | -02.22 | -13.33 | 23.33 | 05.56 | 23.89 | 17.78 | 10.56 | 13.33 |
| | **7** | 36.39 | 36.74 | 30.95 | 43.54 | 31.97 | 39.80 $^{a}$ | 39.79 $^{a}$ | 24.83 | 41.16 | 38.78 |
| | **10** | 60.14 | 52.30 | 50.92 | 53.69 | 54.61 | 58.07 $^{b}$ | 57.83 $^{b}$ | 49.08 | 58.07 $^{b}$ | 56.91 |
| **Method 3** | **3** | 52.78 | 39.44 | -04.44 | 19.44 | 42.22 | 62.78 | 24.44 | 25.00 | 34.44 | 32.22 |
| | **7** | 76.53 | 55.10 | 37.75 | 57.82 $^{a}$ | 49.66 | 71.78 | 40.83 | 41.16 | 55.44 | 50.00 |
| | **10** | 84.79 | 74.88 | 57.83 $^{a}$ | 70.05 $^{b}$ | 66.13 | 81.34 | 64.75 | 63.83 | 70.05 $^{b}$ | 74.65 |

**Method 1:** 5-cm distance in between the two strains (the *Aspergillus* and *non-Aspergillus* strain); **Method 2:** 3-cm distance; **Method 3**: 3-cm distance with delay of three days for OTA-PF inoculation; **J1:** *Penicillium expansum*, **J2:** *Penicillium* sp; **J3:** *P.* sp.; **J4:** *Penicillium* sp.; **J7:** *P.* sp.; **J8:** *Fusarium* sp., **S3:** *P. adametzioides*; **S4:** *P. brevicompactum*; **S5:** *P.* sp.; **S6:** *P.* sp. *Statistically analysed using ANOVA (Duncan at $P \leq 0.05$) [STD err (M1= 0.106); (M2= 0.043); (M3= 0.036)]*. $X^{a-i}$ indicates reduction but no significant differences.

### 3.2.2. *Effect on the OTA production*

All tested strains affected the OTA amount produced by *Aspergillus carbonarius* using the three different methods in different ways. With the first method (range from 8.4 to 322.6 ng OTA/g of dry weight), OTA production was affected in different ways. J7 (*Penicillium* sp.) and J8 (*Fusarium* sp.) significantly increased the amount of OTA produced by OTA-PF. Certain *Penicillium* strains isolated from GJ, such as J2, J1 (*P. expansum*) and J3 significantly reduced the OTA production at certain times (three, seven and 10 days, respectively) (Fig. 8 A). However, for the strains isolated from GS, only *P. adametzioid*es (S3) significantly reduced the OTA produced at three, seven and 10 days (Fig. 8; B). The rest of the tested strains isolated from GS significantly increased the OTA production by *A. carbonarius* (Fig 8 B).

In general, the OTA produced amount with GJ strains was decreased with the second tested method (range from 16.3 to 223.7 ng OTA/g of dry weight) regarding the first method. Other strains produced significantly reductions of OTA including J4 (*Penicillium* sp.), J3 (*Penicillium* sp.) and J8 (*Fusarium* sp.) (Fig. 8 C). In addition, the same pattern of influence on the produced OTA for the strains isolated from GS, with lower rates of OTA increase and reduction, was observed. S3 (*P. adametzioides*) (at three and seven days) and S6 (*Penicillium* sp.) (10 days) significantly decreased OTA (Fig. 8 D).

With the third tested method (range 22.6 to 538.2 ng OTA/g of dry weight), the OTA production was significantly positively affected in by certain strains at 10 days, including S3 (*P. adametzioides*) and J2 (*Penicillium* sp.) (Fig. 8 E and F), and they had pigment production ability (Fig. 6 I and 7 C, respectively). Other strains highly increased the OTA production, such as S4 (*Penicillium* sp.) at three days. The GJ isolated strains reduced the produced OTA (Fig 8 E), but, *P. adametzioides* (S3) only reduced OTA production at 10 days (Fig. 8 D).

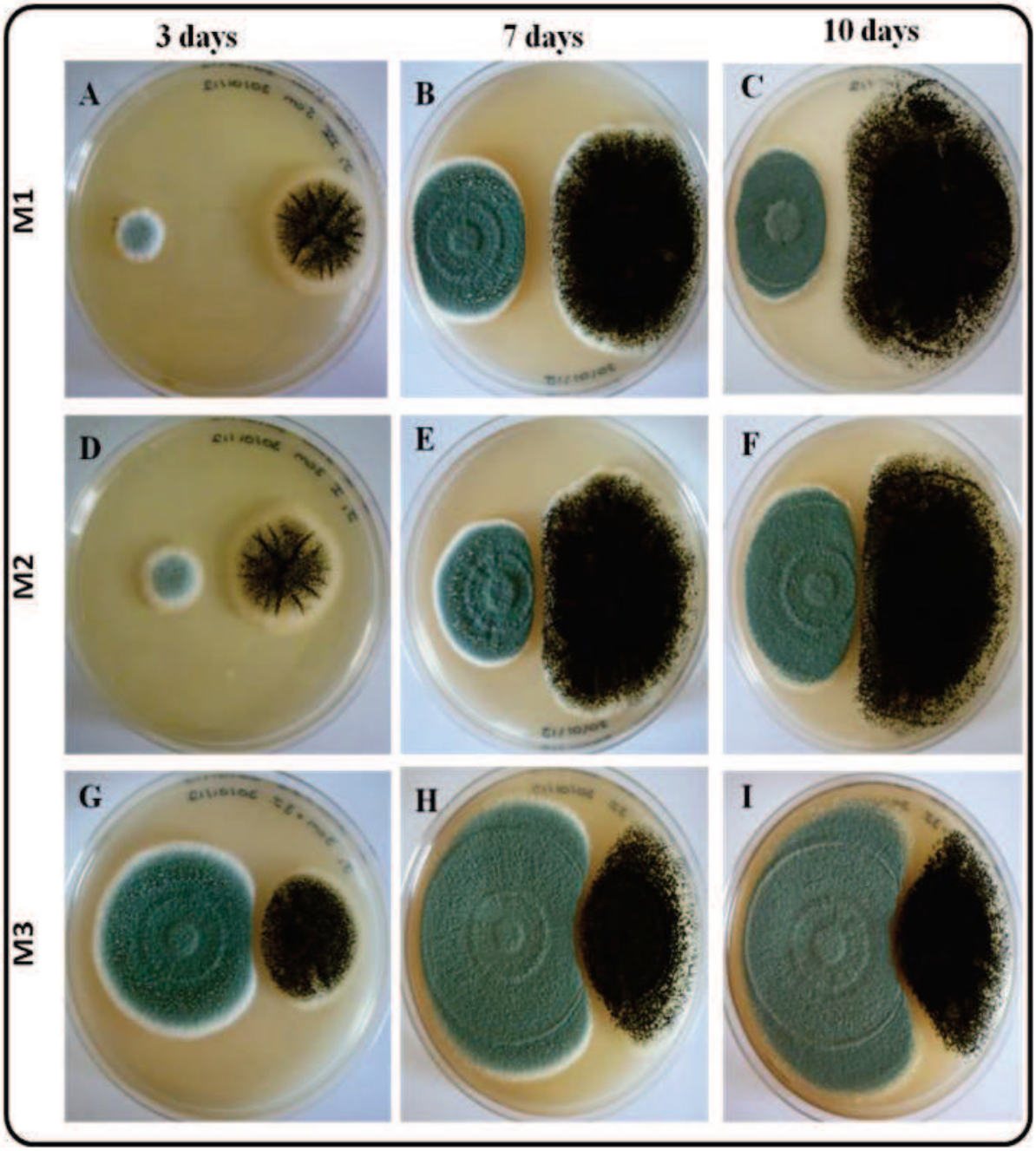


**Figure 4: Direct challenge between *A. carbonarius* and *P. expansum* on PDA media with different distances and inoculation times**

***M 1*:** 5-cm distance; ***M 2*:** 3-cm distance; ***M 3*:** 3-cm distance and inoculation delay of OTA-PF of three days (three, seven, 10 days post-inoculation).

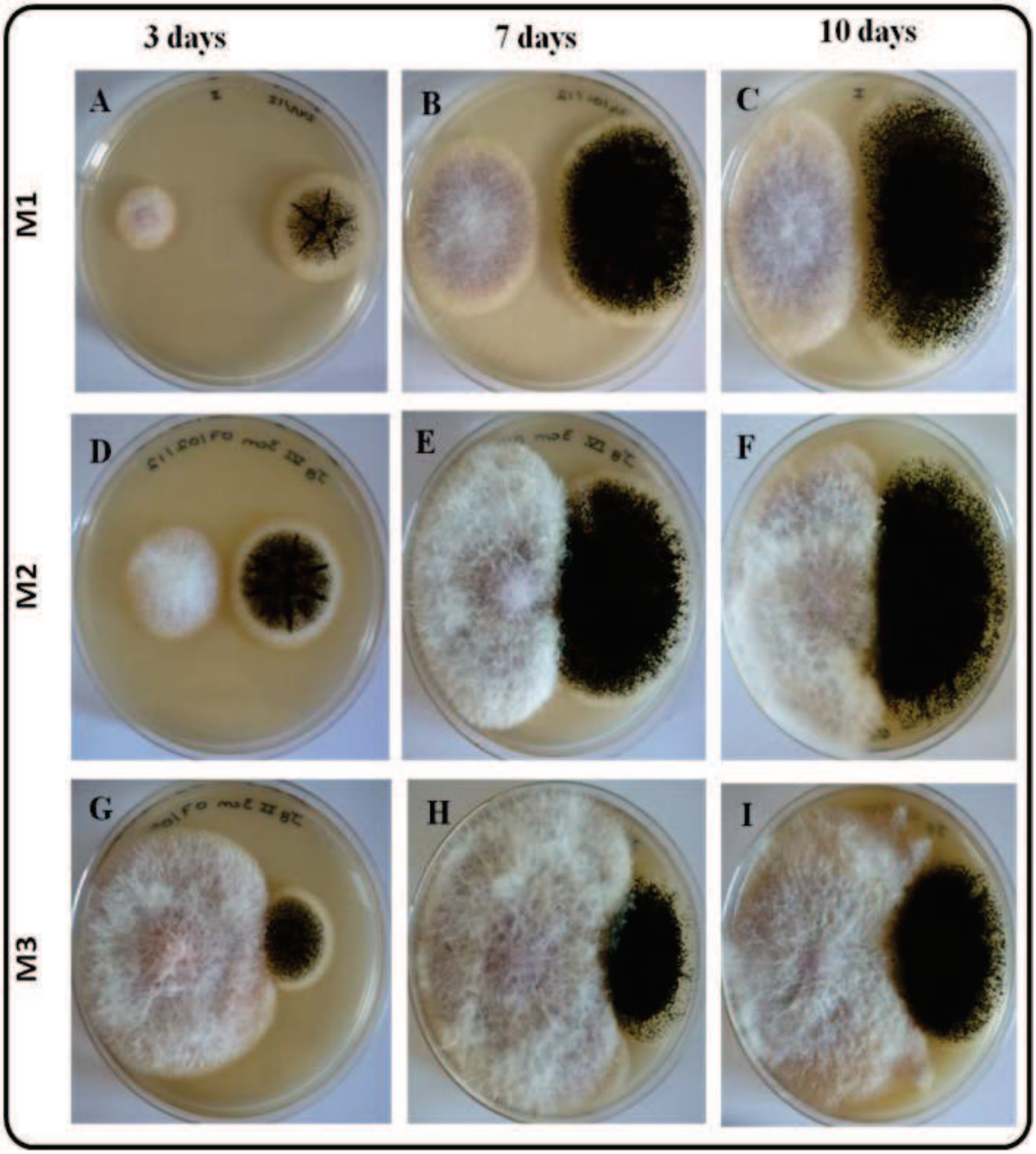


**Figure 5: Direct challenge between *A. carbonarius* and *Fusarium* sp. on PDA media with different distances and inoculation times**.

***M 1*:** 5-cm distance; ***M 2*:** 3-cm distance; ***M 3*:** 3-cm distance and inoculation delay of OTA-PF of three days (three, seven, 10 days post-inoculation).

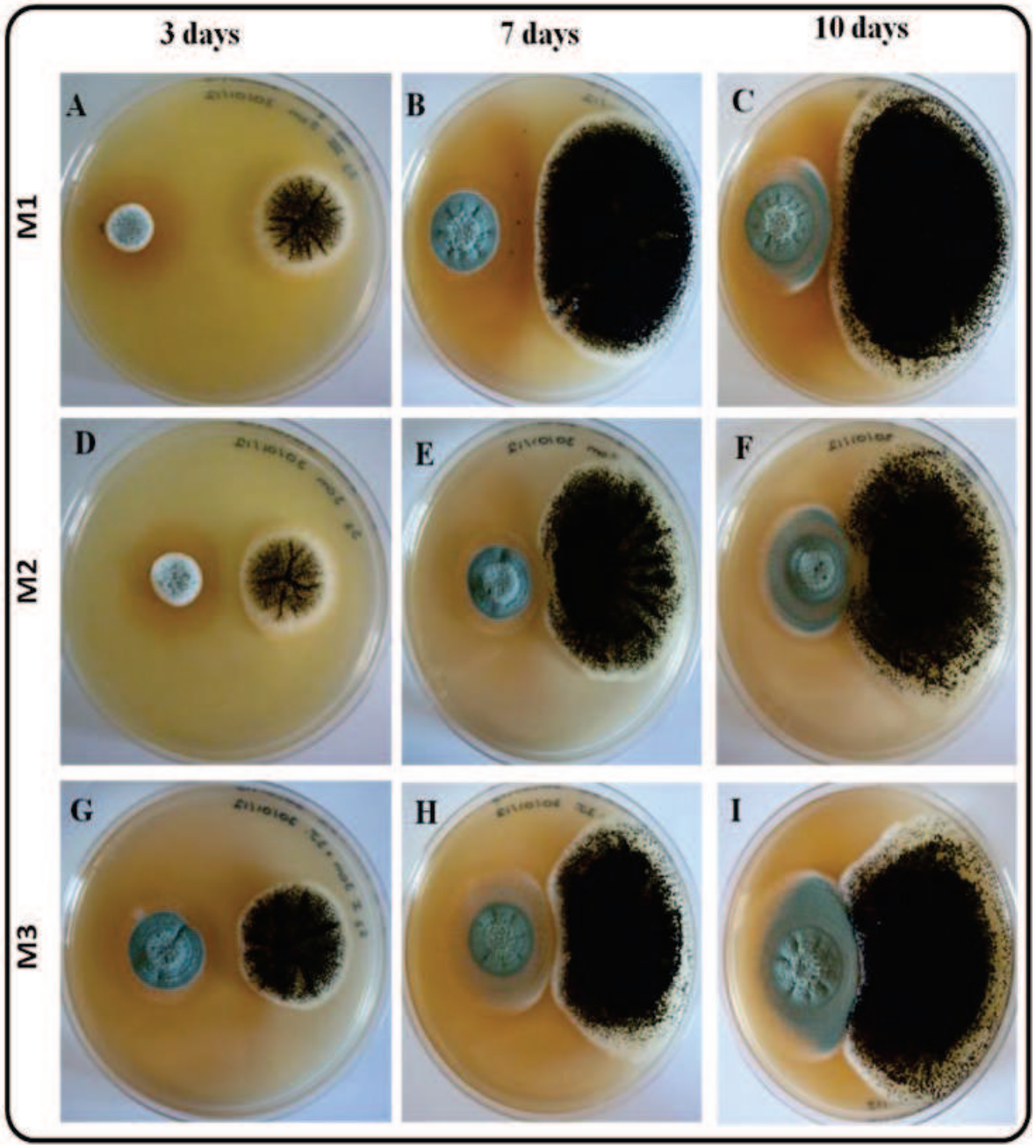


**Figure 6: Direct challenge between *A. carbonarius* and *P. adametzioides* on PDA media with different distances and inoculation times**.

***M 1*:** 5-cm distance; ***M 2*:** 3-cm distance; ***M 3*:** 3-cm distance and inoculation delay of OTA-PF of three days (three, seven, 10 days post-inoculation).

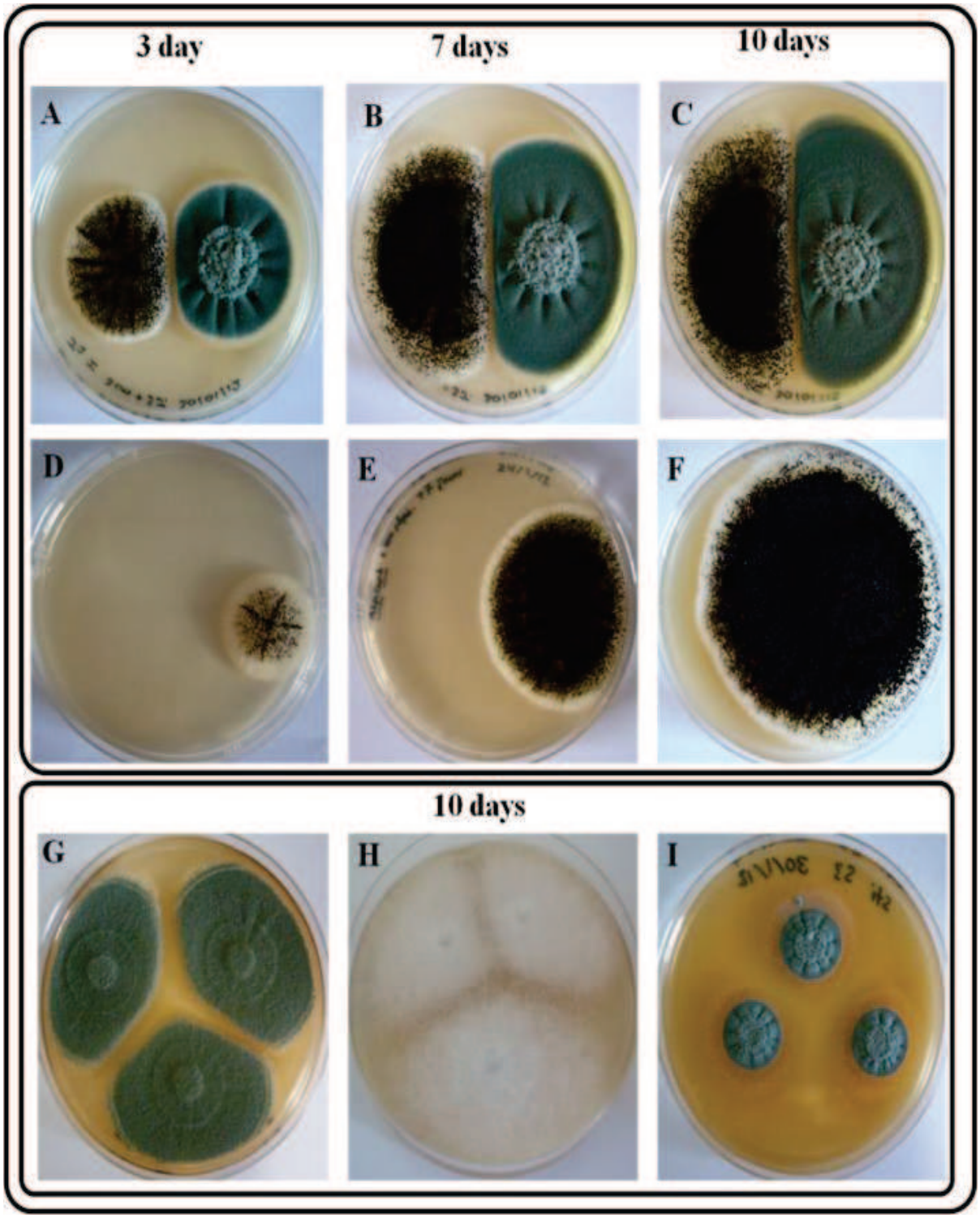


**Figure 7: Fungal cultute on PDA of: A, B,** and **C: Direct challenge between *A. carbonarius* and J2 (*Penicillium* sp.)** *in M3 (3-cm distance & inoculation delay of OTA-PF by three days) at three, seven and 10 days of inoculation*; **D, E** and **F:** *A. carbonarius* control colony control coloniesat three, seven and 10 days of inoculation; **G:** J1 (*P. expansum*); **H:** J8 (*Fusarium* sp.); **I:** S3 (*P. adametzioides*).

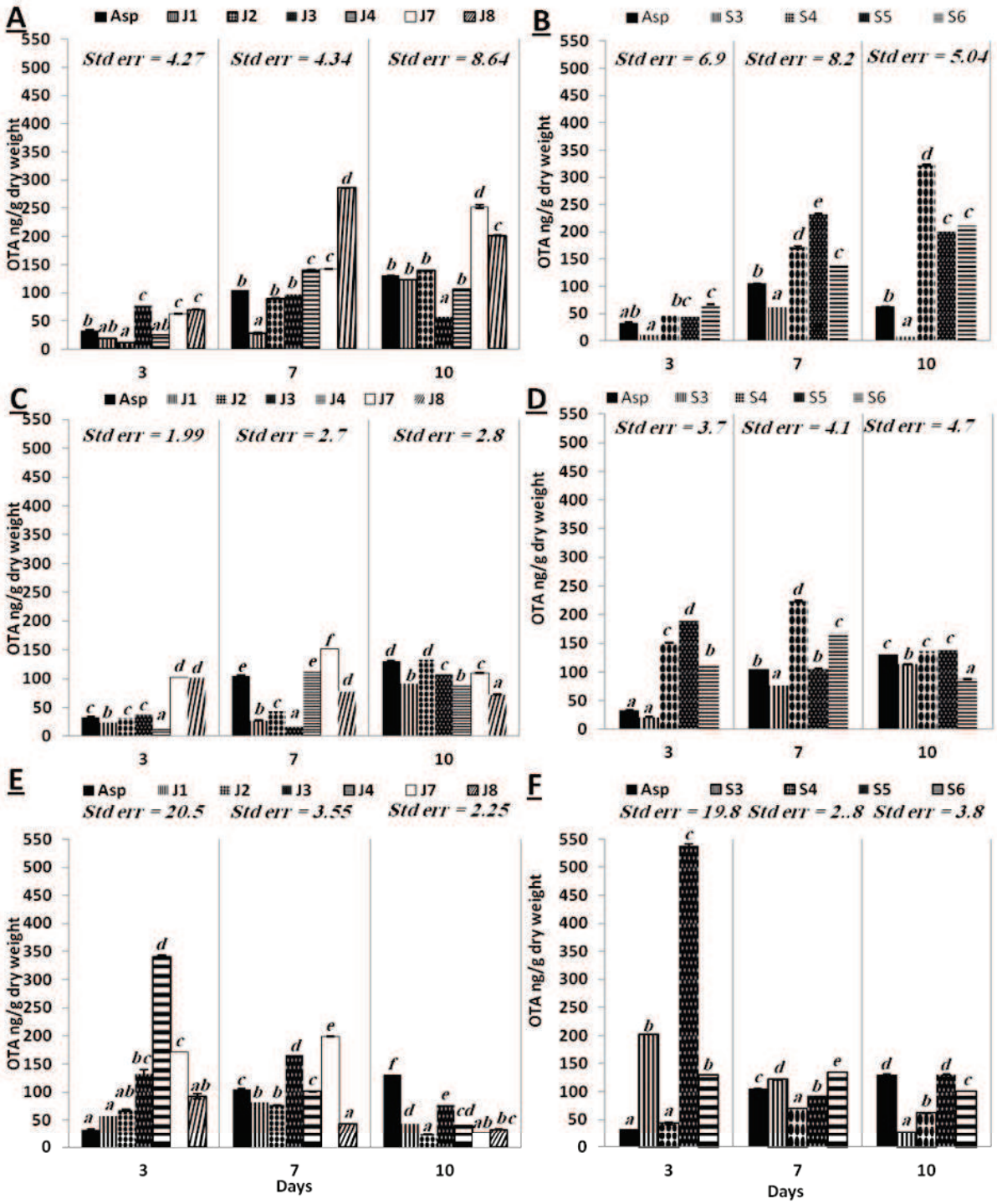


**Figure 8: OTA production of *A. carbonarius* on PDA medium with different methods at different incubation times**. **A:** challenged with strains isolated from GJ at 5-cm distance; **B:** challenged with strains isolated from GS at 5-cm distance, **C:** challenged with strains isolated from GJ at 3-cm distance**; D:** challenged with strains isolated from GS at 3 cm distance; **E:** challenged with strains isolated from GJ at 3-cm distance and delaying inoculation of OTA-PF for three days **; F:** challenged with strains isolated from GJ at 3-cm distance and delaying inoculation of OTA-PF for three days; ***J1, J2, J3, J4, J7*** and ***J8*****:** are *non-Aspergillus* strains isolated from GJ; ***S3, S4, S5*** and ***S6****:* are *non-Aspergillus* strains isolated from GS; ***Asp:*** *A. carbonarius* isolate used for field inoculation.

*Statistically analysed using ANOVA Duncan at $P \leq 0.05$.*

## 4. Discussion

The mechanisms of biological control are generally classified as competition, parasitism or predation, and antibiosis (Fravel, 1988, Rishbeth *et al*., 1988). The organisms involved in the biocontrol of plant aerial diseases include the pathogen, the host, the biocontrol agent and the other microorganisms of all plant ecosystems. These are all affected by each other, by culture practices and by pesticides (Yigal, 1996).

The ability to produce pigments may increase the suppression of fungi mycelial growth (Türkel and Ener, 2009; and Magan *et al.*, 2010), as obseved in certain tested strains such as *P. adametzioides* (S3) and J2.

The reduction effect of certain strains on *A. carbonarius* growth may be caused by their low hyphal growth rate, such as J3 (absence of nutrient competition especially with low incubation periods; three and seven days), or because certain strains secreted molecules later in the culture media that had resulted in growth reduction of OTA-PF as J2. The reduction of produced OTA by *A. carbonarius* as a result of direct challenge with the *Fusarium* sp. (J8) the tested strain is likely due to competition for nutrients (Magallon-Andalon *et al., 2012*) rather than the production of antifungal compounds as the distance between the two fungi was only 3 cm. Moreover, its high mycelial growth rate led to a reduction in the colony size of OTA-PF, which consequently influenced its OTA production.

The ability of *P. expansum* (J1) to reduce OTA-PF mycelial growth and OTA production with the good colonisation and a close distance has a negative effect on another grape post-harvest pathogen, *Botrytis cinerea*, resulting in enhancement of mycelial growth (Morales *et al*., 2013), but its production of mycotoxins [patulin and citrinin; (Watanabe, 2008)] and pathogenicity during the post-harvesting of different fruit [apples (Calvo *et al.*, 2007)] products has reduced its importance as a biological control agent

The increased amount of OTA (by OTA-PF) as observed with certain *Penicillium* strains, such as S4 (*P. brevicompactum*), S5 and S6 may be due to cases of mutualism or commensalism or could be related to their capacity to produce OTA given that they are members of the *Penicillium* genus.

The colonisation (on cultural medium) of certain tested strains had resulted in a reduction in the *A. carbonarius* mycelial growth as observed with the third method as related to the secretion of antifungal metabolites into media cultures or other toxic metabolites or enzymes production. It may have also been due to nutritional competition. The higher distance of challenge between certain strains, such as *P. adametzioides* (S3), and other *Penicillium*

strains (as J1, J2, J3 and J4; at three and seven days) reduced the secretion of OTA by OTA-PF. This OTA reduction possibly lead to the prevention of OTA contamination in the case of epidemic-favorable conditions by reducing the amount of OTA produced in grape post-harvest products (i.e., juice) via the application of the elicitor and resulting modification of the ecosystem and generation of antagonistic effects by endogenous strains. This potentially could also be accomplished by applying one of the tested fungi that displayed antagonistic effects on OTA production, such as *P. adametzioides* (at 10 days).

Certain strains, such as *P. adametzioides* and J2 (that had to be identified), require additional investigation into the details of their mechanism of OTA reduction and their ecosystem effects in the context of *in vivo* application.

**Acknowledgements**

The financial support by the Egyptian government is gratefully acknowledged. We also thank to R Ratomahenina and JC Baccou for their intellectual input and help. We also appreciate SOFT (for providing the Stifénia® product) and IFV, Pech Rouge (for providing the experimental field).

**References**

AFNOR (2002). *Microbiologie Alimentaire*. Saint Denis La Plaine Cedex.(8e éd). Tome, vol.1. p. 64.

Atoui, A., Mathieu, F., & Lebrihi, A. (2005). Targeting a polyketide synthase gene for Aspergillus carbonarius quantification and ochratoxin A assessment in grapes using real-time PCR. *International Journal of Food Microbiology*, 115, 313-318.

Bejaoui, H., Mathieu, F., Taillandier, P., & Lebrihi, A. (2006). Black aspergilli and Ochratoxin A production in French vineyards. *International Journal of Food Microbiology*, 111, S46-S52.

Bendahmane, B. S., Mahiout, D., Benzohra, I. E., & Benkada, M. Y. (2012). Antagonism of three *Trichoderma* species against *Botrytis fabae* and *B. cinerea,* the causal agents of chocolate spot of faba bean (*Vicia faba* L.) in Algeria. *World Applied Sciences Journal*, 17(3), 278-283.

Boyraz, N., & Özcan, M. (2005). Antifungal effect of some spice hydrosols. *Fitoterapia*, 76, 661- 665.

Cabañes, F. J., Accens, F., Bragulat, M. R., Abarca, M. L., Castella, G., Minguez, S., & Pons, A. (2002). What is the source of Ochratoxin A in wine? *International Journal of Food Microbiology*, 79, 213-215.

Calvo, J., Calvente, V., Edith de Orellano, M., Benuzzi, D., & Sanz de Tosetti, M. I. (2007). Biological control of postharvest spoilage caused by *Penicillium expansum* and *Botrytis cinerea* in apple by using the bacterium *Rahnella aquatilis*. *International Journal of Food Microbiology*, 113, 251–257.

Dachoupakan, C., Ratomahenina, R. M., Martinez, V., Guiraud, J. P., Baccou, J. C., & Schorr-Galindo, S. (2009). Study of the phenotypic and genotypic biodiversity of potentially ochratoxigenic black aspergilli isolated from grapes. *International Journal of Food Microbiology*, 132(1), 14-23.

El Sheikha, A. F., Condur, A., Métayer, I., Le Nguyen, D. D., Loiseau, G., & Montet, D. (2009). Determination of fruit origin by using 26S rDNA fingerprinting of yeast communities by PCR-DGGE: Preliminary application to *Physalis* fruits from Egypt. *Yeast*, 26(10), 567-573.

European Commission, European Commission Regulation No. 105/2010 (2010). Amending Regulation (EC) No. 1881/2006 setting maximum levels for certain in foodstuffs as regards ochratoxin A. *Official Journal of European Union*, 35, 7–8.

European Commission, European Commission Regulation No. 1881/2005 (2006). Amending Regulation (EC) No. 1881/2006 setting maximum levels for certain in foodstuffs as regards ochratoxin A. *Official Journal of European Union*, 364, 5–24.

Favilla, M., Pascale, M., Ricelli, A., Evidente, A., Amalfitano, C., & Altomare, C. (2008). Inhibition of Species of the *Aspergillus* Section *Nigri* and Ochratoxin A Production in Grapes by Fusapyrone. *Applied and environmental microbiology*, 74(7), 2248–2253.

Fierer, N., Jackson, J. A., Vilgalys, R., & Jackson, R. B. (2005). Quantitative PCR Assays Structure by Use of Taxon-Specific Assessment of Soil Microbial Community. *Applied Environment Microbiology*, 71(7), 4117-4120.

Fravel, D. R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology*, 26, 75-91.

Galvano, F., Piva, A., Ritieni, A., & Galvano, G. (2001).Dietary strategies to counteract the effects of mycotoxins: A review. *Journal of Food Protection*, 64, 120- 131.

Hope, J. H., & Hope, B. E. (2012). A review of the diagnosis and treatment of Ochratoxin A inhalational exposure associated with human illness and kidney disease including focal segmental glomerulosclerosis. *Journal of Environmental and Public Health*, vol. 2012, Article ID 835059, 10 pages.

Irkin, R., & Korukluoglu, M. (2007). Control of *Aspergillus niger* with garlic, onion and leek extracts. *African Journal of Biotechnology*, 6 (4), 384-387.

Kabak, B., Dobson, A.D.W., & Var, I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed. *Critical Reviews in Food Science and Nutrition*, 46, 593–619.

Magallon-Andalon, C.G., Luna-Solano, G., Ragazzo-Sanchez, J. A., & Calderon-Santoyo, M. (2012). Parasitism and Substrate Competitions Effect of Antagonistic Yeasts for

Biocontrol of *Colletotrichum gloeosporioides* in papaya (*Carica papaya* L.) var Maradol. *Mexican Journal of Scientific Research*, 1(1), 2-9.

Magan, N., Aldred, D., Hope, R., & Mitchell, D. (2010). Environmental Factors and Interactions with Mycobiota of Grain and Grapes: Effects on Growth, Deoxynivalenol and Ochratoxin Production by *Fusarium culmorum* and *Aspergillus carbonarius*. *Toxins*, *2*, 353-366.

Medina, A., Jiménez, M., Mateo, A. R., & Magan, N. (2007). Efficacy of natamycin for control of growth and ochratoxin A production by *Aspergillus carbonarius* strains under different environmental conditions. *Journal of Applied Microbiology*, 103(6), 2234-2239.

Wisniewski, M. E., & Wilson, C. L. (1992). Biological Control of Postharvest Diseases of Fruits and Vegetables: Recent Advances. *Hortscience*, 27(2), 94-98.

Morales, H., Paterson, R. R., Venâncio, A., & Lima, N. (2013). Interaction with Penicillium expansum enhances Botrytis cinerea growth in grape juice medium and prevents patulin accumulation in vitro. *Letters in Applied Microbiology*, 56(5), 356-360.

Pandey, K. D., Tripathi, N. N., Tripathi, D. R., & Dixit, S. N. (1982). Fungitoxic and phytotoxic properties of essential oils of *Hyptis suaveolens*. *Zeits Pflanzenkrank Pflanzenschutz*, 89, 344-349.

Pfohl-Leszkowicz, A., Petkova-Bocharova, T., Chernozemsky, I. N., & Castegnaro M. (2000). Balkan endemic nephropathy and the associated urinary tract tumors: review on etiological causes, potential role of mycotoxins. *Food Additives & Contaminants*, 19, 282-302.

Pitt, D. B., Plestina, J. I., Shepard, R., Solfrizzo, G., Vergere, M., & Walker, P. J. P. (2001). Safety Evaluation of Certain Mycotoxins in Food. In *Joint FAO/WHO Expert Committee on Food Additives (JECFA)* (Series 47, pp 281-415). Rome: Food and Agriculture Organization.

Reddy, L., & Bhoola, K. (2010). Ochratoxins-food contaminants: impact on human health. *Toxins*, 2, 771–779.

Rishbeth, J., Lumsden, R. D., Gibbs, J. N., Hamilton, W. D., & Cook, R. J. (1988). Biological Control of Air-Borne Pathogens. *Philosophical Transactions of the Royal Society of London,* 318(1189), 265-281.

Skarkova, J., Ostry, V., Malir, F., & Roubal, T. (2013). Determination of Ochratoxin A in Food by High Performance Liquid Chromatography.*Analytical Letters*, 46(10), 1495-1504.

Soliman, K. M., & Badeaa, R. I. (2002). Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food Chemistry Toxicology,* 40, 1669-1675.

Suarez-Quiroz, M. L., Gonzalez-Rios, O., Barel, M., Guyot, B., Schorr-Galindo, S., & Guiraud, J. P. (2004). Study of ochratoxin A-producing strains in coffee processing. *International Journal of Food Science and Technology,* 39, 501–507.

Sukorini, H., Sangchote, S., & Khewkho, N. (2013). Plant crude extracts and yeast as alternative to synthetic fungicide for controlling postharvest green mould on citrus fruit. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, LXI (3), 795–801.

Türkel, S., & Ener, B. (2009). Isolation and characterization of new *Metschnikowia pulcherrima* strains as producers of the antimicrobial pigment pulcherrimin. *Zeitschrift für Naturforschung C.*, 64(5-6), 405-410.

Varga, J., & Kozakiewicz, Z. (2006). Ochratoxin in grapes and grape-derived products. *Trends Food Science Technology,* 17, 72–81.

Varga, J., Kocsubé, S., Péteri, Z., Vágvölgyi, C., & Tóth, B. (2010). Chemical, Physical and Biological Approaches to Prevent Ochratoxin Induced Toxicoses in Humans and Animals. *Toxins*, *2*, 1718-1750.

Watanabe, M. (2008). Production of mycotoxins by *Penicillium expansum* inoculated into apples. *Journal of Food Protection*, 71(8), 1714-1719.

Yigal, E. (1996). Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. *European Journal of plant pathology*, 102, 719-732.

Yin, M. C., & Tsao, S. M. (1999). Inhibitory effect of seven *Allium* plants upon three *Aspergillus* species. *International Journal of Food Microbiology,* 49, 49-56.

# Plant Defense Stimulation against OTA-Producing Fungus (*Aspergillus carbonarius*) on Grapes

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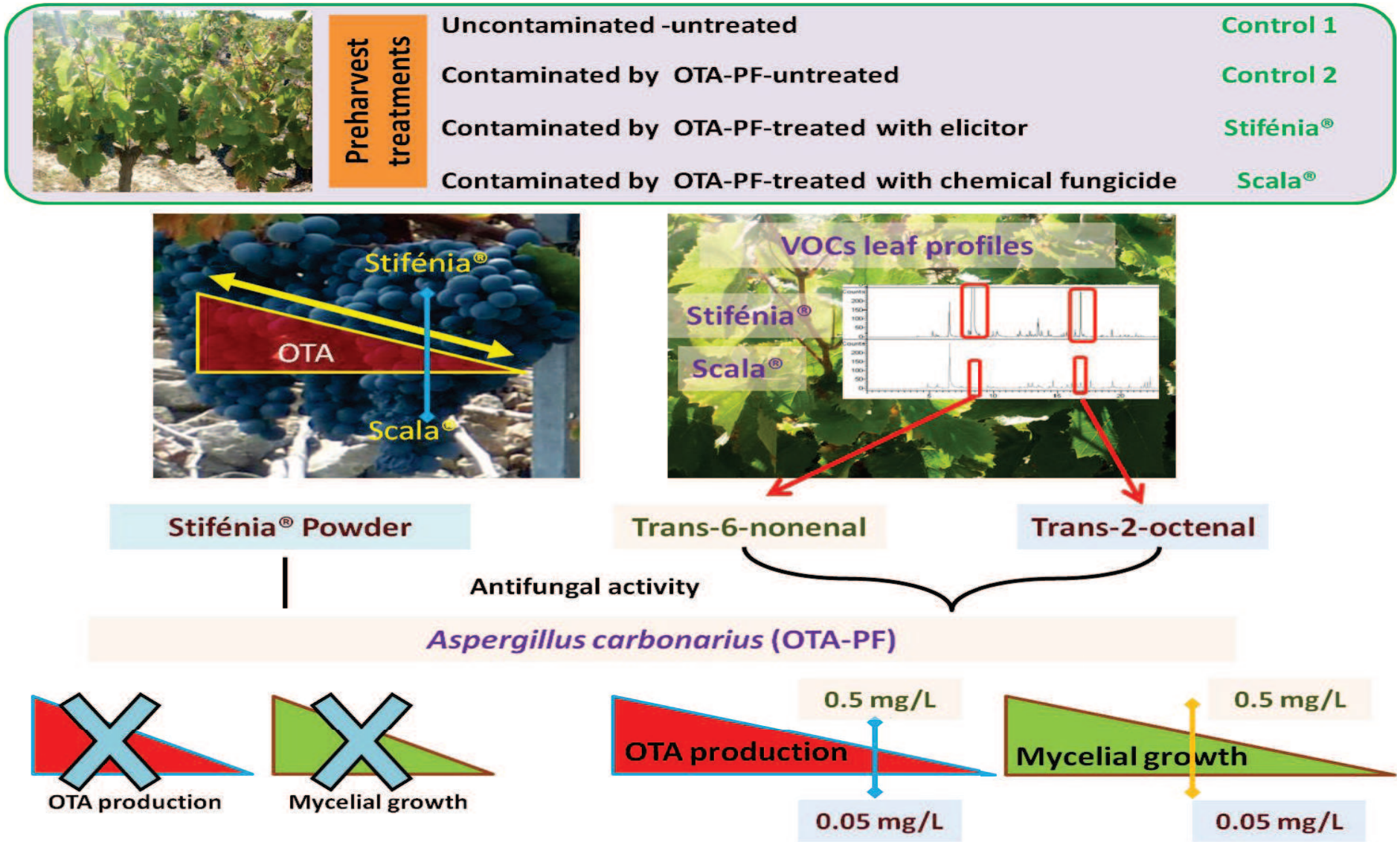
CROPRO-D-13-00784


Preharvest treatments
Uncontaminated -untreated
Control 1
Contaminated by OTA-PF-untreated
Control 2
Contaminated by OTA-PF-treated with elicitor
Stifénia®
Contaminated by OTA-PF-treated with chemical fungicide
Scala®
Stifénia®
OTA
Scala®
VOCs leaf profiles
Stifénia®
Scala®
Stifénia® Powder
Trans-6-nonenal
Trans-2-octenal
Antifungal activity
Aspergillus carbonarius (OTA-PF)
OTA production
Mycelial growth
0.5 mg/L
OTA production
0.05 mg/L
0.5 mg/L
Mycelial growth
0.05 mg/L

**Abstract**

Because the vine culture global importance, particularly in Europe (for grape production and wine industry), pre-harvest treatments were performed as an assay to find an alternative control method for OTA-producing fungus (OTA-PF, *Aspergillus carbonarius*), which is considered as a limited factor for the wine and grape juice consumption consequently. The alternative proposed preharvest treatment [Stifénia® (plant extract)] had equally reduced the contamination of Mourvèdre grape cultivar juices with 33% regarding the chemical fungicide treatment (Scala®). The leaf volatile organic compound (VOC) profiles of these two pre-harvest treatments were assessed using HS-SPAM-GC-MS. Certain VOCs had significantly increased in the plant extract treatment regarding the chemical one. Trans-6-nonenal and trans-2-octenal, which recognized in Stifénia® treatment leaves with the highest significant concentration regarding to their concentration with the chemical treatment, have antifungal activity against the *A. carbonarius* growth and OTA production with low concentrations (0.5 and 0.05 mg/L, respectively). No antifungal activity of the Stifénia® powder against the OTA-PF mycelial growth or its OTA produced amounts were measured. That may partially explain the mode of action of plant defence by producing leaf VOCs that induce positive changes on the OTA-PF and its OTA contents in grapes.

***Key words:*** *elicitor, Stifénia, OTA, Aspergillus carbonarius, volatile compounds, natural defense stimulator.*

**Introduction**

The grape and wine industry is affected by the presence of Ochratoxin A (OTA) in their products by the grape contamination mainly with fungal strains belonging to *Aspergillus* section Nigri. Grape juice and wine are considered as the second contributor in Europe to the ingestion of this mycotoxin with nephrotoxic, neurotoxic and teratogenic effects, and classified as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC, 2003). The European Commission has set the maximum limit for OTA in wine and grape juice to 2 mg/Kg (EC No. 1881/2005, 2006).

Several strategies have been proposed to prevent the toxic effects of mycotoxins in general, and of ochratoxins in particular, in food and feed: (i) prevention of mycotoxin contamination; (ii) decontamination or detoxification of foods contaminated with mycotoxins; and (iii) inhibition of the absorption of consumed mycotoxin in the gastrointestinal tract (Kabak *et al.,* 2006). The prevention of mycotoxin contamination in the field is a main goal of the agricultural and food industries. As mycotoxin-producing moulds can usually colonize damaged parts of plants, crops must be protected against damage caused by either mechanical processes or insects. Field treatment with fungicides is the traditional prevention technique (Vagar *et al.,* 2010).

The vine culture is the most fungicide-consuming culture in France, which contribute mainly to environmental pollution and prescribe effect on producer and consumer safety. The plan "Ecophyto 2018" was designed which aims to progressively half the use of pesticides (*i.e.,* fungicides) in France by 2018, while maintaining the economic performance of French agriculture (MFAP, 2008).

In recent decades, advances spectacular of our knowledge of the mechanisms involved in plant induced resistance (Benhamou and Rey, 2012a; Bavaresco *et al.,* 2003) led the scientific community to consider the exploitation of this natural defence strategy in a context combining the preservation of the environment and the intensive agriculture production. Today, several factors favour the use of natural defence stimulators (NDS) in order to increase the efficacy of plant response to biotic and / or abiotic diseases (Benhamou and Rey, 2012b; Kabak *et al*., 2006)

The active ingredients of NDS could be fully organic or synthetic substances derived from biological molecules: The NDS have several advantages as retaining their biological

activity over a long period with an appropriate formulation because of their ability that give the plants a systemic and sustainable resistance, reduction or absence of phytotoxicity and environmental toxicity. In addition, they are biodegradable (The NDS exerts a potential effect on reducing the occurrence of resistance phenomena frequencies), their application is relatively simple and integrates seamlessly with standard cultural practices. Last not least, The NDS are safe for human and animal health (Benhamou and Rey, 2012b).

Certain commercial natural plant extracts; such as Stifénia® (seed extract of the *Trigonella foenum graecum* L. plant) which was recently approved in France for its efficiency against the grapevine powdery mildew pathogens (*Uncinula necator* or *Erysiphe necator*) (Pajot and Regnault-Roger, 2008; Amézqueta *et al.,* 2012). The Stifénia® efficacy depends on the grape cultivar and the physiological phase of the treated plants, which plays a significant role in the expression of plant defence stimulation (that should be applied before the grape flowering stage) (Regnault-Roger, 2012). Stifénia® seems also to be effective against other diseases such as peach leaf curl [caused by *Taphrina deformans*], and other various downy mildew diseases (Regnault-Roger, 2012). Numerous comparative studies were conducted evaluating Stifénia® and its mechanisms for disease resistance stimulation, such as Gindro *et al.* (2007) study which evaluated the effectiveness of various organic products on grape downey mildew [caused by *Plasmopara viticola*], found that extracts of fenugreek seeds were less effective than extracts of rhubarb and buckthorn. And proposing that spraying leaves may weakly induce only the synthesis of phytoalexins and slowing the pathogen sporulation with 40% rather than stimulating callus formation at pathogen penetration sites.

Durand *et al.* (2009) mentioned that Stifénia affect the pathosystem of coffee berry disease (*Colletotrichum kahawae*), which causes fruits rots on Arabica coffee leading to harvest losses of around 40%., along with caffeine and chlorogenic acid (CGA) concentrations were monitored in the seedling hypocotyls of two varieties of *Coffea arabica* with different degrees of susceptibility, contaminated by *C. kahawae* and treated with Stifénia. After observing symptoms on seedlings. However, it did not seem to stimulate the alkaloid (caffeine) or polyphenol (CGA) biosynthesis pathways.

Camele *et al*. (2009) had performed another study on the evaluation of fighting means against melon powdery mildew [caused by *Sphaerotheca fuliginae*], which has demonstrated that the Stifénia® was significantly less effective than sulfur or fungicide chemicals in addition to induce significant yield losses as a result of repeated applications.

Grape (*Vitis vinifera* L.) volatiles include a great number of compounds, among which monoterpenes, $C_{13}$ norisoprenoids, alcohols, esters and carbonyls are found. Grape may be divided into aromatic and non-aromatic varieties (El Hadi, *et al.,* 2013). Volatile organic compounds (VOCs) may help plant to prevent disease and insect damage by eliciting induced resistance, even in open fields, as demonstrated by Song and Ryu (2013). Where they had found that the 3-pentanol and 2-butanone resulted a significant increscent in the number of ladybird beetles, a natural enemy of aphids infecting cucumber, and protecting the cucumber against the biotrophic bacterial pathogen [*Pseudomonas syringae* pv. *lachrymans*]. Several studies were carried out determining the VOCs effect against *A. niger*; such as the study conducted by Özar *et al* (2009) on onion, the presence VOCs in soils inhibited fungus spore germination. Nonanal, produced by cotton leaves, is responsible for the production of the unique aerial hyphae and decrease in the aflatoxin production of *Aspergillus flavus* and *A. parasiticus* (Green- McDowelle *et al.,* 1999).

This work was conducted to assess a probable explanation for the Stifénia® resistance stimulation against OTA-PF corresponding to the grape leaves volatile compounds profile changes. And to determine the presence of direct influence for the Stifénia® elicitor on OTA-PF or not.

## 1. Material and Methods

### 1.1. *In-vivo* experiment to provide an alternative safe method to control OTA contamination in grapes and its products and ensure stakeholder health.

Four pre-harvest treatments were applied on the cultivar Mourvèdre, in IFV (French Institute of vine and wine), Pech Rouge, Narbonne, France. Two control modalities were performed, the first one (Control 1) which vines were untreated and not contaminated with *Aspergillus carbonarius* [OTA-PF that was previously isolated from French vineyards (Dachoupakan *et al.*, 2009)]. The second control modality (Control 2) was artificially contaminated with OTA-PF at the version stage but not treated. And the other two treatments were also contaminated with OTA-PF by inoculation at the veraison stage and treated; one was multiply treated by Stifénia® (SOFT, France; 15mg/mL) that started at the green-tip stage till harvest with 15 days intervals (as recommended by manufactures to encourage the eliciting effect). And the other was treated twice by Scala® (chemical fungicide with pyrimethanil 400 g/L as active ingredients, Bayer, France) one at green-tip stage and the other

at output stag. Samples were harvest in late September 2010, during the grape harvest period. Grape bunches (5 kg/ treatments) were randomly taken and placed in two previously sterilized bags, which were kept at 4°C until analysis. Mycological analysis was immediately done and the remaining samples were kept frozen at -20°C.

### 1.2. *In-vitro* direct effect of elicitor treatment on OTA-PF

Two concentrations of Stifénia ® solutions; 15 mg/mL (the used concentration for the *in-vitro* treatment) and 150 mg/mL were examined for their antifungal activity against the fungus *A. carbonarius*.

#### 1.2.1. Direct effect of elicitor treatment on the mycelial growth.

Agar dilution method was performed by adding different concentration of Stifénia® (UV sterilize) solution to PDA (Biokar diagnostics, Beauvais, France) media before pouring (at 45°C). Consequently, after cultural media solidification, five µl of $10^6$ spore/mL of OTA-PF were inoculated at plate centers (Fenner *et al.,* 2005; Cabañas *et al*., 2009). Normal PDA culture media inoculated by OTA-PF were used as control. Triplicates were performed for all the treatments.

#### 1.2.2. Direct effect of elicitor treatment on the produced OTA

Saturated filter membranes (SFM) of six millimeter diameter (Filter Durieux n°268) with thirty microlitres of pre-sterilized sterilized Stifénia® solutions were centrally placed on pre-inoculated PDA (Biokar diagnostics, Beauvais, France) plates with 100 µL of *A. carbonarius* spore suspension ($10^6$ conidia/mL) (Ramamurthy *et al*., 2012). Two controls were used; SFMs with 35 µg/L of 5-flourocytosine (Sigma Aldrich, France) solution as positive control (Waldorf and Polak, 1983; Ribeiro *et al*., 2006), and SFMs with sterilized distilled water as a negative control. Triplicates were performed for all the treatments.

### 1.3. OTA content

#### 1.3.1. OTA extraction from grape juice

Fifty grams of each sample was homogenized with 200 mL of 70% methanol using a blender at high speed for 1 min and filtered. The filtered extracts were diluted (1: 5) by PBS/0.01% Tween 20 (washing buffer) (Sigma-Aldrich, France). Twenty millilitres of each filtered diluted extracts were passed through OchraTest™ affinity columns at a rate of

approximately 1 drop/sec until air passed through the column. The columns were then washed with washing buffer (1-2 drops/sec) followed by 10 mL of purified water (1-2 drops/sec). The OTA content were collected in 1.5 mL of methanol:acetic acid (98:2, v:v) (1 drop/sec) (Skarkova *et al.*, 2013).

### 1.3.2. OTA extraction from *Aspergillus* cultural media

#### *1.3.2.1.* From single fungal colony

Colony diameters were measured and three agar plugs of five millimetres of diameter were removed from the *A. carbonarius* colonies (in the area, which confronted the VOC) at three, seven and 10 days of incubation. The plugs were weighed, introduced into 3 mL vials and extracted with 2.5 millilitres of solvent (methanol:formic acid, 25:1) under sonication for 15 min. The solvent was then evaporated under a nitrogen stream at 40 °C. The dried extracts were re-suspended in the mobile phase of the subsequent HPLC (49.5% deionized water, 49.5% acetonitrile, 1% acetic acid) then filtered with a syringe (MiniSart SRP 4 of diameter 0.45 µm, Sartorius, Germany) (Suarez-Quiroz et al., 2004; Barberis *et al,* 2010; Dachoupakan *et al.,* 2009).

#### *1.3.2.2.* From the whole plates

The same protocol described in the section 2.3.2.1. was used with modification: that the OTA content was extracted from all the agar into the plates in a 100 mL vials and extracted by 40 mL of (methanol:formic acid, 25:1) under sonication for 15 min.

### 1.3.3. OTA analysis by HPLC

OTA was analyzed by HPLC (Pump, Shimadzu LC/9A, Japan) with fluorescence detection ($\lambda_{exc}$333 nm; $\lambda_{em}$460 nm, Shimadzu RF-10AXL, Japan), using a C18 column (25 x 4.6 mm, 5 µm, Phenomenex®, USA). The mobile phase [49% acetonitrile (Carlo Erba Reagent, France, P00637G21), 49% Milli-Q water (Quantum® Ex, MILLIPORE), and 1% acetic acid] was pumped at 1 mL/min. The injection volume was 20 µL, and the retention time was approximately 16.3 min. The calibration curve was prepared with OTA solution (Sigma Aldrich, France, 34037-2ML-R) from 0.05 to 5 ng/ mL. The limit of detection (LOD) and limit of quantification (LOQ) of this method which was applied to grape juice samples, were established at 0.023 µg/L and 0.076 µg /L, respectively (Dachoupakan *et al.*, 2009). OTA amounts were presented in µg for g of the fungal dry weight.

## 1.4. Volatile compound profile modifications in Vine leaves

### 1.4.1. Identification of vine leaf volatile compound composition

The volatile compounds of grape leaves that treated with the two OTA-PF control treatments (elicitor (Stifénia) and chemical (SCALA®) treatments) and the volatile compounds present naturally in the Stifénia® powder were identified using the HS-SPME-GC-MS technique [the best SPME technique when target analyses are volatile organic compounds (Marengo *et al*. 2001)] as performed by Tesniere *et al.* (2006) with certain modifications. SPME parameters were optimized: fiber type was 4 CAR/PDMS (Supelco, Bellefonte, Pennsylvania, USA); trans-2-Nonenal (Sigma, France) as internal standard; 60°C for incubation; 10 min as equilibrium period and 20 min for the extraction. (Sánchez-Palomo *et al*., 2005; Haddada *et al*., 2007). The Mass spectrum of the volatile compounds which were found at the HS was compared with the Wiley mass spectral data, consequent by their calculated relative retention (CRR). The CRR were calculated according to the retention time of the separated compounds and the alkaline negative control of the standard solution (Retention Index Standard, Sigma). The analyses of free and bound compounds were performed in triplicate (Pozo-Bayón *et al.,* 2007; Cayot *et al.,* 2008).

### 1.4.2. *In-vitro* experiment to assess the antifungal activity of volatile compounds identified from the Stifénia® treatment leaves on fungi isolated from the same treatment including the *Aspergillus carbonarius*

The antifungal activity of three volatile compounds was measured against the strain used for contamination (*A. carbonarius*). Two of them were highly concentrated in the elicitor treatment grape leaves [Trans-6-Nonenal (13) (Sigma, France) and Trans-2-Octenal (6) (Fulka, France)] and the last Trans-2-Hexenal (Fulka, France) was chosen as a bibliographic study reference (Utto *et al.*, 2008). Four concentrations of each (100, 1, 0.5 and 0.05 µg/mL in alcoholic water) were examined. The 10% ethanol solution was used as a negative control and 130 ng/L of 5-fluorocytosine as a positive control for its recognized antifungal activity (Waldorf and Polak, 1983).

Three different methods were conducted to assess the antifungal activity; solid diffusion method (SDM), vapor diffusion method (VDM), and agar diffusion method (ADM). The SDM was conducted as Ramamurthy *et al*. (2012) described by centrally placing a SFM of 6 mm diameter with different concentration to pre-inoculated PDA Petri dishes with 100

µL of $10^6$ spore/mL of OTA-PF) (the VOCs efficiency on mycelial growth was assessed according to the presence of an inhibition zone or not, and on the OTA production according to OTA extraction from 3 cultural plugs of 5mm as described above in 2.3.2.1. section)

VDM was performed by adding a SFM of 13 mm diameter with different concentration to the plate covers of PDA plates with pre-weighted and sterilized filter membranes (that cover all the medium surface), which were consequently inoculated with 100µl of $10^6$ spore/mL of OTA-PF) (Lopez *et al.,* 2005).

ADM was carried out by mixing the different concentration of the tested volatile compounds to PDA media before pouring (at 45°C). Consequently, after cultural media solidification, sterilized pre-weighted filter membranes were placed onto the agar surface, then 100 µl of $10^6$ spore/mL of OTA-PF were inoculated) (Fenner *et al.,* 2005; Cabañas *et al*., 2009). VOCs affecting OTA-PF mycelial growth and OTA production were expressed for VDM and ADM corresponding to the dry weight of the OTA-PF in each treatment.

## 3. Results

### 3.1. OTA juice content

The OTA content was significantly reduced in the grape juice at the same rate (33%) using the two Stifénia and Scala (1.5 µg OTA/L) treatments in comparison to the OTA content of the contaminated and not-treated treatment (2.6 µg/L) (Tab. 1)

**Table 1: Juice OTA content of Mourvèdre grape cultivar**

| Treatment | OTA content (µg/ml) |
|---|---|
| **Control 1**(uncontaminated-untreated) | $1.1^{a}$ (± 0.01) |
| **Control 2** (OTA-PF contaminated-untreated) | $2.6^{c}$ (± 0.07) |
| **Chemical fungicide** (OTA-PF contaminated and treated by Scala®) | $1.5^{b}$ (± 0.10) |
| **Elicitor** (OTA-PF contaminated and treated by Stifénia®) | $1.5^{b}$ (± 0.07) |

*Statistically analysed using ANOVA, Tukey at 0.05% (STD err = 0.05).*

### 3.2. *In-vitro* direct effect of elicitor treatment on OTA-PF

No OTA-PF mycelial reduction was observed in both Stifénia® (FEN 560) tested concentrations (Fig. 1). Slight decrement of the OTA-PF mycelial growth was marked on the PDA culture media with Stifénia® only at 5 days old.

However, the presence of Stifénia product into the cultural media had significantly modified the OTA produced amounts. The cultural media containing Stifénia® with high concentration (150 mg/L) caused significant increments of OTA produced amounts regarding the control treatment (Fig. 1). Whereas, the low concentration of Stifénia® (15 mg/L) had certain increments at the early aging of the OTA-PF (three, five and 10 days) and other significantly decrements synchronize with the long aging of the OTA-PF on the cultural media (fifteen and twenty days old) were observed (Fig. 1).

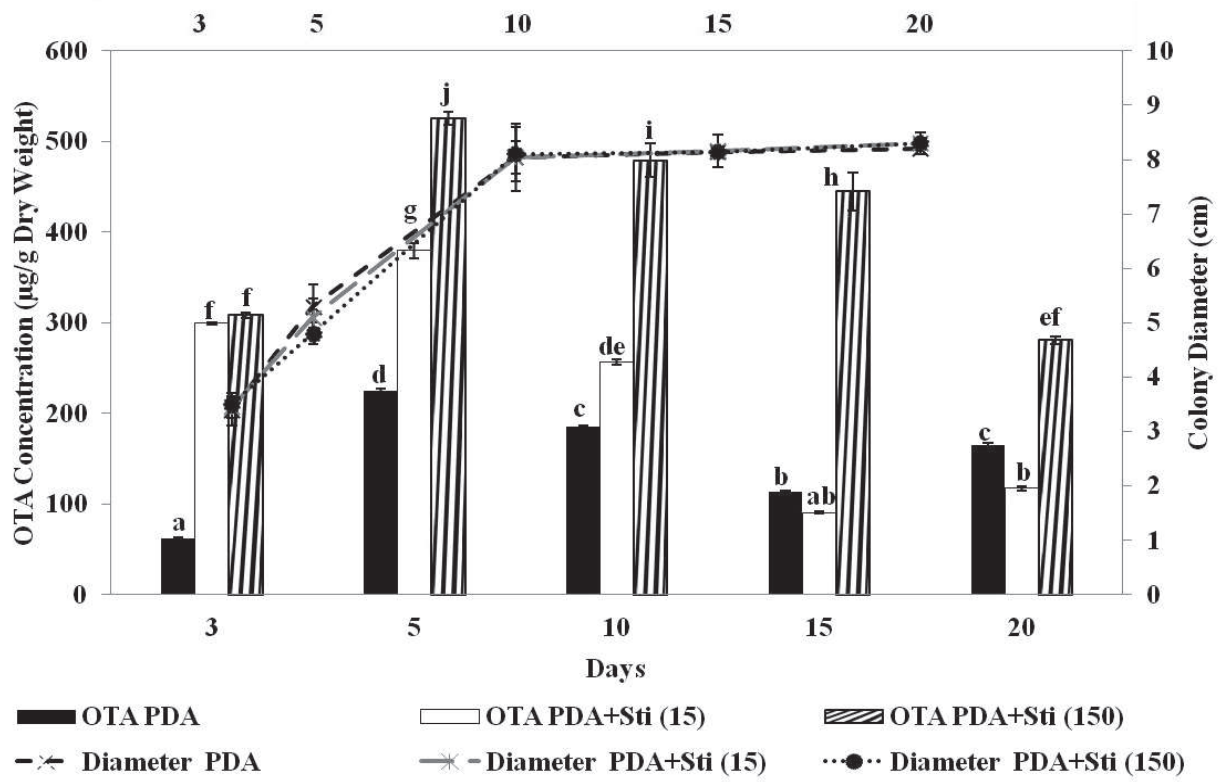



**Figure 1**: **Direct effect of Stifénia® on the *A. carbonarius* growth and OTA production at 15 and 150 mg/L.**

*OTA concentrations were statistically analyzed by ANOVA (Tukey test, P≤ 0.05), Std err= 5.69*

### 3.3. Volatile compounds profile modifications

Twenty-eight VOCs were identified from the treated Mourvèdre grape leaves, half of these identified VOCs were aldehydes and alcohols (Tab. 2). The VOC profile of the grape cultivar was affected by the type of the applied pre-harvest treatment (Fig. 2). Certain VOC amounts were significantly increased in the elicitor treatment leaves comparing with those treated with Scala® treatment [benzaldhyde *(2)*; benzyl alcohol *(4)*; 2-octenal *(6)*; trans-2-undecen-1-ol *(10)*; trans-6-nonenal *(13)*; eugenol *(21)* and cis-9-octadecenal *(22)*] (Fig. 2). Others of the identified VOCs had significant lower quantities in the elicitor treatment than the chemical one, such as trans-trans-2, 4-hexadienal. *(1)*; limonene *(3)*; 5-méthy cyclohexanone *(12)*; methyl salicylate *(14)*; cyclodecanol *(15);* propanoic acid-4-hexen-1-yl ester *(17)*; 1, 6-Octadien-3-ol.3, 7-dimethyl *(18;* caryophyllene *(23)*; cis-5, 9-Undecadien-2-one-6, 10-dimethyl *(18)* and ß-Ionone *(26)* (Fig. 2).

### 3.4. Antifungal activity of the most important volatile compounds identified from the Stifénia® treatment leaves

The two aldehydes, trans-2-Octenal and trans-6-Nonenal were the significant highest identified VOCs in the Stifénia® treated leaves (2.5 and 5.5 times more, respectively) regarding their concentration in the chemically treated leaves (Fig. 2). Therefore, their antifungal activity against OTA-PF was examined.

Neither inhibition zones of the OTA-PF mycelial growth nor significant reduction of its OTA produced amounts were observed in the Solid diffusion method (SDM) (Tab.3) for all the tested VOCs.

The low concentrations of the tested VOCs (2-hexenal, 6-nonenal, and 2-octenal) (0.5,0.5, and 0.05 mg/L concentrations, respectively) had significant reduced the OTA-PF mycelial growth using the other methods, VDM (176.5 ± 4.3, 190.4 ± 0.33, and 164.1 ± 0.6 µg OTA/g dry weight, respectively) and ADM (126.2 ± 1.6, 141.6 ± 1.78, and 140.3 ± 3.6, respectively) methods which were approximately close to the PC effect (134.4 ± 0.5 and 136.2 ± 0.8 mg dry weight, respectively) (Tab. 3).

**Table 2: The identified volatile compounds of Mourvèdre grape cultivar leaves**

| N° (order) | Identified compounds by CPG-MS | Retention time | KI |
|---|---|---|---|
| 1 | 2.4-Hexadienal. (E.E)- | 10.266 | 912 |
| 2 | Benzaldehyde | 12.092 | 964 |
| 3 | Limonene | 14.303 | 1029 |
| 4 | Benzyl alcohol | 14.523 | 1035 |
| 5 | 3-Carene | 14.976 | 1049 |
| 6 | 2-Octenal. (E)- | 15.293 | 1059 |
| 7 | Cyclopropane. pentyl- | 15.717 | 1072 |
| 8 | 4-Nonanol (IS)- | 16.304 | 1090 |
| 9 | 1.6-Octadien-3-ol. 3.7-diméthyl- | 16.635 | 1100 |
| 10 | Trans-2-undecen-1-ol | 16.793 | 1105 |
| 11 | Phenylethyl alcohol | 17.046 | 1113 |
| 12 | 5-méthy. Cyclohexanone | 18.406 | 1157 |
| 13 | 6-Nonenal. (E)- | 18.502 | 1161 |
| 14 | Methyl Salicylate | 19.487 | 1193 |
| 15 | Cyclodecanol | 19.887 | 1206 |
| 16 | 1-Cyclohexene-1-carboxaldehyde. 2.6.6-trimethyl- | 20.291 | 1220 |
| 17 | Propanoic acid.4-hexen-1-yl ester | 20.575 | 1230 |
| 18 | 1.6-Octadien-3-ol.3.7-dimethyl -(-/+) | 21.157 | 1250 |
| 19 | 13-tetradecenal | 21.510 | 1263 |
| 20 | 2.6-Octadienal. 3.7-dimethyl | 21.659 | 1268 |
| 21 | Eugenol | 23.980 | 1352 |
| 22 | 9-Octadecenal. (Z)- | 24.226 | 1361 |
| 23 | Caryophyllene | 25.828 | 1422 |
| 24 | 5.9-Undecadien-2-one. 6.10-dimethyl-. (Z) | 26.424 | 1446 |
| 25 | Butanedioic acid. bis (2-methylpropyl) | 27.167 | 1475 |
| 26 | ß-Ionone | 27.265 | 1479 |
| 27 | Diethyl phthalate | 29.860 | 1586 |
| 28 | Phytol | 40.653 | 2109 |

*IS: Internal Standard; KI: calculated Kovacs Index for each VOC*

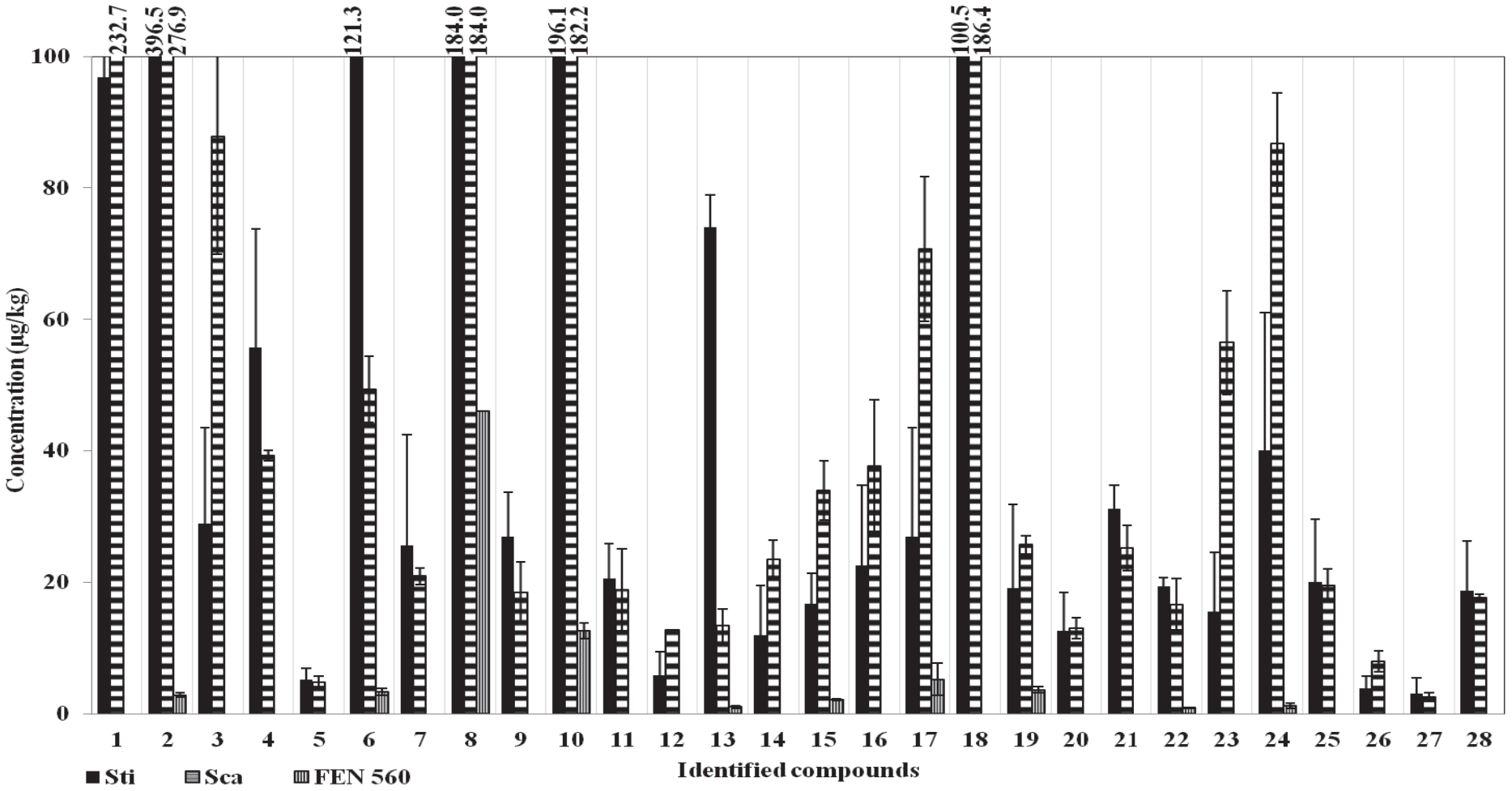



**Figure 2: Concentrations of major identified volatile compounds from Mourvèdre leaves (treated by Stifénia®, and by Scala®) and Stifénia® powder (FEN 560)**

**Table 3: Antifungal activity of the tested VOCs against Aspergillus carbonarius mycelial growth and OTA produced amounts.**

| | Anti-microbial Methods | Volatile compounds: 2-hexenal 100 | 2-hexenal 1 | 2-hexenal 0.5 | 2-hexenal 0.05 | 6-nonenal 100 | 6-nonenal 1 | 6-nonenal 0.5 | 6-nonenal 0.05 | 2-octenal 100 | 2-octenal 1 | 2-octenal 0.5 | 2-octenal 0.05 | NC | PC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mycelial Growth | SDM * | - | - | - | - | - | - | - | - | - | - | - | - | - | + 27.3 ± 0.15 |
| | VDM ** | $267.8^{f}$ ±3.3 | $237.6^{e}$ ±4.3 | $176.5^{c}$ ±4.3 | $190.9^{d}$ ±4.3 | $235.9^{e}$ ±0.8 | $193.3^{d}$ ±0.7 | $190.4^{d}$ ±0.33 | $256.8^{f}$ ±1.6 | $257.9^{f}$ ±2.2 | $187.9^{d}$ ±1.03 | $175.3^{c}$ ±1.5 | $164.1^{b}$ ±0.6 | $258.9^{f}$ ±0.09 | $134.4^{a}$ ± 0.5 |
| | ADM ** | $246.2^{c}$ ± 5.6 | $240.6^{c}$ ± 3.5 | $126.2^{a}$ ± 1.6 | $145.3^{b}$ ± 0.8 | $245.3^{c}$ ± 2.6 | $143.9^{b}$ ± 2.8 | $141.6^{b}$ ± 1.78 | $247.3^{c}$ ± 5.4 | $236.7^{c}$ ± 3.7 | $146.2^{b}$ ± 4.4 | $143.2^{b}$ ± 2.2 | $140.3^{b}$ ± 3.6 | $259.1^{d}$ ±0.06 | $136.2^{a}$ ± 0.8 |
| OTA (µg/g dry weight) | SDM | $60.07^{b}$ ± 1.5 | $61.07^{b}$ ± 0.9 | $59.07^{b}$ ± 2.5 | $62.07^{b}$ ± 0.7 | $58.70^{b}$ ± 2.7 | $62.50^{b}$ ± 0.1 | $60.97^{b}$ ± 1.4 | $65.50^{b}$ ± 3.7 | $59.99^{b}$ ± 3.9 | $61.46^{b}$ ± 0.6 | $59.07^{b}$ ± 1.7 | $59.79^{b}$ ± 2.5 | $62.07^{b}$ ± 0.7 | $32.57^{a}$ ± 1.8 |
| | VDM | $70.0^{e}$ ± 5.6 | $57.9^{d}$ ± 4.1 | $43.2^{bc}$ ± 2.1 | $46.4^{c}$ ± 0.1 | $59.9^{d}$ ± 0.3 | $46.5^{c}$ ± 0.4 | $46.0^{c}$ ± 0.1 | $64.7^{d}$ ± 1.5 | $65.8^{de}$ ± 3.1 | $44.4^{c}$ ± 1.4 | $43.2^{bc}$ ± 0.5 | $41.8^{b}$ ± 0.4 | $63.02^{d}$ ± 0.5 | $29.7^{a}$ ± 0.5 |
| | ADM | $57.87^{e}$ ± 3.0 | $56.94^{e}$ ± 2.4 | $23.49^{a}$ ± 0.1 | $27.23^{b}$ ± 0.6 | $57.09^{e}$ ± 3.4 | $34.1^{cd}$ ± 0.7 | $32.01^{c}$ ± 1.1 | $59.33^{e}$ ± 2.3 | $58.79^{e}$ ± 0.6 | $36.91^{d}$ ± 2.1 | $35.4^{cd}$ ± 0.5 | $31.19^{c}$ ± 1.4 | $62.09^{e}$ ± 1.1 | $26.01^{a}$ ± 1.1 |

**: Inhibition zone (mm), (-: absence of inhibition zone, +: presence of inhibition zone), **: dry weight of mycelial growth (mg), **NC:** negative control, **PC:** positive control, **SDM:** Solid diffusion method, **VDM:** Vapor diffusion method, **ADM:** Agar dilution method.*

*Statistically analyzed by ANOVA (Tukey test, P≤0.05) STD errs for: VDM-MG (=0.45), ADM-MG (=0.78), SDM-OTA (=0.15), VDM-OTA (=0.62), ADM-OTA (=0.65).*

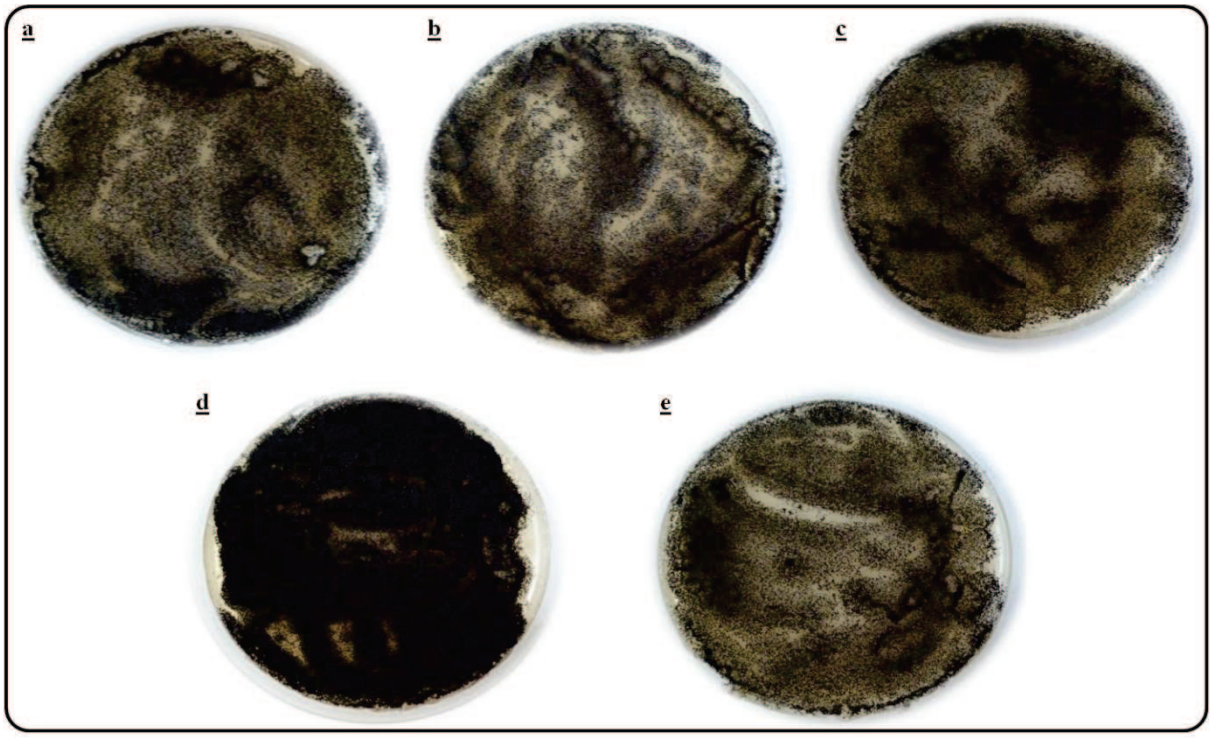



**Figure 3: Antifungal activity of VOCs on OTA-PF colonization** using agar diffusion method: **a:** Negative control, **b:** Positive control, **c:** trans-2-Hexenal 0.05mg/L, **d:** trans-6-Nonenal(13) 0.5mg/L, **e:** trans-2-Octenal(6) 0.05mg/L

The same effective concentrations of the tested VOCs (2-hexenal, 6-nonenal, and 2-octenal) (0.5,0.5, and 0.05 mg/L concentrations, respectively) against the OTA-PF mycelial growth had also significantly reduced the OTA produced amounts using the other methods, vapor diffusion(VDM) (43.24 ± 2.1, 46.0 ± 0.1, and 44.42 ± 1.4 µg OTA/g dry weight, respectively) and agar dilution (ADM) (23.49 ± 0.1, 32.01 ± 1.1, and 31.19 ± 1.4, respectively) methods , which were approximately close to the PC effect (29.7 ± 0.5 and 26.01 ± 1.1 µg OTA/g dry weight, respectively) (Tab. 3).

The most effective method used is ADM which showed the highest significant antifungal activity of the volatile compounds under study (Fig 3).

## 4. Discussion

Stifénia® has no direct antifungal activity on mycelial growth and OTA production ability of the tested OTA-PF. At the low elicitor concentration, The increment of the OTA amounts at early stages of OTA-PF aging may be due to enrichment of PDA cultural media by the fenugreek seed powder [rich in nitrogen and carbon sources and in other growth promoting nutrients (Gopalan *et al.*, 1996), which suggest that the Stifénia® was used as a source of nutrients for the OTA production. The consequent decrement of OTA amounts with the

fungal long aging confirms the absence of Stifénia® direct antifungal activity on OTA production ability, which may be explained as a result to the consumption of the available nutrients in the cultural media and/or reuse part of OTA amount as a source of nutrient (Dachoupakan *et al.*, 2009) and/or OTA self-inhibitory metabolites production as found by Vining *et al. (*1962) and Hurst and Kruse (1972) with other organisms. The absence of OTA decrements at the high elicitor concentration unlike those found at the low elicitor concentration (with long aging), may prove that this decrements was a result of the depletion of the available nutrients for OTA production at low Stifénia® concentration.

Half of the identified volatile compounds were aldehydes and alcohols that were produced by the plant leaves during the plant defense stimulation, which agreed with Dudareva *et al.,* (2006) findings. As demonstrated by Trombetta *et al*. (2002) and Kubo *et al*. (2003) the unsaturated aldehydes have a broad antimicrobial spectrum and show similar activity against bacteria, which acts on the plasmatic membrane, by perturbation its lipidic fraction, and on intracellular targets. Also these aldehydes (particularly, 2-octenal and 6-nonenal) have antifungal activity against human skin pathogenic fungi (Battinelli *et al.,* 2006). This result agreed with the antifungal activity demonstrated against OTA-PF. Certain studies such as Bisignano *et al.* (1999) and Battinelli *et al.* (2006) that highlighted the importance of double bond presence in the aldehydes molecule for the antimicrobial activity, (particularly, antifungal activity). Hildebrand (1989) highlighted the assumed role of plant volatile aldehydes (such as 2-hexenal, 2-octenal and 6-nonenal), that are produced during wounding, through the lipoxygenase enzyme system, in the plant defense mechanism.

Although the effectiveness of Stifénia® is the subject of controversy (Regnault-Roger, 2012), as marked on different crops and pathogens [such as, Gindro *et al.*, 2007 (grape downey mildew) and Camele *et al*., 2009 (melon podery mildew)] rather than the grape powdery mildew (Pajot and Regnault-Roger, 2008) a feature which is noteworthy: it seems that vine treatment with Stifénia®, leads to a reduction of the OTA-produced by *Aspergillus carbonarius* with 33%. Another effect of the plant stimulation by Stifénia for the presence of beneficial bio-control agents as *Penicillium adamatezoides*; resulting of Stifénia® pre-harvest treatment (H. Ahmed *et al.* (a), unpublished data) and certain positive changes of grape and juice physical (increment of grape skin thickness, particularly, wax and cuticle mass layers) and chemical characteristics (increment of the polyphenols as antioxidants) were observed (H. Ahmed *et al.* (b), unpublished data).

**Acknowledgments**

The financial support by the Egyptian government is gratefully acknowledged. We also thank to R Ratomahenina and JC Baccou for their intellectual input and help. We also appreciate SOFT (for providing the Stifénia® product) and IFV, Pech Rouge (for carrying out the field experiment).

**References**

Ahmed, H., Hilaire, F., Strub, C. Schorr-Galindo, S. (a). First report: *Penicillium adametzioides*, an emerging biocontrol agent for ochratoxin-producing fungus in grapes, resulting from natural product pre-harvest treatment. Food Cont. FOODCONT-D-13-01559.

Ahmed, H., Stemmelen, A., Durand, N., Strub, C., Schorr-Galindo, S., (b). Influence of preharvest treatments (chemical, fungal and yeast bio agents, and elicitor) on the OTA juice content and the physical and chemical characteristics of the Mourvèdre grape cultivar. J Agric and Food chem. DOI: 1449199-18816167.

Amézqueta S., Schorr-Galindo, S., Murillo-Arbizu, M., González-Peñas, E., López de Cerain, A., Guiraud, J. P., 2012. OTA-producing fungi in foodstuffs: A review. Food Cont. 26(2), 259-268. DOI:10.1016/j.foodcont.2012.01.042.

Barberis, C., Astoreca, A., Asili, R., Fernandez-Juri, G., Chulze, S., Magnoli, C., Dalcero, A. 2009. *In vitro* control of growth and ochratoxin A production by butylated hydroxyanisole in *Aspergillus* section *Nigri* species. Food Cont. 20(8), 709–715. DOI:10.1016/j.foodcont.2008.09.003.

Battinelli, L., Daniele, C., Cristiani, M., Bisignano, G., Saija, A., Mazzanti, G., 2006. *In vitro* antifungal and anti-elastase activity of some aliphatic aldehydes from *Olea europaea* L. fruit. Phytomed. 13, 558–563. DOI:10.1016/j.phymed.2005.09.009

Bavaresco, L., Vezzulli, S., Battilani, P., Giorni, P., Pietri, A., Bertuzzi, T. 2003. Effect of ochratoxin A-producing *Aspergilli* on stilbenic phytoalexin synthesis in grapes. J Agri Food Chem. 51, 6151-6157. DOI: 10.1021/jf0301908

Benhamou, N., Rey, P., 2012a. Stimulateurs des défenses naturelles des plantes: une nouvelle stratégie phytosanitaire dans un contexte d'écoproduction durable. : II. Intérêt des SDN en protection des cultures. Phytoprotection, 92(1), 24-35. DOI: 10.7202/1013299ar

Benhamou, N., Rey, P., 2012b. Stimulateurs des défenses naturelles des plantes: une nouvelle stratégie phytosanitaire dans un contexte d'éco-production durable. I. Principes de la résistance induite. Phytoprotection 92, 1-23. DOI: 10.7202/1012399ar

Bisignano, G., Tomaino, A., Lo Cascio, R., Crisafi, G., Uccella, N., Saija, A., 1999. On the *in vitro* antimicrobial activity of oleuropein and hydroxytyrosol. J Pharma. Pharmaco. 51(8), 971–974. DOI: 10.1211/0022357991773258

Cabañas, R., Abarca, M.L., Bragulat, M.R., Cabañes, F.J., 2009. *In vitro* activity of imazalil against *Penicillium expansum*: Comparison of the CLSI M38-A broth microdilution method with traditional techniques. Inter J Food Microbio. 129(1), 26-29. DOI: 10.1016/j.ijfoodmicro.2008.10.025

Camele, I., Campanelli, G., Ferrari, V., Viggian,i G., Candido, V., 2009. Powdery mildew control and yield response of inodorus melon. Ital J Agron. 4:19-26.

Cayot, N., Dury-Brun, C., Karbowiak, T., Savary, G. Voilley, A., 2008. Measurement of transport phenomena of volatile compounds: A review. Food Res Inter. 41, 349-362. DOI: 10.1016/j.foodres.2008.02.002

Dachoupakan, C., Ratomahenina, R., Martinez, V., Guiraud, J.P., Baccou, J.C., Schorr-Galindo, S., 2009. Study of the phenotypic and genotypic biodiversity of potentially ochratoxigenic black aspergilli isolated from grapes. Inter J Food Microbio. 132(1), 14:23. DOI: 10.1016/j.ijfoodmicro.2009.03.011

Dudareva, N., Negre, F., Nagegowda, D.A., Orlova, I., 2006. Plant volatiles: Recent advances and future perspectives. Crit Rev Plant Sci. 25, 417-440. DOI: 10.1080/07352680600899973

Durand, N., Bertrand, B., Guyot, B., Guiraud, J.P., Tachon, A.F., 2009. Study on the *Coffea arabica*/*Colletotrichum kahawae* pathosystem: Impact of a biological plant protection product. J Plant Dis Protec. 116(2), 78-85.

El Hadi, M.A.M., Zhang, F.J., Wu, F.F., Zhou, C.H., Tao, J., 2013. Advances in Fruit Aroma Volatile Research. Molecules *18*, 8200-8229; DOI: 10.3390/molecules18078200

European Commission, European Commission Regulation No. 1881/2005 (2006). Amending Regulation (EC) No. 1881/2006 setting maximum levels for certain in foodstuffs as regards ochratoxin A. Offi J Euro U. 364, 5-24.

Fenner, R., Sortino, M., Kuze Rates, S.M., Dall'Agnol, R., Ferraz, A., Bernardi, A.P., Albring, D., Nör, C., von Poser, G., Schapoval, E., Zacchino, S., 2005. Antifungal activity of

some Brazilian *Hypericum* species. Phytomedicine 12, 236-240. DOI: 10.1016/j.phymed.2003.11.004

Gindro, K., Godard, S., De Groote, I., Viret, O., 2007. Peut-on stimuler les mécanismes de défense de la vigne? Une nouvelle méthode pour évaluer le potentiel des éliciteurs. Revue Suisse Viti Arbori Horti. 39, 377-383.

Gopalan, C., Sastri, R.B.V., Balasubramaniam, S.C., Narasinga Rao, B.S., Deosthale, Y.G., Pant, K.C. 1996. Nutritive value of Indian foods. National Institute of Nutrition, Indian Council for Medical Research, Hyderabad, India

Green-McDowelle, D., Ingber, M.B., Wright, M.S., Zeringue Jr., H.J., Bhatnagar, D., Cleveland, T.E., 1999. The effect of selected cotton-leaf volatiles on the growth, development and aflatoxin production of *Aspergillus parasiticus*. Toxicon 37, 883-893.

Haddada, F.M., Manai, H., Daoud, D., Fernandez, X., Lizzani-Cuvelier, L, Zarrouk, M., 2007. Profiles of volatile compounds from some mono varietal Tunisian virgin olive oils. Comparison with French PDO. Food Chem.103, 467-476. DOI: 10.1016/j.foodchem.2006.08.023

Hildebrand, D.F., 1989. Lipoxygenases. *Physiologia Plantarum* 76, 249-253.

Hurst, A., Kruse, H., 1972. Effect of Secondary Metabolites on the Organisms Producing Them: Effect of Nisin on *Streptococcus lactis* and Enterotoxin B on *Staphylococcus aureus*. Antimicrob Agents Chemoth. 1(3), 277-279, DOI, 10.1128/AAC.1.3.277

International Agency for Research on Cancer (IARC) (2003). Ochratoxin A. In IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans; IARC Press: Lyon, France, 2003; 56, pp. 489-521.

Kabak, B., Dobson , A.D.W., Var, I., 2006. Strategies to prevent mycotoxin contamination of food and animal feed. Crit Rev Food Sci Nutri. 46, 593-619. DOI: 10.1080/10408390500436185.

Kubo, I., Fujita, K., Kubo, A., Nihei, K., Lunde, C.S., 2003. Modes of antifungal action of (2E)-alkenals against *Saccharomyces cerevisiae*. J Agric Food Chem. 51(14), 3951–3957. DOI: 10.1021/jf0211884

López, P., Sánchez , C., Batlle, R., Nerín, C., 2005. Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. J Agric Food Chem. 53(17), 6939-46. DOI: 10.1021/jf050709v

Marengo, E, Aceto, M, Maurino, V., 2001. Classification of Nebbiolo-based wines from Piedmont (Italy) by means of solid-phase microextraction-gas chromatography-mass spectrometry of volatile compounds. J Chromatogr A 943, 123-137.

Ministère Française de l'Agriculture et de la pèche (MFAP) (2008). http://agriculture.gouv.fr/IMG/pdf/PLAN_ECOPHYTO_2018.pdf (09/10/2013).

Özer, N., Koç, M., Der, B., 2009. The sensitivity of *Aspergillus niger* and *Fusarium oxysporum* f.sp. *cepae* to fungistasis in onion growing soils. J Plant Patho. 91(2), 401-410.

Pajot, E., Regnault-Roger, C., 2008. Stimulation des défenses naturelles des plantes et résistance induite: une nouvelle approche phytosanitaire? Biopesticides d'origine végétale-Editions Tec & Doc -Lavoisier.

Pozo-Bayón, M.A., Ruíz-Rodriguez, A., Pernin, K., Cayot, N., 2007. Influence of eggs on the aroma composition of a sponge cake and on the aroma release in model studies on flavored sponge cakes. J Agri. Food Chem. 55, 1418-1426. DOI: 10.1021/jf062203y

Ramamurthy, V., Raveendran , S., Thirumeni, S., Krishnaveni, S., 2012. Antimicrobial activity of Heterocytic Cyanobacteria. Inter J Adv Life Sci. 1, 32-39.

Regnault-Roget, C., 2012. Trends for commercialization of biocontrol Agent (Biopesticide) Products. *In:* Mérillon J.M., Ramawat, K.G., (Eds). *Plant Defence: Biological control*. Springer Dordrecht Heidelberg London New-York, DOI: 10.1007/978-94-007-1933-0. Pp (139-160).

Ribeiro, E.L., Scroferneker, M.L., Cavalhaes, M.S., Campos, C.C., Nagato, G.M., Souza, N.A., Ferreira, W.M., Cardoso, C.G., Dias S.M.S., Pimenta, F.C., Toledo, O.A., 2006. Phenotypic aspects of oral strains of *Candida albicans* in children with Down's syndrome. Braz J Biolo. 66(3), 939-944. http://dx.doi.org/10.1590/S1519-69842006000500020

Sánchez-Palomo, E.M. Díaz-Maroto C., Pérez-Coello M.S., 2005. Rapid determination of volatile compounds in grapes by HS-SPME coupled with GC-MS. Talanta 66, 1152-1157. DOI: 10.1016/j.talanta.2005.01.015

Skarkova, J., Ostry, V., Malir, F., & Roubal, T., 2013. Determination of Ochratoxin A in Food by High Performance Liquid Chromatography. Analyt Lett. 46(10), 1495-1504. DOI: 10.1080/00032719.2013.771266

Song, G.C., Ryu, C.M., 2013. Two volatile organic compounds trigger plant self-defense against a bacterial Pathogen and a sucking insect in cucumber under open field conditions. Inter J Mol Sci, *14:*9803-9819; DOI: 10.3390/ijms14059803.

Tesniere, C., Torregrosa, L., Pradal, M., Souquet, J.M., Gilles, C., Santos, K.D., Chatelet, P., Gunata, Z., 2006. Effects of genetic manipulation of alcohol dehydrogenase levels on the response to stress and the synthesis of secondary metabolites in grapevine leaves. J Experimen Bota. 57(1), 91-99. DOI:10.1093/jxb/erj007

Trombetta, D., Saija, A., Bisignano, G., Arena, S., Caruso, S., Mazzanti, G., Uccella, N., Castelli, F., 2002. Study on the mechanisms of the antibacterial action of some plant α, β unsaturated aldehydes. Lett App Microbio. 35(4), 285-290. DOI: 10.1046/j.1472-765X.2002.01190.x

Utto, W., Mawson, A.J., Bronlund, J.E., 2008. Hexanal reduces infection of tomatoes by *Botrytis cinearea* which maintain quality. Postharvest bio techno. 47, 434-437.

Varga, J., Kocsubé, S., Péteri, Z., Vágvölgyi, C., Tóth, B., 2010. Chemical, Physical and Biological Approaches to Prevent Ochratoxin Induced Toxicoses in Humans and Animals. Toxins 2, 1718-1750. DOI: 10.3390/toxins2071718

Vining, L.C., Kelleher, W.J., Schwarting, A.E., 1962. Oosprein production by a strain of *Beauveria bassiana* originally identified as *Amanita muscaria*. Revue canad microbio. 8(6), 931-933. DOI: 10.1139/m62-122

Waldorf, A.R., Polak, A., 1983. Mechanisms of Action of 5-Fluorocytosine. Antimicrob agents chromate. 23(1), 79-85.

# SUPPLEMENTARY RESULTS - PART 2

This part of result concerns the characterization of *Penicillium adametzioides* and its activity.

## 1. *Morphological characterization*

The morphological and microscopic studies combined with the molecular identification conducted in the Article 3 were agreed with Deng *et al* (2012) for the identification of the isolated strain S3 from the grape stalk treated by Stifénia as *Penicillium adametzioides*: conidiophores varies from 19.44-26.24 µm and the dimension of the conidia are 2.4 µm long and 1.85 µm width with a oval shape and a concave side (as shown in Fig. 1C).

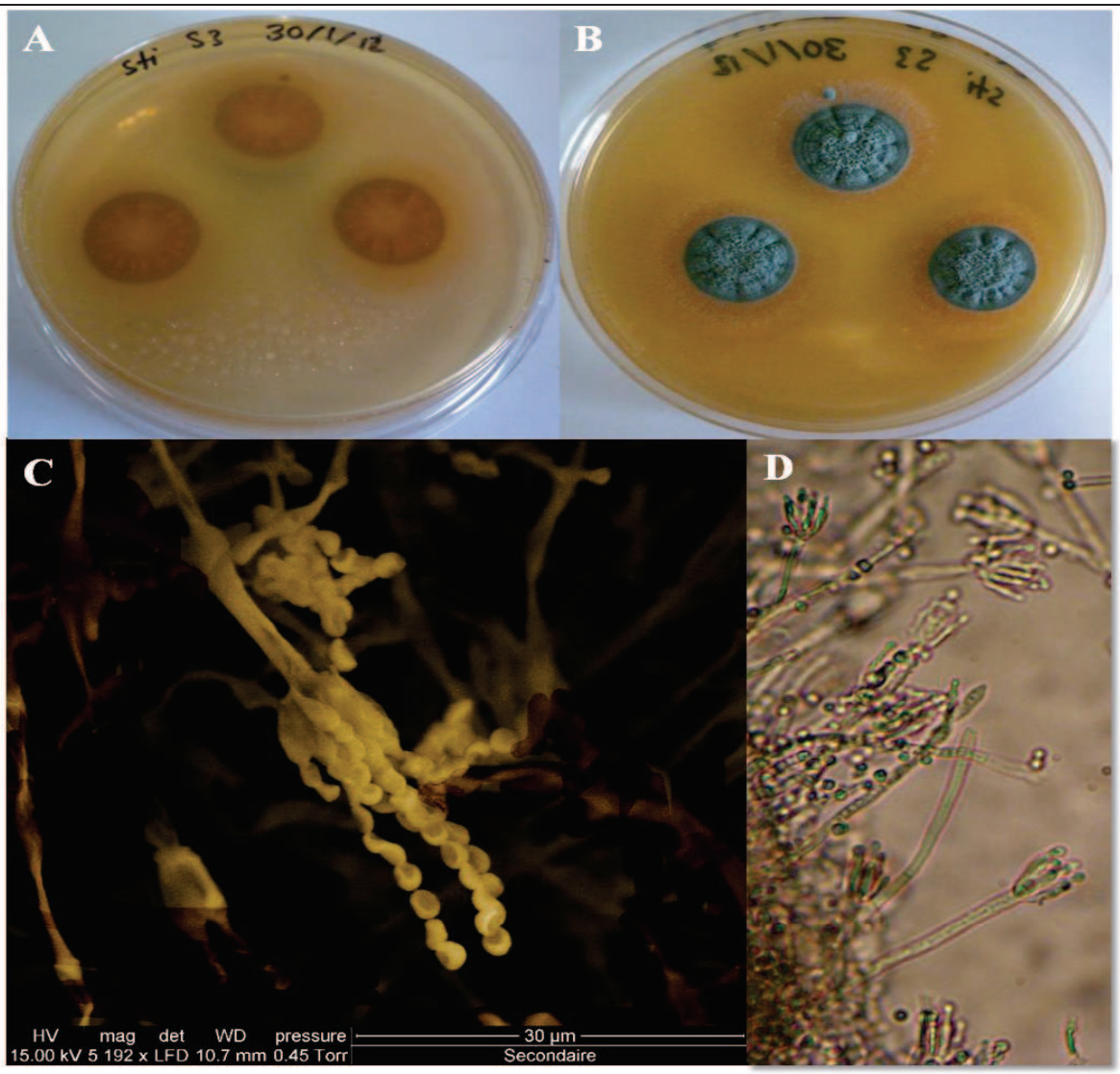



**Figure S2.1: Morphology of the isolate S3 (*Penicillium adametzioides*) Colonies grown on PDA media (A-B)** after 10 days at 25°C, microscopic structure of penicilli and conidia and conidia, **C:** electon microscopy (scale bare: 30µm), **D:** Light microscopies (scale bars: 10 µm).

Further studies were conducted in order to understand the biological control mechanism of *Penicillium adametzioides* against OTA-PF (*A. carbonarius*) as the indirect effect of the *P. adametezioide*s metabolites (culture filtrates or compounds).

## 2. Indirect antifungal activity of *P. adametzioides* against *A. carbonarius*

### 2.1. Antagonistic test using cultural filtrates of *P. adametzioides* (PDB)

The *P. adametzioides* cultures grown on different PBD were filtrated and stored at -20°C until analyzing (Fig. S2.2.).

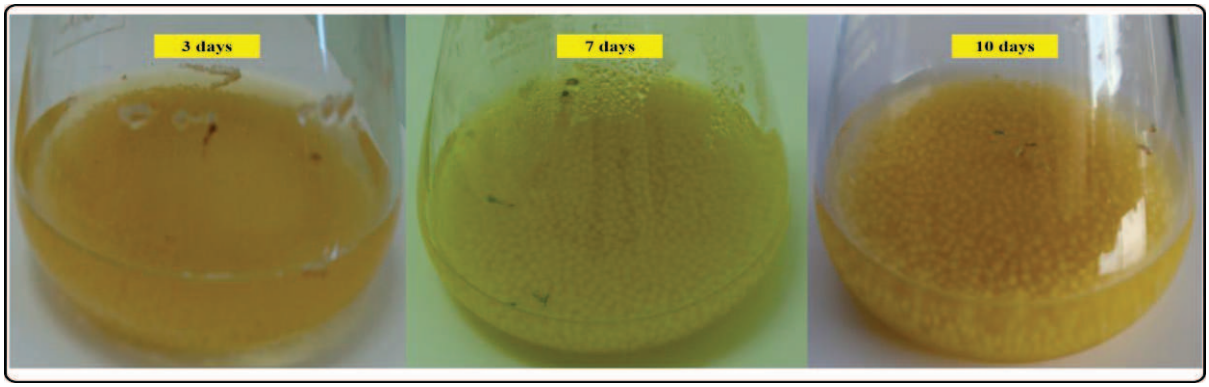



**Figure S2.2: *P. adametzioides* cultures grown on PBD at different**

No antifungal activity of the *P. adametzioides* culture filtrates was observed on the OTA-PF mycelial growth (Fig. S2.3.) (no inhibition zones were observed.

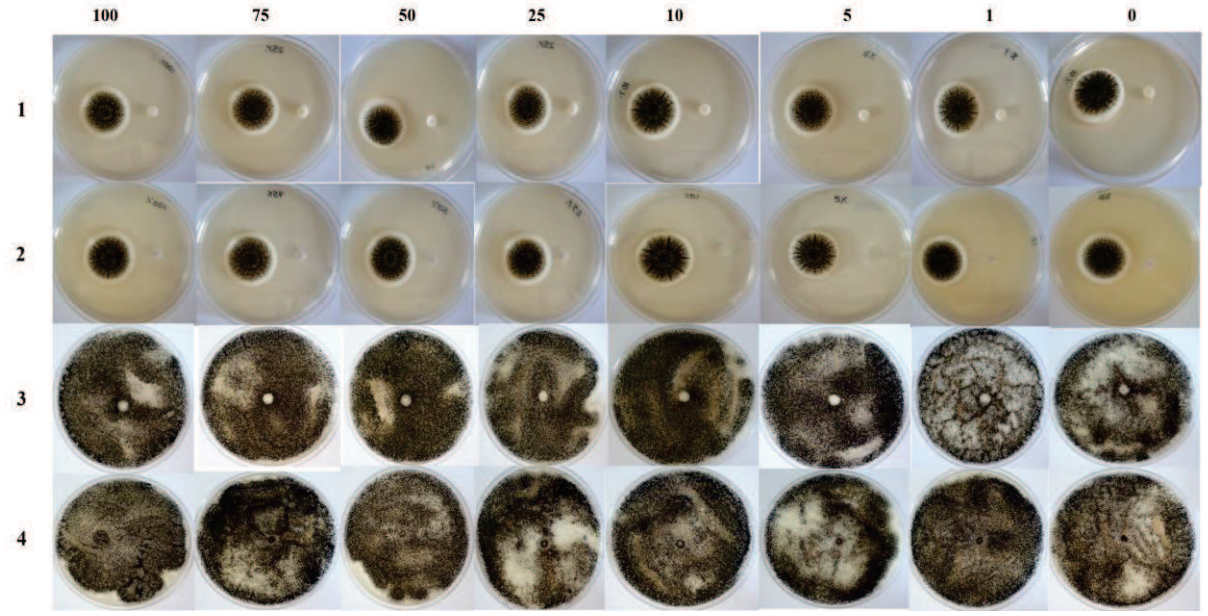



**Figure S2.3: Antifungal activity tests of the *P. adametzioides* 3 days-PBD culture filtrates using different tested methods against OTA-PF on PDA.**

**1:** Side Solid diffusion method, **2:** Side liquid diffusion method, **3:** Center Solid diffusion method, **4:** Center liquid diffusion method

No antifungal activity was observed in the OTA amounts produced by OTA-PF in all the dilutions tested except a slight reduction in the side solid diffusion method. Which was the motive to concentrate the culture filtrates.

### *2.2.* Antagonistic test using compactin produced by *P. adametzioides*

Compactin (also known as mevastatin) is a statin which is well known in the pharmacology as cholesterol inhibitor by catalyzing the conversion of HMG-CoA (3-hydroxy-3-methylglutaryl) to mevanolate. That statin was mainly produced by fungal strains such as *P. citrinum*, *P. brevicompactum*, *A. terreus*, *P. cyclopium* and *P. adametzioides* (Harsha *et al.,* 2013). This mechanism is involved in antifungal activity against certain strains of yeasts and other fungal species such as *Aspergillus* (Nyilasi *et* al., 2010; Galgóczy *et al.,* 2011). This potential actifungal activity was thus tested against the OTA-PF.

No significant antifungal activity of the compactin (using agar dilution method) was observed on the mycelial growth of OTA-PF (Fig. S2.A) or on its ability to produce OTA (Fig. S2.B).

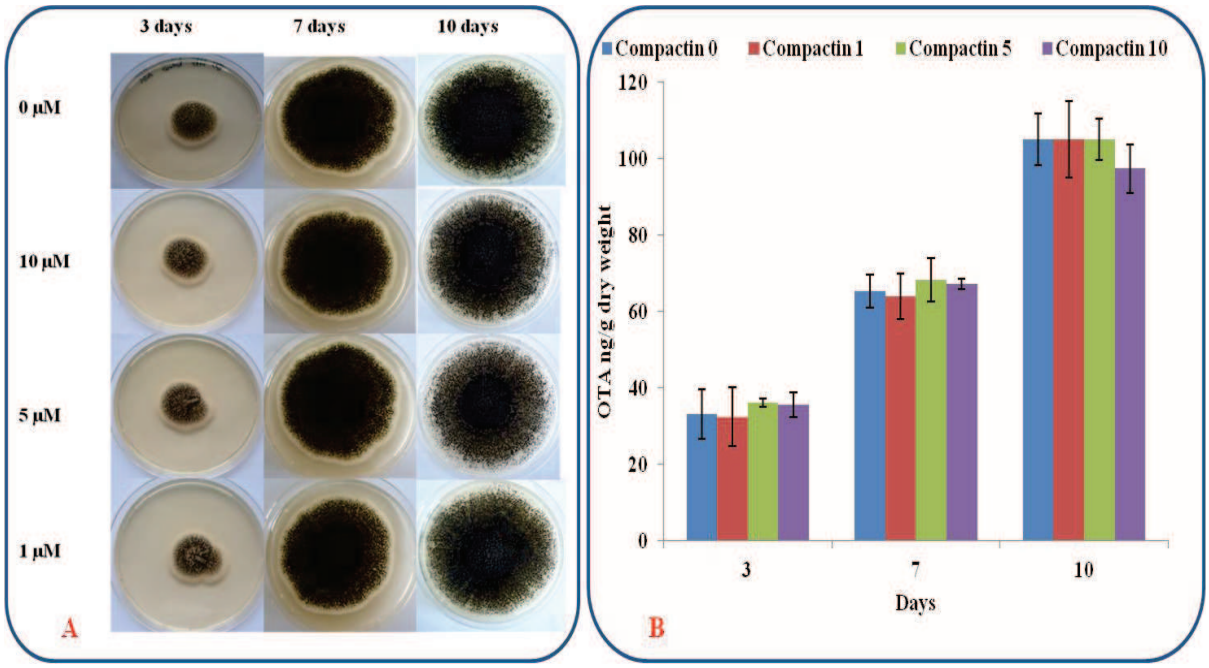



**Figure S2.4 : Antifungal activity of compactin against OTA-PF mycelial growth (A) and its OTA production ability (B), using different concentrations (0, 1, 5 and 10 µM)**

Hence the known metabolite produced by *P. adametzioides*, compactin, has no antifungal activity on OTA-PF. Another study was performed using the *thin layer chromatography* (TLC) in order to separate and identifying the potential metabolite of *Penicillium* responsible for OTA production inhibition as shown in precedent chapter.

## 2.1. Separating P. adametzioides metabolites using TLC

This part of results remains unachieved because certain metabolites were separated but have to identified (as shown in Fig. S2.5)

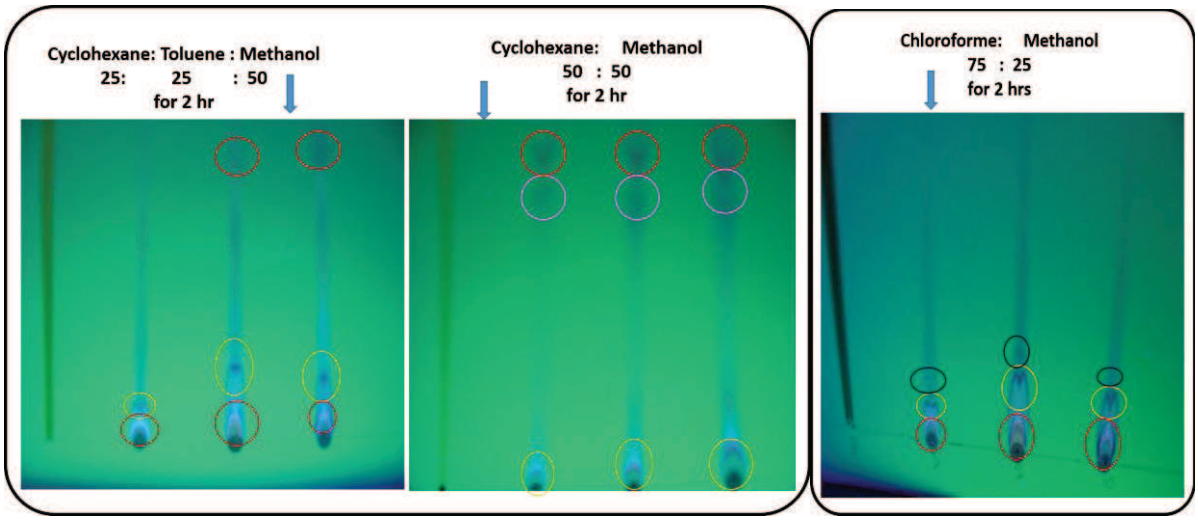



**Figure S2.6: TLC qepartion of 3, 7 and 10 days PDB *P. adametzioides* concentrated filtrates using different soulvant mixtures, and β-carotine as reference.**

# CONCLUSION OF PART-2

Although, the Stifénia® treatment had reduced the OTA amounts produced by OTA-PF, no direct antifungal activity of the Stifénia powder aganist *Aspergillus carbonarius* (OTA-PF) mycelial growth and the OTA produced amounts was measured. Certain strains of the ten NBA strains isolated from the elicitor treatment, had a positive mycelial growth effect on *A. carbonarius* colonies, such as *Penicillium* spp. and *Fusarium* sp. Other strains displayed a reduction effect on OTA production of OTA-PF, such as *Penicillium* spp. (J2, J3). *P. adametzioides* (S3) and *P. expansum* (J1) (at certain stages) that reduced the OTA production and mycelial growth. *P. expansum* was excluded as a potential biocontrol agent because of its mycotoxin production ability.

The higher challenge distance between certain strains of *P. adametzioides* (S3) and other *Penicillium* strains (as J1, J2, J3 and J4; at three and seven days) reduced the secretion of OTA by OTA-PF. Also the Stifénia treatment had affected the VOC leaves profile regarding the VOC chemical fungicide profile with significally increase of two VOCs (trans-6-nonenal and trans-2-octenal. These two VOCs have antifungal activity against the *A. carbonarius* growth and OTA production with low concentrations (0.5 and 0.05 mg/L, respectively). That may partially explain the mode of action of plant defence by producing leaf VOCs that induce positive changes on the OTA-PF metabolism and thus reduction of OTA contents in grapes.

This reduction of OTA production by toxignic fungi could possibly prevent OTA contamination of grapevine products in the case of epidemic favourable conditions. This could be accomplished by applying as elicitor one of the tested fungi with an antagonistic effect on OTA production, such as *P. adametzioides* (at 10 days). Certain strains, such as *P. adametzioides* (S3) and J2 (*P.* sp.) should be further investigated to determine the details of the underlying mechanism of their reduction of OTA production and their effects on vine ecosystem in cases of *in vivo* application.

The metabolites of *P. adametzioides* produced in liquid medium culture have no indirect antifungal activity in low concentrations, which proposing either that the *P. adametzioides* antifungal effect is a direct action or that the metabolites need to be concentrated or combined with other active mode.

## References

**Amézqueta S., González-Peñas, E.; Murillo-Arbizu, M.; López de Cerain, A. (2009).** Ochratoxin A decontamination: A review. *Food Control*, **20**:326-333.

**Benhamou N. and P. Rey (2012).** Stimulateurs des défenses naturelles des plantes: une nouvelle stratégie phytosanitaire dans un contexte d'écoproduction durable. : II. Intérêt des SDN en protection des cultures. *Phytoprotection*, **92(1)**:24-35.

**Camele, I., G. Campanelli, V. Ferrari, G. Viggiani and V. Candido. (2009).** Powdery mildew control and yield response of inodorus melon. *Italian Journal of Agronomy*, **4**:19-26.

**Deng J. X., N. C. Paul, H. K. Sang, J. H. Lee, Y. S. Hwang and S. H. Yu (2012).** First Report on Isolation of *Penicillium adametzioides* and *Purpureocillium lilacinum* from Decayed Fruit of Cheongsoo Grapes in Korea. *Mycobiology*, **40(1)**:66-70.

**European Commission** (**2008).** Commission regulation (EC) No. 299/2008 amending regulation (EC) No. 396/2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin, as regards the implementing powers conferred on the Commission. *Official Journal of the European Union*, **97**:67-71.

**Galgóczy L., I. Nyilasi, T. Papp and C. Vágvölgyi (2011).** Statins as antifungal agents. *World Journal of Clinical Infectious Diseases,* **1(1)**: 4-10.

**Harsha N., S. Subbarao, V. Sridevi, M. V. V. C. Lakshmi and T K. Kiran (2013).** Production of Mevastatin by Solid State Fermentation Using Sesame Oil Cake. *Research Journal of Pharmaceutical, Biological and Chemical Sciences,* **4(1)**:429-436.

**Kabak B. and A. D. W. Dobson (2009).** Biological strategies to counteract the effects of mycotoxins. *Journal of Food Protection*, **72**:2006-2016.

**Nyilasi I., S. Kocsubé, L. Galgóczy, T.Papp, M.Pesti and C.Vágvölgyi (2010).** Effect of different statins on the antifungal activity of polyene antimycotics. *Acta Biologica Szegediensis,* **54(1)**:33-36.

**Ponsone M. L., M. L. Chiotta, J. M. Palazzini, M. Combina and S. Chulze (2012).** Control of ochratoxin A production in grapes. *Toxins*, **4**:364-372. DOI: 10.3390/toxins4050364.

**Simon C. (2007).** Stifénia doit faire ses preuves. *Revue La Vigne*, **191**:79.

# Chapter 4

# Final Conclusion

# FINAL CONCLUSION

As concluded by Lima *et al.,* (2008) and Droby *et al*., (2009), an integrated strategy based on the combination of biological control agents with natural compounds or reduced dosage of fungicides appears to be one of the most reliable options for large-scale utilization of microbial antagonists in the control of ochratoxigenic fungi and reduction of the entry of OTA to the food chains.

According to this previously illustrated, the increase need to find an alternative and safe OTA-controling methods that could substitute chemical fungicides use or alternatives that might be accompanied with low concentration of these chemicals. *In-vivo* experiment using different suggested preharvest-natural OTA-control treatments (fungal bioagent, yeast bioagent and plant natural product that could act as an elicitor) with comparison to a traditional chemical fungicide treatment and two untreated control modalities [one of them was *A. carbonarius* (OTA-PF) contaminated and the other was not contaminated]. This expermint had conducted in field with favouring climate condition for the OTA-PF colonization and affecting the grape bunches and berries.

The Stifénia® and yeast bioagent treatment had approximately the equal reduction percent of the chemical fungicide treatment, for the OTA content contamination of their juices to be within the European authorized in the grape and its products. While, the fungal bioagent treatment had reduced the OTA contamination but had over the maximum OTA limit. Which suggested the use of the elicitor and yeast bioagent as OTA prevention control treatments regardingthe improvement the juice secuirity.

The qualitative and quantitative microbiological analysis of the mycoflora isolated from the different treatments and modalities, had demonstrated that the Stifénia® treatment had significantly affected the fungal ecosystem in quantity and quality. Where, it had significantlly reduced the total mycoflora in grape juices and stems (66.7% and 71.4, respectively) and the black aspergilli incidence (9938% and 83%, respectively). All other treatments have a higher incidence regarding the uncontaminated treatment. In addition, the Stifénia® treatment had emerged new strains that was not isolated from the other treatments and modalities.

Even though, the qualitative fingerprinting, PCR-DGGE technique, had rapidly monitored and classified the different treatments according to their fungal biodiversity (on

the base of using universal fungal primers at the ITS region), its result had a partial incorrect profile. PCR-DGGE had demonstrated, that the Stifénia treatment had the highest similarity to the uncontaminated modality, and the microbiological analysis, had mentioned the opposite, where, the Stifénia had the highest number of emerged new strains (qualitative analysis), and the lowest CFU (quantitative analysis). This might be due to the PCR-DGGE that could not differentiate in between the vial and dead cells spores.

A promising result was obtained with the separation of higher ds-DNA size more than the maximum limit by changing the gel concentrations. This could be used to improve the applications of PCR-DGGE in evaluation and differentiation between different microflora ecosystems by increasing the microflora sequence variance.

The combination of the specific qPCR for the ochratoxigenic contaminant stain (PKS region) with the universal qPCR for total fungi, had approximately the same estimations and ratios for the different preharvest treatments effect on the black-aspergilli-strains and total mycoflora, with a slight modifications. Accordingly, the both qPCR (specific and universal primers) could be used as a fast detector (relative estimations) and evaluator (absolute estimations) technique, for the comparative studies as done in this study. However, for the fungal biodiversity, it could not be a useful technique (this disadvantages could be partially overcome by using a various qPCR analysis for the same extracted DNA using many specific primers for certain species or group of fungi).

The elicitor (Stifénia®) and yeast (*Saccharomyces cerevisiae*) treatments appeared to be the most effective treatments in the enhancement of disease resistance or plant defense barriers [passive defense (wax and cuticle layers and skin thickness) and chemical barriers (pH, acids and sugar contents)] of grape berries against certain pathogens. The majority of the plant defense passive barrier's enhancements were significantly higher in the Stifénia® treatment (skin thickness, cuticle mass), however, the yeast treatment had slightly higher enhancement in the plant chemical defense barriers (sugar, pH, phenolic compounds). In addition these two treatments (elicitor and yeast bioagent) had significantly improved the grape juice quality observed regarding the high content of the polyphenolic content, which was two times more than that in the chemical treatment. That could increase the positive changes in the grape juice quality and berry disease resistance combined to improvement of the juice safety.

*Final Conclusion*

Given to the previous findings, the yeast bioagent and elicitor treatments have improved the juice quality and safety. The Stifénia treatment has showed the highest effect on the fungal ecosystem and the black aspergilli one, including the OTA-PF and allowed the isolation of new fungal strains that were not isolated from other treatments. That was the motive to study the effect of these non-black *Aspergillus* (NBA) strains on the OTA-PF. The performed *in vitro* antagonistic tests between the ten Stifénia® NBA isolates and the OTA-PF using three antagonistic tests (at two different distances, two inoculation times, and three incubation periods) had showed that certain strains had positively reduced the OTA-PF mycelial growth, such as *Penicillium* spp. and *Fusarium* sp. Other strains displayed a reduction effect on OTA production of OTA-PF, such as *Penicillium* spp. (J2, J3). *P. adametzioides* (S3) and *P. expansum* (J1). Tha last one reduced both the OTA production and mycelial growth. *P. expansum* was however excluded to be used as a bio-control agent because of its mycotoxin production ability. The higher challenge distance between certain strains of *P. adametzioides* (S3) and other *Penicillium* strains (as J1, J2, J3 and J4; at three and seven days) reduced the secretion of OTA by OTA-PF. This OTA production reduction could possibly prevent OTA contamination of grape post-harvest products in the case of epidemic favorable conditions for ochratoxigenic fungi by reducing their toxigenic metabolism abilities.

This could be accomplished by applying as elicitor one of the tested fungi with an antagonistic effect on OTA production, such as *P. adametzioides* (at 10 days). Certain strains, such as *P. adametzioides* (S3) and J2 (*P.* spp.) should be further investigated to determine the details of the underlying mechanism of their OTA reduction and their ecosystem effects in cases of *in vivo* application.

The Stifénia treatment had affected the volatile compound profile of the treated leaves with comparison to the leaf volatile organic compound (VOC) profile of fungicide pre-harvest treatment (which, were assessed using HS-SPME-GC-MS). Certain VOCs had significantly increased in the plant extract treatment regarding the chemical one. Trans-6-nonenal and trans-2-octenal, which recognized in Stifénia® treatment leaves with the highest significant concentration regarding to their concentration with the chemical treatment, have antifungal activity against the *A. carbonarius* growth and OTA production with low concentrations (0.5 and 0.05 mg/L, respectively).

*Final Conclusion*

No antifungal activity of the Stifénia® powder against the OTA-PF mycelial growth or its OTA produced amounts were measured.

The improvement of juice safety (reducing the OTA content), juice quality, berry passive and chemical defense barriers, could be achieved by replacing the field fungicide treatment by one of the elicitor or the yeast bioagent treatment.

The Stifénia treatment could positively affect the fungal ecosystem (by introducing a possible fungal biocontrol agent reducing the OTA production and mycelial growth, such as *Penicillium adametzioides*). In addition, the Stifénia treatment could modify the leaf volatile organic compounds profile with increasing certain compounds that have a positive antifungal activity against the OTA-PF fungal growth and its OTA production. That could highlight a part of the eliciting process enhanced by the Stifénia treatment against the OTA-PF. And highly suggesting the possibility of chemical fungicide replacement, at the first level by Stifénia followed by yeast bioagent.

## Perspectives

Hence, the future use of biological agents for controlling ochratoxigenic fungi and ochratoxin production will depend on the cost production and the field effectiveness of the formulated product. Therefore, further studies should be conducted for the optimization of *Penicillium adametzioides* biocontrol efficacy and other study should be conducted testing the survival and colonization of biological control agents in wounded and unwounded grape surfaces and in the presence of low quantities of fungicides applied separately or in combination with microbial antagonists. Moreover, the widespread diffusion of fungal pathogens resistant to fungicides used for a long time in the field and/or in packinghouses (e.g., benzimidazoles), has led to the need of assessing the compatibility and efficacy of biological control agents with new and recently developed fungicides (Lima *et al.,* 2011; Ponsone *et al*., 2012). Another study should be performed in order to identify the corresponding metabolic compound(s) produced by *Penicillium adametzioides* affecting the OTA production of the *Aspergillus carbonarius* and to possibly study the metabolic pathway involved in order to produce active substances that could be used in biological control.

# REFERENCES

**Abarca M. L., F. Accensi, M. R. Bragulat, G. Castellá and F. J. Cabañes (2003)** *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection,* **66**:504-506.

**Abarca M. L., M. R. Bragulat, G. Castella, F. Accensi and F. J. Cabañes (1997).** New ochratoxigenic species in the genus *Aspergillus*. *Journal of Food Protection,* **60**:1580-1582.

**Abarca M. L., M. R.Bragulat, G. Castella and F. J. Cabañes (1994).** Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. *Applied and Environmental Microbiology,* **60**:2650-2652.

**Abarca M.L., F. Accensi, J. Cano and F. J. Cabanes (2004).** Taxonomy and significance of black aspergilli. *Antonie van Leeuwenhoek*, **86**:33-49.

**Abdulkadar A. H. W., A. A. Al-Ali, A. M. Al-Kildi and J. H. Al-Jedah (2004).** Mycotoxins in food products available in Qatar. *Food Control,* **15**:543-548.

**Abouzied M. M., A. D. Horvath, P. M. Podlesny, N. P. Regina, V. D. Metodiev, R. M. Kamenova-Tozeva, N. D. Niagolova, A. D. Stein, E. A. Petropoulos and V. S. Ganev (2002).** Ochratoxin A concentrations in food and feed from a region with Balkan endemic nephropathy. *Food Additives and Contaminants*, **19**:755–764. DOI: 10.1080/02652030210145036.

**Abrunhosa L. and E. A. Venancio (2007).** Isolation and purification of an enzyme hydrolyzing ochratoxin A from *Aspergillus niger*. *Biotechnology Letters*, **29**:1909 –1914.

**Abrunhosa L., R. R. M. Paterson and A. Venâncio (2010).** Biodegradation of Ochratoxin A for Food and Feed Decontamination. *Toxins*, **2**:1078-1099; DOI: 10.3390/toxins2051078

**Abrunhosa L., R. R. M.Paterson, Z. Kozakiewicz, N. Lima and A. Venancio (2001).** Mycotoxin production from fungi isolated from grapes. *Letters in Applied Microbiology*, **32**:40-242.

**Abrunhosa L., R. Serra and A. Venancio (2002).** Biodegradation of ochratoxin A by fungi isolate from grapes. *Journal of Agricultural and Food Chemistry,* **50**:7493-7496.

**Accensi F., M. L. Abarca and F. J. Cabañes (2004).** Occurrence of *Aspergillus* species in mixed feeds and component raw materials and their ability to produce ochratoxin A. *Food Microbiology,* **21**:623-627.

**Accensi F., M. L. Abarca, J. Cano, L. Figuera and F. J. Cabañes (2001).** Distribution of Ochratoxin A producing strains in the *A. niger aggregate*. *Antonie van Leeuwenhoek,* **79**:365-370.

*References*

**Adamek P., B. Bergstrom, T. Borjesson and U. Stollman (1992).** Determination of volatile compounds for the detection of moulds. *Developments in food science*, **31**:327-336.

**Adams D. O. (2006).** Phenolics and Ripening in Grape Berries. *American Journal of Enology and Viticulture*, **57**:249-256.

**AFNOR (2002).** Microbiologie Alimentaire. Saint-Denis La Plaine Cedex, 8ème éd. Tome, vol. 1, p. 64.

**Agrios G.N. (1988).** Plant Pathology. 3rd Ed. Academic Press, San Diego, CA, USA.

**Ahmed-Hoda M.H., M. Digiaro and G.P. Martelli (2004).** Viruses and virus diseases of grapevine in Egypt. *EPPO Bulletin*, **34(3)**:395-398.

**Amézqueta S., S. Schorr-Galindo, M. Murillo-Arbizu, E. González-Peñas, A. López de Cerain, J. P. Guiraud (2012).** OTA-producing fungi in foodstuffs: A review. *Food Control,* **26(2)**:259-268.

**Amponsah N. T., E. E. Jones, H. J. Ridgway and M. V. Jaspers (2009).**First report of *Neofusicoccum australe* [*Botryosphaeria australis*], a cause of grapevine dieback in New Zealand. *Australasian Plant Disease Notes*, **4(1)**:6-8.

**Anglade P. and J. Puisais (1987).** Vin et vignobles de France. Edition Larousse, collection 'Savour Club', Paris.

**Araujo F. J. and L. E. Williams (1988).** Dry matter and nitrogen partitioning and root growth of young field-grown Thompson Seedless grapevines. Vitis, **27**:21-32.

**Arzumanov T., N. Jenkins and S. Roussos, (2005).** Effect of aeration and substrate moisture content on sporulation of *Metarhizium anisopliae* var. *acridum*. *Process Biochemistry*, **40**:1037-1042.

**Atalla, M.M. and K. N. El-Din (1993).** Production of ochratoxin A in a semisynthetic medium. *Egyptian Journal of Microbiology* **28**, 271-279.

**Atoui A., F. Mathieu and A. Lebrihi (2007).** Targeting a polyketide synthase gene for *Aspergillus carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. *International Journal of Food Microbiology*, **115**:313-318.

**Atoui A., H. P. Dao, F. Mathieu and A. Lebrihi (2006).** Amplification and diversity analysis of ketosynthase domains of putative polyketide synthase genes in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. *Molecular Nutrition and Food Research,* **50(6)**: 488-493. DOI: 10.1002/mnfr.200500165

**Aziz A., B. Poinssot, X. Daire, M. Adrian, A. Bézier, B. Lambert, J. M. Joubert and A. Pugin (2003).** Laminarin elicits defense responses in grapevine and induces protection

against *Botrytis cinerea* and *Plasmopara viticola*. *Molecular Plant-Microbe Interactions,* **16(12)**:1118-1128.

**Bååth E., K. Arnebrant andA. Nordgren (1991).** Microbial biomass and ATP in smelter-polluted forest humus. *Bulletin of Environmental Contamination and Toxicology*, **47(2)**:278-282.

**Bacha N., A. Atouia, F. Mathieu, T. Liboz and A.Lebrihi (2009).** *Aspergillus westerdijkiae* polyketide synthase gene "aoks1" is involved in the biosynthesis of ochratoxin A. *Fungal Genetics and Biology*, **46(1)**:77-84.

**Baiano, A. and C. Terracone (2011).** Varietal differences among the phenolic profiles and antioxidant activities of seven table grape cultivars grown in the South of Italy based on chemometrics. *Journal of Agricultural and Food Chemistry*, **59**:9815-9826.

**Battilani P. and A. Pietri (2002).** Ochratoxin a in Grapes and Wine. *European Journal of Plant Pathology*, **108(7)**:639-643.

**Battilani P., A. Pietri, A. T. Bertuzzi, L. Languasco, P. Giorni and Z. Kozakiewicz, (2003).** Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. *Journal of Food Protection,* **66**:633-636.

**Battilani P., N. Magan and A. Logrieco (2006).** European research on ochratoxin A in grapes and wine. *International Journal of Food Microbiology*, **111(S1)**:S2-S4.

**Bau M., G. Castellá, M. R. Bragulat and F. J. Cabañes (2006).** RFLP characterization of *Aspergillus niger aggregate* species from grapes from Europe and Israel. *International Journal of Food Microbiology,* **111(S1)**:S18-S21.

**Bau M., M. R. Bragulat, L. A. Abarca, S. Mínguez and F. J. Cabañes (2005).** Ochratoxigenic species from Spanish wine grapes. *International Journal of Food Microbiology,* **98(2)**:125-130.

**Bavaresco L. and C. Fregoni (2001).** Physiological role and molecular aspects of grapevine stilbenic compounds. In Molecular Biology and Biotechnology of the GrapeVine; Roubelakis-Angelakis, K. A., (Ed.); Kluwer Academic Publishers: Dordrecht, The Netherlands, 2001; pp 153-182.

**Bavaresco L., S. Vezzulli, P. Battilani, P. Giorni, A. Pietri and T. Bertuzzi (2003).** Effect of OTA-producing aspergilli on stilbenic phytoalexin synthesis in grapes. *Journal of Agricultural and Food Chemistry,* **51**:6151-6157.

**Bavaresco L., S. Vezzulli, S. Civardi, M. Gatti, P. Battilani, A. Pietri and F. Ferrari (2008).** Effect of lime-induced leaf chlorosis on ochratoxin A, *trans*-resveratrol, and ε-

viniferin production in grapevine (Vitis Vinifera L.) berries infected by *Aspergillus carbonarius*. *Journal of Agricultural and Food Chemistry,* **56**:2085-2089.

**Bayman P., J. L. Baker, M. A. Doster, T. J. Michailiades and N. E. Mahoney (2002)**. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. *Applied and Environmental Microbiology,* **68**:2326-2329.

**Bejaoui H., F. Mathieu, P. Taillandier and A. Lebrihi (2006).** Biodegradation of ochratoxin A by *Aspergillus* section Nigri species isolated from French grapes: a potential means of ochratoxin A decontamination in grape juices and musts. *FEMS Microbiology Letters*, **255**:203-208. DOI: 10.1111/j.1574-6968.2005.00073.x

**Belhadj A., C. Saigne , N. Telef , S. Cluzet , J. Bouscaut , M. F. Corio-Costet and J. M. Mérillon (2006).** Methyl Jasmonate induces defense responses in grapevine and triggers protection against *Erysiphe necator*. *Journal of Agricultural and Food Chemistry*, **54(24)**:9119–9125. DOI: 10.1021/jf0618022

**Belhadj A., N. Telef, C. Saigne, S. Cluzet, F. Barrieu, S. Hamdi and J. M. Merillon (2008).** Effect of methyl jasmonate in combination with carbohydrates on gene expression of PR proteins, stilbene and anthocyanin accumulation in grapevine cell cultures. *Plant Physiology Biochemistry*, **46**:493-499.

**Bellemain E., T. Carlsen, C. Brochmann, E. Coissac, P. Taberlet and H. Kauserud (2010).** ITS as an environmental DNA barcode for fungi: an *in silico* approach reveals potential PCR biases. *BMC Microbiology*, **10**:189-179. DOI: 10.1186/1471-2180-10-189.

**Bellí N., D. Mitchell, S. Marín, I. Alegre, A. J. Ramos, N. Magan and V. Sanchis (2005).** OTA-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. *European Journal of Plant Pathology,* **113(3)**:233-239. DOI: 10.1007/s10658-005-5547-4.

**Bellí N., E. Pardo, S. Marín, G. Farré, A. J. Ramos and V. Sanchis (2004).** Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *Journal of the Science of Food and Agriculture*, **84**:541-546.

**Bellí N., M. Bau, S. Marín, M. L. Abarca, A. J. Ramos and M. R. Bragulat (2006a).** Mycobiota and ochratoxin A producing fungi from Spanish wine grapes. *International Journal Food Microbiology*, **111 (S1):**S40-S45.

**Bellí N., S. Marín, V. Sanchis and A. J. Ramos (2006b).** Impact of fungicides on *Aspergillus carbonarius* growth and ochratoxin A production on synthetic grape-like medium and on grapes. *Food Additives and Contaminants*, **23**:1021-1029.

**Bellí N., S. Marín, V. Sanchis, and A. J. Ramos (2002).** Review: Ochratoxin A in wines, musts and grape juices: occurrence, methods of analysis and regulations. *Food Science and Technology International,* **8:**325-335.

**Bendahmane B. S., D. Mahiout, I. E. Benzohra and M. Y. Benkada (2012).** Antagonism of three *Trichoderma* species against *Botrytis fabae* and *B. cinerea,* the causal agents of chocolate spot of faba bean (*Vicia faba* L.) in Algeria. *World Applied Sciences Journal*, **17(3)**:278-283.

**Benhamou N. and P. Rey (2012).** Stimulateurs des défenses naturelles des plantes: une nouvelle stratégie phytosanitaire dans un contexte d'écoproduction durable. : II. Intérêt des SDN en protection des cultures. *Phytoprotection*, **92(1)**:24-35. DOI: 10.7202/1013299ar.

**Bennett J. W. (1987).** Mycotoxins, mycotoxicoses, mycotoxicology and mycopathology. *Mycopathlogia,* **100**:3–5.

**Bennett J. W. and M. Klich (2003).** Mycotoxins. Clinical Microbiology Reviews, **16(3)**:497-516. DOI: 10.1128/CMR.16.3.497-516.2003

**Berlese A.N., 1898.** Saggio di una monografia delle peronosporacee. *Rivista di Patologia Vegetale*, **6**:79-110.

**Berna A. (2010).** Metal oxide sensors for electronic noses and their application to food analysis. *Sensors*, **10**:3882-3910. DOI: 10.3390/s100403882.

**Betina V. (1989).** Mycotoxins: Chemical biological and environmental aspects. Betina, V. (Ed.). Elsevier, Amsterdam, The Netherlands.

**Bezuidenhout S. C., W. C. A. Gelderblom, C. P. Gorst-Allman, R. M. Horak, W. F. O. Marasas; G. Spiteller and R. Vleggaar (1988).** Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme.* Journal of the Chemical Society, Chemical Communications, **1988**:743-745.

**Blaeser M. and H.C. Weltzien (1979).** Epidemiologische Studien an *Plasmopara viticola* zur Verbesserung der Spritzterminbestimmung. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz*, **86**: 489-98.

**Blesa J., H. Berrada, J. M. Soriano, J. C. Moltó and J. Mañes (2004).** Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography. *Journal of Chromatography A*, **1046**:127-131.

**Bleve G., F. Grieco, G. Cozzi, A. Logrieco and A. Visconti (2006).** Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. *International Journal of Food Microbiology*, **108**:204-209.

*References*

**Blouin J. and J. Cruège (2003).** Analyse et composition des vins. Editions de la vigne, Dunod, Paris.

**Boudra H., P. Le Bars and J. Le Bars (1995).** Thermostability of ochratoxin A in wheat under two moisture conditions. *Applied and Environmental Microbiology*, **61**:1156-1158.

**Boulton R (1980).** The general relationship between Potassium, Sodium and pH in grape juice and wine. *American Journal of Enology and Viticulture*, **31(2)**:182-186.

**Boutrif E. and C. Bessy (2001).** Global significance of mycotoxins and phycotoxins. *In:* Koe W. J., R. A. Samson, H. P. van Egmond, J. Gilbert and M. Sabino (Eds), *Mycotoxins and phycotoxins in perspective at the turn of the millennium*.. Ponsen and Looyen, Wageningen, The Netherlands, 3-16.

**Braidot E., M. Zancani, E. Petrussa, C. Peresson, A. Bertolini, S. Patui, F. Macrì and A. Vianello (2008).**Transport and accumulation of flavonoids in grapevine (*Vitis vinifera* L.). *Plant Signaling and Behavior*, **3(9)**: 626–632.

**Brakhage A. A. and V. Schroeckh (2011).** Fungal secondary metabolites – Strategies to activate silent gene clusters. *Fungal Genetics and Biology*, **48**:15-22

**Bredenkamp M. W., J. L. M. Dillen, P. H. Van Rooyen and P. S. Steyn (1989).** Crystal structures and conformational analysis of ochratoxin A and B: Probing the chemical structure causing toxicity. *Journal of the Chemical Society,* **2**:1835-1839.

**Breitholtz-Emanuelsoon A., M. Olsen, A.Oskarsson, I. Palminger and K. Hult (1993).** Ochratoxin A in cow's milk and in human milk with corresponding human blood samples. *Journal of AOAC International,* **76**:842-846.

**Bresch H., M. Urbanek and K. Hell (2000a).** Ochratoxin A in coffe, tea and beer. *Archiv für Lebensmittelhygiene,* **51**:89-94.

**Bresch H., M. Urbanek and M. Nusser (2000b).** Ochratoxin A in food containing liquorice. *Nahrung,* **44**:276-278.

**Bridge P. D., D. L. Hawksworth, Z. Kozakiewicz, A. H. S. Onions., R. R. M. Paterson, M. J. Sackin and P. H. A. Sneath, (1989).** A reappraisal of terverticillate Penicillia using biochemical, physiological and morphological features. 1. Numerical taxonomy. *Journal of General Microbiology,* **135**:2941-2966.

**Brooka P. J. (1973).** Epidemiology of grapevine anthracnose, caused by *Elsinoe ampelina*. *New Zealand Journal of Agricultural Research*, **16(3)**:333-342. DOI:10.1080/00288233.1973.10421113

**Bryden W. L. (2007).** Mycotoxins in the food chain: human health implications. *Asia Pacific Journal of Clinical Nutrition*, **16 (S1)**:95-101.

**Bucheli P., I. Meyer, A. Pittet, G. Vuataz and R. Viani (1998).** Industrial storage of green robusta coffee under tropical conditions and its impact on raw material quality and ochratoxin A content. *Journal of Agricultural and Food Chemistry,* **46**:4507-4511.

**Bulit J. and R. Lafon (1978).** Powdery Mildew of the Vine. In: Spencer. D.M. (Ed.).The powdery mildews. Academic Press. New York. pp. 525-548.

**Burdaspal P. A. and T. M. Legarda (1998).** Ochratoxin A in roasted coffee and soluble coffees marketed in Spain. *Alimentaria,* **296**:31-35.

**Burdaspal P. A. and T. M. Legarda (1999).** Ochratoxin A in wines and grape products originated from Spain and other European countries. *Alimentaria* **299**: 107-113.

**Cabañas R., M.L. Abarca, M.R. Bragulat and F.J. Cabañes (2009).** *In vitro* activity of imazalil against *Penicillium expansum*: Comparison of the CLSI M38-A broth microdilution method with traditional techniques. *International Journal of Food Microbiology,* **129(1)**:26-29.

**Cabañes F. J., F. Accensi, M. R. Bragulat, M. L. Abarca, G. Castellá, S. Minguez and A. Pons (2002).** What is the source of ochratoxin A in wine? *International Journal of Food Microbiology*, **79**:213-215.

**Caffi T., V. Rossi, R. Bugiani, F. Spanna, L. Flamini, A. Cossu and C. Nigro (2009).** A model predicting primary infections of *Plasmopara viticola* in different grapevine-growing areas of Italy. *Journal of Plant Pathology*, **91(3)**:535-548.

**Calvo A. M., R. A. Wilson, J. W. Bok and N. P. Keller (2002).** Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews,* **66**:447-459.

**Campagnoli A., F. Cheli, C. Polidori, M. Zaninell, O. Zecca, G. Savoini, L. Pinotti and V. Dell'Orto (2011).** Use of the Electronic Nose as a Screening Tool for the Recognition of Durum Wheat Naturally Contaminated by Deoxynivalenol: A Preliminary Approach. *Sensors*, **11**:4899-4916. DOI: 10.3390/s110504899.

**Candolfi-Vasconcelos M. C. and W. Koblet (1990).** Yield, fruit quality, bud fertility and starch reserves of the wood as a function of leaf removal in Vitis vinifera - Evidence of compensation and stress recovering. Vitis, **29**:199-221.

**Carbonneau A., A. Deloire and B. Jaillard (2007).** La vigne physiologie, terroir, culture. Dunod, Paris.

**Carette J. (2005).** De la grappe au verre. Editions Publibook, Paris.

**CAST (1989).** Mycotoxins: Economic and Health Risks. Report No. 116. Council for Agricultural Science and Technology, Ames, Iowa, USA.

*References*

**Castellá G. and F.J. Cabañes (2011).** Development of a Real Time PCR system for detection of ochratoxin A-producing strains of the *Aspergillus niger aggregate. Food Control,* **22(8)**:1367-1372.

**Castellá G., T. O. Larsen, F. J. Cabañes, H. Schmidt, A. Alboresi, L. Niessen, P. Farber and R. Geisen, (2002).** Molecular characterization of ochratoxin A producing strains of the genus *Penicillium*. *Systematic and Applied Microbiology,* **25**:74-83.

**Castellari M., A. Versari, U. Spinabelli, S. Galassi and A. Amati (2000).** An improved HPLC method for the analysis of organic acids, carbohydrates and alcohols in grape. *Journal of Liquid Chromatography & Related Technologies*, **23(13)**:2047-2056.

**Castellarin S. D., L. Bavaresco, L. Falginella, M. I. V. Z. Gonçalves and G. di Gaspero (2012).** Phenolics in Grape Berry and Key Antioxidants. In: Gerós H., M. Chaves and S. Delrot (Eds). The Biochemistry of the grape berry. Bentham Science; Bussum, The Netherlands: pp. 89–110.

**Cawthon D. L. and J. R. Morris (1982).** Relationship of seed number and maturity to berry development, fruit maturation, hormonal changes, and uneven ripening of 'Concord' (*Vitis labrusca* L.) grapes. *Plant Molecular Biology*, **107**:1097-1104.

**Cawthon D. L. and J. R. Morris (1982).** Relationship of seed number and maturity to berry development, fruit maturation, hormonal changes, and uneven ripening of 'Concord' (*Vitis labrusca* L.) grapes. *Plant Molecular Biology*, **107**:1097-1104.

**Cawthon D. L. and J. R. Morris (1982).** Relationship of seed number and maturity to berry development, fruit maturation, hormonal changes, and uneven ripening of 'Concord' (*Vitis labrusca* L.) grapes. *Plant Molecular Biology*, **107**:1097-1104.

**Cayot N., C.Dury-Brun, T. Karbowiak, G. Savary and A. Voilley (2008)** Measurement of transport phenomena of volatile compounds: A review. *Food Research International*, **41**:349-362.

**Cerutti G., A. D'amato and M. Zucchetti (2000).** Sulla presenza di ocratossina A, nitrato e nitrito nel vino. *Imbottigliamento,* **23**: 39-43.

**Chelkowski J., R. A. Samson, M. Wiewiorowska and P. Golinski (1987).** Ochratoxin A formation by isolated strains of the conidial stage of *Aspergillus glaucus* Link ex Grey (=*Eurotium herbariorum* Wiggers Link ex Grey) from cereal grains. *Nahrung,* **31**:267-270.

**Chellemi D. O. & J. J Marois (1991).** Sporulation of *Uncinula necator* on grape leaves as influenced by temperature and cultivar. *Phytopathology*, **81**:197-201.

**Chellemi D. O. and J. J Marois (1991).** Sporulation of *Uncinula necator* on grape leaves as influenced by temperature and cultivar. *Phytopathology*, **81**:197-201.

*References*

**Chernin L., A. Brandis, Z. Ismailov and I. Chet (1996).** Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. *Current Microbiology*, **32**:208-212.

**Christensen L. P., and W. L. Peacock (2000).**The Raisin Drying Process. Pages 207-216. In: Raisin Production Manual. University of California, Agricultural and Natural Resources Publication 3393, Oakland, CA.

**Chulze S. N., C. E. Magnoli and A. M. Dalcero (2006).** Occurrence of ochratoxin A in wine and ochratoxigenic mycoflora in grapes and dried vine fruits in South America. *International Journal of Food Microbiology,* **111**:S5-S9.

**Ciegler A. (1972).** Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. *Canadian Journal of Microbiology,* **18**:631-636.

**Ciegler A., D. J. Fennell, H. J. Mintzlaff and L. Leistner (1972).** Ochratoxin synthesis by *Penicillium* species. *Naturwissenschaften,* **59**:365-366.

**Ciegler A., R. W. Detroy and E. B. Lillejoj (1971).** Patulin, penicillic acid and other carcinogenic lactones, p. 409-434. *In* A. Ciegler, S. Kadis and S. J. Ajl (Eds), Microbial toxins, vol. VI: fungal toxins. Academic Press. New York, N.Y.

**Conde, C., S. Paulo, N. Fontes, A. C. P. Dias, R. M. Tavares, M. J. Sousa, A. Agasse, S. Delrot, H. Gerós (2007).** Biochemical changes throughout grape berry development and fruit and wine quality. *Food*, **1(1)**:1-22. ISSN 1749-7140.

**Conradie W. J. (1980).** Seasonal uptake of nutrients by Chenin blanc in sand culture: I. Nitrogen. *South African Journal for Enology and Viticulture*, **1**:59-65.

**Considine J. A. and P. E. Kriedemann (1972).** Fruit splitting in grapes: determination of the critical turgor pressure. *Australian Journal of Agricultural Research*, **23**:17-24.

**Coombe B. (2001).** Ripening berries - a critical issue. *Australian Viticulture*, **5**:28-34.

**Coombe B. G. (1976).** The development of fleshy fruits. *Annual Review of Plant Biology*, **27**:507-528.

**Coombe B. G. (1989).** The grape berry as a sink. *Acta Horticulturae*, **239**:149-158.

**Coombe B.G. (1987).** Distribution of solutes within the developing grape berry in relation to its morphology. *American Journal of Enology and Viticulture*, **38**:120-127.

**Cotty P. J., P. Bayman, D. S. Egel and K. S. Elias (1994).** Agriculture, aflatoxins and *Aspergillus*, p. 1–27. *In* K. A. Powell, A. Renwick and J. F. Peberdy (Ed.), The genus *Aspergillus*. Plenum Press, New York, N.Y.

**Covarelli L., G. Beccari, A. Marini and L. Tosi (2012).** A review on the occurrence and control of ochratoxigenic fungal species and ochratoxin A in dehydrated grapes, non-fortified

dessert wines and dried vine fruit in the Mediterranean area. *Food Control*, **26**:347-356. DOI: 10.1016/j.foodcont.2012.01.044

**Cozzi G., S. Somma, M. Haidukowski and A. F. Logrieco (2013).** Ochratoxin A management in vineyards by *Lobesia botrana* biocontrol. *Toxins*, **5**:49-59. DOI: 10.3390/toxins5010049

**Creasy G. L., Price S. F. and P. B. Lombard (1993).** Evidence for xylem discontinuity in Pinot Noir and Merlot: Dye uptake and mineral composition during berry maturation. *American Journal of Enology and Viticulture*, **44**:187-192.

**Creppy E. E. (1999).** Human ochratoxicosis. *Journal of Toxicology -Toxin Reviews,* **18**:277-293.

**Da Rocha Rosa C. A., V. Palacios, M. Combina, M. E. Fraga, A. De Oliveira Rekson, C. E. Magnoli and A. M. Dalcero (2002)** Potential ochratoxin A producers from wine grapes in Argentina and Brazil. *Food Additives and Contaminants*, **19**:408-414.

**Dachoupakan C., R. Ratomahenina, V. Martinez, J. P. Guiraud, J. C. Baccou, S. Schorr-Galindo (2009).** Study of the phenotypic and genotypic biodiversity of potentially ochratoxigenic black aspergilli isolated from grapes. *International Journal of Food Microbiology*, **132(1)**:14-23. DOI: 10.1016/j.ijfoodmicro.2009.03.011.

**Dai G. H., C. Andary, L. Mondolot-Cosson and D. Boubals (1995).** Involvement of phenolic compounds in the resistance of grapevine callus to downy mildew (Plasmopara viticola). *European Journal of Plant Pathology*, **101(5)**:541-547. DOI: 10.1007/BF01874479

**Dalcero A., C. Magnoli, C. Hallak, S. M. Chiacchiera, G. Palacio, and C. A. R. Rosa (2002).** Detection and ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section *Nigri* in Argentina. *Food Additives and Contaminants,* **19**:1065-1072.

**Davies C., T. Wolf and S. P. Robinson (1999).** Three putative sucrose transporters are differentially expressed in grapevine tissues. *Plant Science*, **147**:93-100.

**Davis N. D., U. L. Diener, and D. W. Eldridge (1966).** Production of Aflatoxins B1 and G1 by *Aspergillus flavus* in a Semisynthetic Medium. *Applied and Environmental Microbiology*, **14(3)**:378-380.

**De Rossi P., M. Reverberi, A. Del Fiore, V. Tolaini, A. Ricelli, A. A. Fabbri and C. Fanelli (2010).** Early identification of Aspergillus carbonarius in artificially and naturally contaminated grape berries by real-time polymerase chain reaction. Quality Assurance and Safety of Crops and Foods, **2(3)**:120-126. DOI: 10.1111/j.1757-837X.2010.00074.x

*References*

**De Rossi P., M. Reverberi, A. Ricelli, A. AFabbri, D. Caputo, G. De Cesare, R. Scipinotti and C. Fanelli (2011).** Early detection of ochratoxigenic fungi in wine grapes and of ochratoxin A in wine. *Analytical Microbiology*, **61**:11-15.

**De Vries R. P., J. C. Frisvad, P. J. I. van der Vondervoort, K. Burgers, A. F. A. Kuijpers and R. A. Samson (2004).** *Aspergillus vadensis*, a new species of the group of black Aspergilli. *Letters in Applied Microbiology,* **38**:440-445.

**Deak T., J. Chen, D. A. Golden, M. S. Tapia, J. Tornai-Lehoczki, B. C. Viljoen, M. T. Wyder and L. R. Beuchat (2001).** Comparison of dichloran 18% glycerol (DG18) agar with general purpose mycological media foe enumerating food spoilage yeasts. *International Journal of Food Microbiology,* **67**:49-53.

**Deberghes P., A. M. Betbeder, F. Boisard, R. Blanc, J. F. Delaby, S. Krivobok, R. Steiman, F. Seigle-Murandi and E. E. Creppy (1995).** Detoxification of ochratoxin A, a food contaminant: Prevention of growth of *Aspergillus ochraceus* and its production of ochratoxin A. *Mycotoxin Research*, **11(1)**:37-47.

**Degelmann P., M. Becker, M. Herderich and H. U. Humpf (1999).** Determination of ochratoxin A in beer by high-performance liquid chromatography. *Chromatographia,* **49**:543-546.

**Demyttenaere J. C. R, R. M Moriñaa, N. De Kimpe and P. Sandra (2004).** Use of headspace solid-phase microextraction and headspace sorptive extraction for the detection of the volatile metabolites produced by toxigenic *Fusarium* species. *Journal of Chromatography A,* **1027(1–2)**:147–154

**Derckel J. P., F. Baillieul, S. Manteau, J. C. Audran, B. Haye, B. Lambert, and L. Legendre (1999).** Differential Induction of Grapevine Defenses by Two Strains of *Botrytis cinerea*. *Biochemistry and Cell Biology,* **89(3)**:197-203. DOI: 10.1094/PHYTO.1999.89.3.197

**Díaz G. A., R. Torres, M. Vega and B. A. Latorre (2009).** Ochratoxigenic *Aspergillus* species on grapes from Chilean vineyards and *Aspergillus* threshold levels on grapes. *International Journal of Food Microbiology*, **133**:195-199.

**Díez B., C. Pedrós-Alio and R. Massana (2001).** Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. *Applied and Environmental Microbiology,* **67**:2932-2941.

**Dimakopoulou M., S. E. Tjamos, P. P. Antoniou, A. Pietri, P. Battilani, N. Avramidis, E. A. Markakis and E. C. Tjamos (2008).** Phyllosphere grapevine yeast *Aureobasidium*

*pullulans* reduces *Aspergillus carbonarius* (sour rot) incidence in wine-producing vineyards in Greece. *Biological Control*, **46**:158-165.

**Dirheimer G. (1996).** Mechanistic approaches to ochratoxin toxicity. *Food Additives and Contaminants,* **13**:45-48.

**Djajakirana G., R. G. Joergensen and B. Meyer (1996).** Ergosterol and microbial biomass relationship in soil. *Biology and Fertility of Soils*, **22(4)**:299-304.

**Dokoozlian N. K. (2000).** Grape Berry Growth and Development. In: Christiansen L P. (Ed) Raisin Production Manual. University of California, Agricultural and Natural Resources Publication 3393, Oakland, CA. pp. 259(p: 30-37)

**Doster M. A., T. J. Michailides and D. P. Morgan (1996).** *Aspergillus* species and mycotoxins in figs from California orchards. *Plant Disease,* **80**:484-489.

**Dragacci S. and J. M. Frémy (1998).** Dosage des mycotoxines. In *Moisissures des aliments peuhydratés*; Cahagnier, B., Ed.; Tec et Doc: Paris, France, 1998; pp. 187–207.

**Droby S. and A. Lichter (2007).** Post-harvest *Botrytis* infection: etiology, development and management. In: Elad Y., B. Williamson, P. Tudzynski and N. Delen (Eds). *Botrytis*: Biology, Pathology and Control, Springer Netherlands, 2007, pp. 349-367. DOI: 10.1007/978-1-4020-2626-3_19.

**Droby S., M. Wisniewski, D. Macarisin and C. Wilson (2009).** Twenty years of postharvest biocontrol research: Is it time for a new paradigm? *Postharvest Biology and Technology*, **52**:137-145.

**Dunsford P. A. and T. N. Sneyd (1989).** Pressing for quality. In: Proceedings of the 7th Australian Wine Industry Technical Conference.

**Ehrlich K. C., P. K. Chang and D. Bhatnagar (2011).** Mycotoxins. *In*: eLS. John Wiley & Sons Ltd, Chichester. DOI:10.1002/9780470015902.a0000373.pub2

**El Adlouni C., M. Tozlovanu, F. Naman, M. Faid and A. Pfohl-Leszkowicz (2006).** Preliminary data on the presence of mycotoxins (Ochratoxin A, citrinin and aflatoxin B1) in black table olives" Greek style" of Moroccan origin. *Molecular Nutrition and Food Research*, **50**:507-512.

**El Sheikha A. F., A. Condur, I. Métayer, D. D. Le Nguyen, G. Loiseau and D. Montet (2009).** Determination of fruit origin by using 26S rDNA fingerprinting of yeast communities by PCR-DGGE: Preliminary application to *Physalis* fruits from Egypt. *Yeast*, **26(10)**:567-573.

**El Sheikha A. F., N. Durand, S. Sarter, J. B. L. Okullo and D. Montet (2012).** Study of the microbial discrimination of fruits by PCR-DGGE: Application to the determination of the

geographical origin of *Physalis* fruits from Colombia, Egypt, Uganda and Madagascar. *Food Control*, **24(1-2)**:57-63.

**El-Banna A., J. I. Pitt and L. Leistner (1987).** Production of mycotoxins by *Penicillium* species. *Systematic and Applied Microbiology,* **10**:42-46.

**El-Halouat A. and J. Debevere (1997).** Molds and yeasts isolated from hydrated prunes and raisins having different water activities. *Siences des Aliments,* **17**:539-545.

**El-Kady I., S. El-Maraghy and A. N. Zohri (1994).** Mycotoxin producing potential of some isolates of *Aspergillus flavus* and *Eurotium* groups from meat products. *Microbiological Research,* **149**:297-307.

**El-Khoury A. and A. Atoui (2010).** Ochratoxin A: General Overview and Actual Molecular Status. *Toxins,* **2**:461-493. DOI: 10.3390/toxins2040461.

**Elmer P. A. G. and T. J. Michailides (2007).** Epidemiology of *Botrytis cinerea* in orchard and vine crops. In: Elad Y., B. Williamson, P. Tudzynski and N. Delen (Eds). *Botrytis*: Biology, Pathology and Control, Springer Netherlands, 2007, pp. 243-272. DOI: 10.1007/978-1-4020-2626-3_14.

**Elsawi N. M., O. M. Elmaghraby, H. S. Mohran, and M. A. Abogharbia (1994).** Abnormal contamination of cottage cheese in Egypt. *Journal of Applied Animal Research,* **6**:81-90.

**Engel G. (2000).** Ochratoxin A in sweets, oil seeds and dairy products. *Archiv für Lebensmittelhygiene,* **51**:98-101.

**Entwistle C. A., A. C. Williams, P. J. Mann, J. Russell, P. Slack and J. Gilbert (2001).** Combined phenyl silane and immunoaffinity column cleanup with liquid chromatography for determination of ochratoxin A in roasted coffee; Collaborative study. *Journal of AOAC International*, **84**:444-450.

**Ercolini D. (2004).** PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56(3)**:297-314.

**Esna-Ashari M. and A. M. Pour (2011).** Effect of methyl jasmonate on resveratrol production in organs and cell suspension cultures of two Iranian grapevine (*Vitis vinifera* L.) cultivars. *Journal of Horticultural Science Biotechnology*, **86**:557-562.

**European Commission, European Commission Regulation No. 1881/2005 (2006).** Amending Regulation (EC) No. 1881/2006 setting maximum levels for certain in foodstuffs as regards ochratoxin A. *Official Journal of European Union*, **364**:5-24.

**FAOSTAT (2010***). http://faostat.fao.org/site/291/default.aspx*. 18/11/2013.

*References*

**Farber P. and R. Geisen (2004).** Analysis of differentially expressed ochratoxin A biosynthesis genes of *Penicillium nordicum*. *European Journal of Plant Pathology*, **110**:661-669.

**Faucet-Marquis V., F. Pont, F. C. Størmer, T. Rizk, M. Castegnaro and A. Pfohl Leszkowicz (2006)**. Evidence of a new dechlorinated ochratoxin A derivative formed in opossum kidney cell cultures after pretreatment by modulators of glutathione pathways: Correlation with DNA-adduct formation. *Molecular Nutrition and Food Research*, **50**:530-542.

**Fenner R., M. Sortino, S. M. Kuze Rates, R. Dall'Agnol, A. Ferraz, A. P. Bernardi, D. Albring, C. Nör, G. von Poser, E. Schapoval and S. Zacchino (2005).** Antifungal activity of some Brazilian *Hypericum* species. *Phytomedicine*, **12**:236-240.

**Ferrandino A., A. Carlomagno, S. Baldassarre and A. Schubert (2012).** Varietal and pre-fermentative volatiles during ripening of *Vitis vinifera* cv Nebbiolo berries from three growing areas. *Food Chemistry*, **135(4)**:2340–2349.

**Fierer N., J. A. Jackson, R. Vilgalys and R. B. Jackson (2005).** Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Applied and Environmental Microbiology*, **71(7)**:4117-4120.

**Filali A., L. Ouammi, A. M. Betbeder, I. Baudrimont, R. Soulaymani, A. Benayada and E. E. Creppy (2001).** Ochratoxin A in beverages from Morocco: a preliminary survey. *Food Additives and Contaminants* **18**: 565-568.

**Fischer G., T. Muller, ¨R. Schwalbe, R. Ostrowski and W. Dott (2000).** Species-specific profiles of mycotoxins produced in cultures and associated with conidia of aireborne fungi derived from biowaste. *International Journal of Hygiene and Environmental Health,* **203**:97-104.

**Fontes N., H. Gerós and S. Delrot (2011).** Grape berry vacuole: A complex and heterogeneous membrane system specialized in the accumulation of solutes. *American Journal of Enology and Viticulture*, **62**:270–278.

**Frisvad J. C. and R. A. Samson (2000).** *Neopetromyces* gen. nov. and an overview of teleomorphs of *Aspergillus* subgenus *Circumdati*. *Studies in Mycology,* **45**:201-207.

**Fukal L. and M. Marek (1991).** A screening method for simultaneous immunochemical detection of aflatoxins and ochratoxin A in cereals and feed. *Veterinary Medicine*, **36**:251-254.

**Fumagalli F., M. Rossoni, M. Iriti, A. di Gennaro, F. Faoro, E. Borroni, M. Borgo, Scienza A., A. Sala, and G. Folco, (2006).** From field to health: A simple way to increase

the nutraceutical content of grape as shown by NO-dependent vascular relaxation. *Journal of Agricultural and Food Chem*istry, **54**:5344-5349.

**Gabler F. M., J. L. Smilanick, M. Mansour, D. W. Ramming and B. E. Mackey (2003).** Correlations of morphological, anatomical, and chemical features of grape berries with resistance to *Botrytis cinerea*. *Phytopathology*, **93(10)**:1263-1273.

**Galbiati C. and G. Longhin (1984).** Indagini sulla formazione e sulla germinazione delle oospore di *Plasmopara viticola*. *Rivista di Patologia Vegetale*, **20**:66-80.

**Galet P. (2000).** General Viticulture. Translated from French by Towey J.,Oenoplurimedia, Chaintre', France.

**Gallo A., G. Perrone, M. Solfrizzo, F. Epifani, A. Abbas, A. D.W. Dobson and G. Mulè (2009).** Characterisation of a pks gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. *International Journal of Food Microbiology,* **129(1)**:8-15

**Gallo A., K. S. Bruno, M. Solfrizzo, G. Perrone, G. Mulè, A. Visconti and S. E. Bakerb (2012).** New insight into the ochratoxin A biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. *Applied and Environmental Microbiology*, **78(23):**8208-8218. DOI: 10.1128/AEM.02508-12.

**Garcia A. A. F., L. L. Benchimol, A. M. M. Barbos, I. O. Geraldi, C. L. S. Jr, A. P. de Souza (2004).** Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. Genetics and Molecular Biology, **24(4)**: 579-588.

**Garcia A. A. F., L. L. Benchimol, A. M. M. Barbosa, I. O. Geraldi, C. L. S. Jr and A. P. de Souza (2002).** Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. Genetics and Molecular Biology, **27(4)**:579-588.

**Gareis M. (1996).** Fate of ochratoxin A on processing of meat products. *Food Additives and Contaminants,* **13**:35-37.

**Gareis M. and R. Scheuer (2000).** Ochratoxin A in meat and meat products. *Archiv für Lebensmittelhygiene,* **51**:102-104.

**Geisena R., Z. Mayerb, A. Karolewieza and P. Färbera (2004).** Development of a Real Time PCR System for Detection of *Penicillium nordicum* and for Monitoring Ochratoxin A Production in Foods by Targeting the Ochratoxin Polyketide Synthase Gene. *Systematic and Applied Microbiology*, **27(4)**:501-507.

**Gelderblom W. C. A., K. Jaskiewicz, W. F. O. Marasas, P. G. Thiel, R. M. Horak, R. Vleggaar and N. P. J. Kriek (1988).** Fumonisins- novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme. Applied and Environmental Microbiology*, **54:**1806-1811.

**Genovesea A., A. Gambutia, S. A. Lamorteb and L. Moioa (2013).** An extract procedure for studying the free and glycosilated aroma compounds in grapes. *Food Chemistry,* **136(2)**:822–834.

**Gessler C., I. Pertot and M. Perazzolli (2011).** *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathologia Mediterranea*, **50**:3−44.

**Glasel J. A. (1995).** Validity of nucleic acid purities monitored by 260 nm/280 nm absorbance ratio. *Biotechnology*, **18**: 62-63.

**Gobbin D., M. Jermini, B. Loskill, I. Pertot, M. Raynal, C. Gessler (2005).** Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. *Plant Pathology*, **54**:522-534.

**Gomez E., A. Martinez and J. Laencinaz (1994).** Localization of free and bound aromatic compounds among skin, juice and pulp fractions of some grape varieties. *Vitis*, **33**:1-4.

**González-Salgado A., B. Patiño, C. Vázquez and M. T. González-Jaén (2005).** Discrimination of *Aspergillus niger* and other *Aspergillus* species belonging to section Nigri by PCR assays. *FEMS Microbiology Letters*, **245(2)**:353-361. DOI: 10.1016/j.femsle.2005.03.023

**González-Salgado A., B. Patiño, J. Gil-Serna, C. Vázquez, M.T. and González-Jaén (2009).** Specific detection of *Aspergillus carbonarius* by SYBR® Green and TaqMan® quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. *FEMS Microbiology Letters*, **295**:57-66.

**Gonzalezsanjose M. L. and C. Diez (1992).** Relationship between anthocyanins and sugars during the ripening of grape berries. *Food Chemistry*, **43**(**3)**:193-197. DOI: 10.1016/0308-8146(92)90172-X.

**Goto T., D. T. Wicklow and Y. Ito (1996).** Aflatoxin and cyclopiazonic acid production by a sclerotium-producing *Aspergillus tamarii* strain. *Applied and Environmental Microbiology*, **62**:4036-4038.

**Goto T., D. T. Wicklow and Y. Ito (1996).** Aflatoxin and cyclopiazonic acid production by a sclerotium-producing *Aspergillus tamarii* strain. *Applied and Environmental Microbiology,* **62**:4036-4038.

**Gourama H. and L. B. Bullerman (1995).** Relationship between aflatoxins production and mould growth as measured by ergosterol and plate count. *Lebensmittel-Wissenschaft Technologie,* **28**:185-189.

**Goyal S., C. Lambert, S. Cluzet, J. M. Mérillon and K. G. Ramawat (2012).** Secondary Metabolites and Plant Defence. pp, 109-138. *In:* Mérillon J. M. and K. G. Ramawat (Eds), Plant Defence: Biological Control. Springer Dordrecht Heidelberg London New York. DOI: 10.1007/978-94-007-1933-0

**Gray D. I. and B. M. Morris (1992).** A low cost video analysis system for the BBC Master computer. *Binary,* **4**:58-61.

**Guerreroa R. F., A. Liazidb, M. Palmab, B. Puertasa, R. González-Barrioc, Á. Gil-Izquierdoc, C. García-Barrosob, E. Cantos-Villara (2009).** Phenolic characterisation of red grapes autochthonous to Andalusia. *Food Chemistry*, 112(4):949–95.

**Haddada F. M., H. Manai, D. Daoud, X. Fernandez, L. Lizzani-Cuvelier and M. Zarrouk (2007).** Profiles of volatile compounds from some mono varietal Tunisian virgin olive oils. Comparison with French PDO. *Food Chemistry*, **103**:467-476.

**Halleen F. and G. Holz (2011).** An Overview of the Biology, Epidemiology and Control of *Uncinula necator* (Powdery Mildew) on Grapevine, with Reference to South Africa. *South African Journal for Enology and Viticulture*, **22(2)**:111-121.

**Hardie W. J., T. P. O'Brien and V. G. Jaudzems (1996).** Morphology, anatomy and development of the peric.arp after anthesis in grape, *Vitis vinifera* L. *Australian Journal of Grape and Wine Research,* **2**:97-142.

**Harris J. M., P. E. Kriedemann and J. V. Possingham (1968).** Anatomical aspects of grape berry development. *Vitis*, **7**:106-119.

**Harris J. P. and P. G. Mantle (2001).** Biosynthesis of ochratoxins by *Aspergillus ochraceus*. Phytochemistry, **558**:709 –716.

**Hayat A., N. Paniel, A. Rhouati, J. L. Marty and L. Barthelmebs (2012).** Recent advances in ochratoxin A-producing fungi detection based on PCR methods and ochratoxin A analysis in food matrices. *Food Control*, **26(2)**:401-415.

**Heenan C. N., K. J. Shaw and J. I. Pitt (1998).** Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. *Journal of Food Microbiology,* **1:**67-72.

**Heidler D. and G. Schatzmayr (2003).** A new approach to managing mycotoxins. *World Poultry*, **19**:12-15.

**Heyndrickx M., L. Vauterin, P. Vandamme, K. Kersters and P. De Vos (1996).** Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *Journal of Microbiological Methods,* **26**:247-259.

*References*

**Ho L. C. (1988).** Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annual Review of Plant Physiology and Plant Molecular Biology,* **39**:355-378.

**Höhler D. (1998).** Ochratoxin A in food and feed: occurrence, legislation and mode of action. *Zeitschrift für Ernahrungswissenschaft,* **37**:2-12.

**Horie Y. (1995).** Productivity of ochratoxin A of *Aspergillus carbonarius* in *Aspergillus* section *Nigri*. *Nippon Kingakkai Kaiho* 36, 73-76.

**Hübner M., T. Vrabcheva and M. Gareis (1998).** Simultaneous detection of ochratoxin A and B in spices and herbs by immunoaffinity column cleanup and HPLC. *Revue de Médecine Vétérinaire,* **149**:507.

**Huff W. E. and Hamilton P. B. (1979).** Mycotoxins-their biosynthesis in fungi: ochratoxins-metabolites of combined pathways. *Journal of Food Protection*, **42**:815-820.

**Huglin P. (1986)**. Biologie et écologie de la vigne. Lavoisier Technique & documentation and P. Laussanne (Eds), Paris.

**IARC (1993).** Some naturally occurring substances, food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risks to humans, vol. 56. IARC, Lyon, France.

**Iriti M., M. Rossoni, M. Borgo and F. Faoro (2004).** Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to *Botrytis cinerea*. *Journal of Agricultural and Food Chem*istry, **52**:4406-4413.

**Iriti M., M. Rossoni, M. Borgo, L. Ferrara and F. Faoro (2005).** Induction of resistance to gray mold with benzothiadiazole modifies amino acid profile and increases proanthocyanidins in grape: Primary *versus* secondary metabolism. *Journal of Agricultural and Food Chem*istry, **53**:9133-9139.

**Iyer M. M., G. L. Sacks and O. I. Padilla-Zakour (2012).** Assessment of the validity of maturity metrics for predicting the volatile Composition of Concord grape Juice. Journal of Food Science, **77(3)**:C319–C325. DOI: 10.1111/j.1750-3841.2011.02592.x

**Jackson D. I. (1986).** Factors affecting soluble solids, acid, pH, and color in grapes. *American Journal of Enology and Viticulture*, **37(3)**:179-183.

**Jaillon O., J. M. Aury, B. Noel, A. Policriti, C. Clepet, A. Casagrande, N. Choisne, S. Aubourg, N. Vitulo, C. Jubin, A. Vezzi, F. Legeai, P. Hugueney, C. Dasilva, D. Horner, E. Mica, D. Jublot, J. Poulain, C. Bruyère, A. Billault, B. Segurens, M. Gouyvenoux, E. Ugarte, F. Cattonaro, V. Anthouard, V. Vico, C. Del Fabbro, M. Alaux, G. Di Gaspero, V. Dumas, N. Felice, S. Paillard, I. Juman, M. Moroldo, S. Scalabrin, A. Canaguier, I.**

**Le Clainche, G. Malacrida, E. Durand, G. Pesole, V. Laucou, P. Chatelet, D. Merdinoglu, M. Delledonne, M. Pezzotti, A. Lecharny, C. Scarpelli, F. Artiguenave, M. E. Pè, G. Valle, M. Morgante, M. Caboche, A. F. Adam-Blondon, J. Weissenbach, F. Quétier, P. Wincker (2007).** The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature*, **449(7161)**:463-467.

**Jeandet P., R. Bessis, M. Sbaghi and P. Meunier (1995).** Production of the phytoalexin resveratrol by grapes as a response to *Botrytis* attack under natural conditions. *Journal of Phytopathology,* **143**:135-139.

**Jeffries P., J. C. Dodd, M. J. Jeger and R. A. Plumbley (1990).** The biology and control of *Colletotrichum* species on tropical fruit crops. *Plant Pathology*, **39(3)**:343–366.DOI: 10.1111/j.1365-3059.1990.tb02512.x

**Jensen J. S., S. Demiray, M. Egebo and A. S. Meyer (2008).** Prediction of Wine Color Attributes from the Phenolic Profiles of Red Grapes (*Vitis vinifera*). *Journal of Agricultural Food Chemistry*, **56(3)**:1105–1115

**Joosten H. M. L. J., J. Goetz, A. Pittet, M. Schellenberg and P. Bucheli (2001).** Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. *International Journal of Food Microbiology,* **65**:39-44.

**Jørgensen K. (1998).** Survey of pork, poultry, coffee, beer and pulses for ochratoxin A. *Food Additives and Contaminants,* **15**:550-554.

**Kamel B., H. Dawson and Y. Kakuda (1985).** Characteristics and composition of melon and grapes seed oil and cakes. *Journal of the American Chemical Society*, **62**:881-883.

**Karlovsky P. (1999).** Biological detoxification of fungal toxins and its use in plant breeding, feed and food production. *Natural Toxins*, **7**:1-23.

**Keller M. (2010).** Managing grapevines to optimise fruit development in a challenging environment: a climate change primer for viticulturists. *Australian Journal of Grape and Wine Research*, **16**:56-69.

**Keller M., K. J. Arnink, G Hrazdina (1998).** Interaction of nitrogen availability during bloom and light intensity during veraison. I. Effects on grapevine growth, fruit development, and ripening. *American Journal of Enology and Viticulture*, **49(3)**:333-340.

**Kennedy J.A., C. Saucier and Y. Glories (2006).** Grape and wine phenolics: History and perspective. *American Journal of Enology and Viticulture*, **3**:20–21.

**Kennelly M. M., D. M. Gadoury, W. F. Wilcox, P. A. Magarey and R. C. Seem (2007).** Primary infection, lesion productivity, and survival of sporangia in the grapevine downy mildew pathogen *Plasmopara viticola*. *Phytopathology,* **97**: 512-522.

**Keshri G. and N. Magan (2000).** Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of two *Fusarium* spp. using volatile production profiles and hydrolytic enzymes. *Journal of Applied Microbiology,* **89(5)**:825-833. DOI: 10.1046/j.1365-2672.2000.01185.x

**Kim J., J. Lim and C. Lee (2013).** Quantitative real-time PCR approaches for microbial community studies in wastewater treatment systems: Applications and considerations. *Biotechnology Advances*, **(in Press)**. DOI: 10.1016/j.biotechadv.2013.05.010.

**Klich M. A. (2007).** *Aspergillus flavus*: the major producer of aflatoxin. *Molecular Plant Pathology,* **8(6)**:713-722.

**Klich M. A., and J. I. Pitt (1988).** Differentiation of *Aspergillus flavus* from *Aspergillus parasiticus* and other closely related species. *Transactions of the British Mycological Society*, **91:**99–108.

**Klich M. A., E. J. Mullaney, C. B. Daly and J. W. Cary (2000).** Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *A. tamarii* and *A. ochraceoroseus*. *Applied and Environmental Microbiology*, **53:**605–609.

**Kliewer W. M., L. Howarth and M. Omori (1967)**. Concentrations of Tartaric Acid and Malic Acids and Their Salts in *Vitis Vinifera* Grapes. *American Journal Enology Viticulture*, **18(1)**:42-54.

**Kowalchuk G. A., J. R. Stephen, W. De Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp. (1997).** Analysis of ammonia-oxidizing bacteria of the β-subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR amplified 16S ribosomal DNA fragments. *Applied Environmental Microbiology*, **63**: 1489-1497.

**Kriedemann P. E. (1968).** Photosynthesis in vine leaves as a function of light intensity, temperature, and leaf age. *Vitis*, **7**:213-220.

**Krivobok S., F. Seigle-Murandi, R. Steiman and E. E. Creppy (1995).** Fungal flora and ochratoxin A production in various food and feed in France. *Systematic and Applied Microbiology,* **18**:455-459.

**Krogh P., B. Hald and E. J. Pedersen (1973).** Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxic porcine nephropathy. *Acta Pathologica et Microbiologica Scandinavica,* **81**:689-695.

**Kuiper-Goodman T. (1996).** Risk assessment of ochratoxin A: an update. *Food Additives and Contaminants,* **13**:53-57.

**Kuiper-Goodman T. (1998).** Food safety: mycotoxins and phycotoxins in perspective. *In:* Miraglia M., H. van Egmond, C. Brera and J. Gilbert (Eds), *Mycotoxins and phycotoxins-developments in chemistry, toxicology and food safety*. *Proceedings of the IX IUPAC, International Symposium*., Fort Collins, Alaken Inc, Colorado, USA, pp. 25-48.

**Kuiper-Goodman T. and P. M. Scott (1989).** Risk assessment of the mycotoxin ochratoxin A. *Biomedical and Environmental Sciences* **2**:179-248.

**Kumar A. and A.K. Purohi (2012).** The Role of Indigenous Knowledge in Biological Control of Plant Pathogens: Logistics of New Research Initiatives. pp, 161-194. *In:* Mérillon J. M. and K. G. Ramawat (Eds), Plant Defence: Biological Control. Springer Dordrecht Heidelberg London New York. DOI: 10.1007/978-94-007-1933-0

**Kupina S. A., C. A. Pohl and J. L. Gannotti (1991).** Determination of Tartaric, Malic, and Citric Acids in Grape Juice and Wine Using Gradient Ion Chromatography. *American Journal Enology Viticulture*, **42(1)**:1-5.

**Kurrein M. B., G. H. Green, and S. L. Rowles (1975).** Localized deposition of calcium oxalate around a pulmonary *Aspergillus niger* fungus ball. *American Journal of Clinical Pathology,* **64**:556-563.

**Kurtzman C. P., B. W. Horn and C. W. Hesseltine (1987).** *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamari. Antonie van Leeuwenhoek,* **53(3)**:147-158

**Kuske M., A. C. Romain and J. Nicolas (2005).** Microbial volatile organic compounds as indicators of fungi. Can an electronic nose detect fungi in indoor environments? *Building and Environment*, **40(6)**:824-831.

**Laforgue R., L. Guérin, J. J. Pernelle, C. Monnet, J. Dupont and M. Bouix (2009)**. Evaluation of PCR-DGGE methodology to monitor fungal communities on grapes. *Journal of Applied Microbiology*, **107**:1208-1218. DOI: 10.1111/j.1365-2672.2009.04309.x

**Landry M. M. and C. W. Parkins (1993).** Calcium oxalate crystal deposition in necrotizing otomycosis caused by *Aspergillus niger*. *Modern Pathology,* **6**:493-496.

**Larcher R. and G. Nicolini (2001).** Survey of ochratoxin A in musts, concentrated musts and wines produced or marketed in Trentino (Italy). *Journal Commodity Science*, **40**: 69-78.

**Larsen T. O., A. Svendsen and J. Smedsgaard (2001).** Biochemical characerization of ochratoxin A-producing strains of the genus *Penicillium*. *Applied and Environmental Microbiology,* **67**:3630-3635.

**Legarda T. M. and P .A. Burdaspal (1998).** Ocratoxina A en cervezas elaboradas en España y otros países europeos. *Alimentaria,* **291**:115-122.

**Legarda T. M. and P. A. Burdaspal (2001).** Presencia de ocratoxina A en muestras de pan comercializado en España y en muestras procedentes de doce países extranjeros. *Alimentaria,* **321**:89-96.

**Leistner L. and W. Rodel (1976).** The stability of imtermediate moisture foods with respect to microorganisms. *In*: Davies R., G. G. Birch and K. J. Parker (Eds) Intermediate Moisture Foods. London: Applied Science Publishers, pp. 120-137.

**León K., D. Mery, F. Pedreschi and J. León (2006).** Color measurement in *L*a*b** units from RGB digital images. *Food Research International*, **39(10)**:1084-1091.

**Leong S., A. D. Hocking, and J. I. Pitt (2004).** Occurrence of fruit rot fungi (*Aspergillus* section *Nigri*) on some drying varieties of irrigated grapes. *Australian Journal of Grape and Wine Research,* **10**:83-88.

**Leoni L. A. B., L. M. Valente Soares and P. L. C. Oliveira (2000).** Ochratoxin A in Brazilian roasted and instant coffees. *Food Additives and Contaminants*, **17(10)**:867-870.

**Letutour B., A. Tantaoui-Elaraki and L. Ihlal (1983).** Simultaneous detection of aflatoxin $B_1$ and ochratoxin A in olive oil. *Journal of the American Oil Chemists Society,* **60**:835-837.

**Levi C. P., H. Trenk and H. Mohr (1974).** Study of the occurrence of ochratoxin A in green coffee beans. *Journal AOAC,* **57**:866-870.

**Li S., R. R. Marquardt, A. A. Frohlich, T. G. Vitti and G. Crow (1997).** Pharmacokinetics of ochratoxin A and its metabolites in rats. *Toxicology and Applied Pharmacology,* **145**:82.

**Lima G., F. De Curtis and V. De Cicco, (2008).** Interaction of microbial biocontrol agents and fungicides in the control of postharvest diseases. *Stewart Postharvest Review*, **1**:1-7.

**Lima G., R. Castoria, F. De Curtis, A. Raiola, A. Ritieni and V. De Cicco (2011).** Integrated control of blue mould using new fungicides and biocontrol yeasts lowers levels of fungicide residues and patulin contamination in apples. *Postharvest Biology and Technology,* **60**:164-172. DOI: 10.1016/j.postharvbio.2010.12.010.

**Livak K. J., S. P. A. Flood, J. Marmejo, W. Giusti, and K. Deetz (1995).** Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR products and nucleic acid hybridization. *PCR Methods and Applications*, **4**:357-362.

**Lo Curto R., T. Pellicano, F. Vilasi, P. Munafo and G Dugo (2004).** Ochratoxin A occurrence in experimental wines in relationship with different pesticide treatments of grapes. *Food Chemistry*, **84**:71-75.

**López P., C. Sánchez, R. Batlle and C. Nerín (2005).** Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. *Journal of Agricultural and Food Chemistry,* **53(17)**:6939-46.

**Mabrouk H., A. Carbonneau and H. Sinoquet (1997).** Canopy structure and radiation regime in grapevine. I. Spatial and angular distribution of leaf area in two canopy systems. *Vitis*, **36**:119-123.

**Mac Donald S. P. Wilson, K. Barnes, A. Damant, R. Massey, E. Mortby and M.J. Shephered (1999).** Ochratoxin A in dried vine fruit: method development and survey. *Food Additives and Contaminants*, **16**:253-260.

**Madhyasta S. M., R. R. Marquardt, A. A. Frohlich, G. Platford and D. Abramson (1990).** Effect of different cereal and oilseed substrates on the growth and production of toxins by *Aspergillus* alutaceus and *Penicillium verrucosum*. *Journal of Agricultural and Food Chemistry,* **38**:1506-1510.

**MAFF, Ministry of Agriculture, Fisheries and Food. (1999).** Food surveillance information sheets No. 185. 1998 survey of retail products for ochratoxin A. UK.

**Magarey R. D., R. W. Emmett, P. A. Magarey and P. R. Franz (1993).** Evaluation of control of grapevine anthracnose caused by *Elsinoe ampelina* by pre-infection fungicides. *Australasian Plant Pathology*, **22(2)**:48-52.

**Magnoli C., A.Astoreca, L. Ponsone, M. Combina, G. Palacio, C. A. R. Rosa, and A. M. Dalcero (2004).** Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Letters in Applied Microbiology,* **39**:326-331.

**Magnoli C., M. Violante, M. Combina, G. Palacio and A. Dalcero (2003).** Mycoflora and ochratoxin-producing strains of Aspergillus section Nigri in wine grapes in Argentina. *Letters in Applied Microbiology,* **37(2)**:179-184.

**Maier T., A. Schieber, D. R. Kammerer, R. Carle (2009).** Residues of grape (*Vitis vinifera* L.) seed oil production as valuable source of phenolic antioxidants. *Food Chemistry*, **116**:551-559.

**Majerus P. and H. Otteneder (1996).** Nachweis und vorkommen von ochratoxin A in wein und traubensaft. *Deutsche Lebensmittel-Rundschau,* **92**:338-390.

**Majerus P., H. Bresch and H. Otteneder (2000).** Ochratoxin A in wines, fruit juices and seasonings. *Archiv für Lebensmittelhygiene,* **51**:81-128.

**Majerus P., I. Cutka, A.Dreyer, S. El-Dessouki, W. Eyrich, H. Reusch, B. Schurer and H. U. Waiblinger (1993).** The burden of OTA in foods and plant origin. *Deutsche Lebensmittel-Rundschau,* **89**:112-114.

**Maldonado M C and A M Strasser de Saad (1998).** Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems. *Journal of Industrial Microbiology and Biotechnology*, **20(1)**:34-38.

*References*

**Maldonado M. C. and D. A. S. Callieri (1989).** Influence of environmental conditions on the production of pectinesterase and polygalacturonase by *Aspergillus niger*. *MIRCEN Journal of Applied Microbiology and Biotechnology*, **5(3)**:327-333.

**Manderville R. A. and A. Pfohl-Leszkowicz (2008).** Bioactivation and DNA adduction as a rationale for ochratoxin A carcinogenesis. *World Mycotoxin Journal*, **1**:357-367.

**Mantle P.G. (2002).** Risk assessment and the importance of ochratoxins. *International Biodeterioration and Biodegradation,* **50**:143-146.

**Marasas W. F. O., J. D. Miller, R. T. Riley and A. Visconti (2001).** Fumonisins-occurrence, toxicology, metabolism and risk assessment, p, 332-359. *In* B. A. Summerell J. F. Leslie, D. Backhouse, W. L. Bryden and L. W. Burgess (Eds), *Fusarium*. Paul E. Nelson Memorial Symposium. APS Press, St. Paul, Minn.

**Marengo, E, M Aceto, V Maurino (2001).** Classification of Nebbiolo-based wines from Piedmont (Italy) by means of solid-phase microextraction-gas chromatography-mass spectrometry of volatile compounds. *Journal of Chromatography A*, **943**:123-137.

**Margaritis E. and M. Jones (2006).** Beyond cereals: crop processing and *Vitis vinifera* L. Ethnography, experiment and charred grape remains from Hellenistic Greece. *Journal of Archaeological Science*, **33**(6):784–805

**Markaki P., C. Delpont-Binet, F. Grosso and S. Dragacci (2001).** Determination of Ochratoxin A in red wine and vinegar by immunoaffinity high-pressure liquid chromatography. *Journal of Food Protection,* **64**: 533-537.

**Martelli G. P. (1993).** Graft-transmissible Diseases of Grapevines: Handbook for Detection and Diagnosis. FAO, Rome.

**Martin K. J. and P. T. Rygiewicz (2005).** Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology*, **5**:28-38. DOI: 10.1186/1471-2180-5-28

**Martínez-Culebras P.V. and D. Ramón (2007).** An ITS-RFLP method to identify black Aspergillus isolates responsible for OTA contamination in grapes and wine. *International Journal of Food Microbiology,* **113(2)**:147-153.

**Masoud W. and C. H. Kaltoft (2006).** The effects of yeasts involved in the fermentation of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*. *International Journal of Food Microbiology*, **106**:229-234.

**Mato I., S. Suárez-Luque and J. F. Huidobro (2006).** Simple determination of main organic acids in grape juice and wine by using capillary zone electrophoresis with UV detection. *Food Chemistry,* **102:104**-112.

**Matthews M. A. and K. A. Shackel (2005).** Growth and water transport in fleshy fruit. *In: Holbrook N.M. and Zwieniecki M.A. (Eds.).* Vascular Transport in Plants. Elsevier Academic Press, Burlington, MA. pp. 181-195.

**Medina A., G. González, J. M. Sáez, R. Mateo, M. Jiménez (2004).** Bee pollen, a substrate that stimulates ochratoxin A production by *Aspergillus ochraceus* Wilh. *Systematic and Applied Microbiology,* **27**:261-267.

**Medina A., R. Mateo, L. López-Ocaña, F. M. Valle-Algarra and M. Jiménez (2005).** Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. *Applied and Environmental Microbiology,* **71**:4696-4702.

**Meng X. C., Y. X. Tang, A. Y. Zhang, X. M. Huang and Z. Q. Zhang (2012).** Effect of oligochitosan on development of *Colletotrichum musae in vitro* and *in situ* and its role in protection of banana fruits. *Fruits*, **67**:147-155.

**Meulenberg E. P. (2012).** Immunochemical Methods for Ochratoxin A Detection: A Review. *Toxins*, **4**:244-266; DOI: 10.3390/toxins4040244

**Miller J. D. (1998).** Global significance of mycotoxins. In: Miraglia M., H. van Egmond, C. Brera and J. Gilbert (Eds) Mycotoxins and Phycotoxins Developments in Chemistry, Toxicology and Food Safety Alaken Inc., Ford Collins, Colorado. 1998, pp.3-15.

**Miller J. D. and J. C. Young (1997).** The Use of Ergosterol to Measure Exposure to Fungal Propagules in Indoor Air. *American Industrial Hygiene Association Journal*, **58(1)**: 39-43. DOI: 10.1080/15428119791013062

**Mills L. J., J. C. Ferguson and M. Keller (2006).** Cold-hardiness evaluation of grapevine buds and cane tissues. *American Journal of Enology and Viticulture,* **57**:194-200.

**Molot B. and D. Solanet (2003).** Ochratoxine: Prévention du risque. In *Etude au vignoble de fongicides actifs contre Aspergillus carbonarius incidences sur la présence aux vendanges*, Proceedings of Les Entretiens Viti-Vinicoles Rhone-Mediterranée, Nimes, France, 16 April 2003; pp. 18–21.

**Moss M. O. (1996).** Mode of formation of ochratoxin A. *Food Additives and Contaminants*, **13**:5-9.

**Moss M. O. (1998).** Recent studies of mycotoxins. *Symposium series (Society for Applied Microbiology)*, **84**:62S-76S.

**Mostert L., P. W. Crous, J. C. Kang and A. J. L. Phillips (2001).** Species of *Phomopsis* and a *Libertella* sp. occurring on grapevines with specific reference to South Africa:

morphological, cultural, molecular and pathological characterization. *Mycologia*, **93(1)**:146-167.

**Mounjouenpou P., N. Durand, J. P. Guiraud, S. A. M. Tetmoun, D. Gueule and B. Guyot (2013).** Assessment of Exposure to Ochratoxin -A (OTA) through Ground Roasted Coffee in two Cameroonian Cities: Yaounde and Douala. *International Journal of Food Science and Nutrition Engineering*, **3(3)**:35-39. DOI: 10.5923/j.food.20130303.03.

**Mühlencoert E., I. Mayer, M. W. Zapf, R. F. Vogel and L. Niessen (2004).** Production of ochratoxin A by *Aspergillus ochraceus*. *European Journal of Plant Pathology,* **110**:651-659.

**Mulè G., A. Susca, A. Logrieco, G. Stea and A. Visconti (2006).** Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. *International Journal of Food Microbiology*, **111**:28-34.

**Müller H. M. (1982).** Decontamination of mycotoxins. I. Physical process. *Übersichten zur Tierernährung*, **10**:95-122.

**Mullins M. G., A. Bouquet and L. E. Williams (2003).** Biology of the grapevine. Cambridge University press, Cambridge, United Kingdom.

**Murphy P. A., S. Hendrich, C. Landgren, and C. M. Bryant (2006).** Food Mycotoxins: An Update. *Journal of Food Science*, **71(5)**: R51-R65. DOI: 0.1111/j.1750-3841.2006.00052.x.

**Muyzer G., A. Teske, C.O. Wirsen and H.W. Jannasch (1995).** Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent sample by denaturing gradient gel electrophoresis of 16S rDNA fragment. *Archives of Microbiology,* **164**:165-172.

**Nakajima M., H. Tsubouchi, M. Miyabe and Y. Ueno (1997).** Survey of aflatoxin $B_1$ and ochratoxin A in commercial green coffee beans by high-performance liquid chromatography linked with immunoaffinity chromatography. *Food and Agricultural Immunology,* **9**:77-83.

**Nally M. C., V. M. Pesce, Y. P. Maturano, C. J. Muñoz, M. Combina, M. E. Toro, L. I. C. de Figueroa, and F. Vazquez (2012).** Biocontrol of *Botrytis cinerea* in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina. *Postharvest Biology and Technology*, **64**:40-48.

**Nally M. C., V. M. Pesce, Y. P. Maturano, M. E. Toro, M. Combina, L. I. C. de Figueroa and F. Vazqueza (2013).** Biocontrol of fungi isolated from sour rot infected table grapes bySaccharomyces and other yeast species. *Postharvest Biology and Technology*, **86**:456-462

**Navarre C. (1994).** L'œnologie. Agriculture d'Aujourd'hui, 3$^{e}$ edition, LAVOISIER. Paris.

**Navarre C. and F. Langlade (2002).** L'œnologie, 5e edition. Edition TEC & DOC, Lavoisier, Paris.

**Navarrete F., E. Abreo, L. Bettucci, S. Martínez and S. Lupo (2009).** First report of *Greeneria uvicola* as cause of grapevine dead-arm dieback in Uruguay. *Australasian Plant Disease Notes*, **4**:117-119.

**Nelson P. E., A. E. Desjardins and R. D. Plattner (1993).** Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry and significance. *Annual Review of Phytopathology,* **31:**233–252.

**Nesheim S.; N. F. Hardin, O. I. Francis, W. S. Langham (1973).** Analysis of ochratoxin A and B and their esters in barley: Using partitions and thin-layer chromatography. I Development of the method. *Journal of AOAC International*, **56**:817-821.

**Nielsen K. F. (2003).** Mycotoxin production by indoor moulds. *Fungal Genetic and Biology,* **39**:103-117.

**Nilsson R. H., L. Tedersoo, K. Abarenkov, M. Ryberg, E. Kristiansson, M. Hartmann, C. L. Schoch, J. A. A. Nylander, J. Bergsten, T. M. Porter, A. Jumpponen, P. Vaishampayan, O. Ovaskainen, N. Hallenberg, J. Bengtsson-Palme, K. M. Eriksson, K. H. Larsson, E. Larsson, U. Kõljalg (2012).** Five simple guidelines for establishing basic authenticity and reliability of newly generated fungal ITS sequences. *MycoKeys*, **4**:37-63. DOI: 10.3897/mycokeys.4.3606.

**O'Brien E. and D. R. Dietrich (2005).** Ochratoxin A: the continuing enigma. *Critical Reviews in Toxicology,* **35**:33-60.

**OIV (2009).** Recueil International des Méthodes d'Analyses. O. I. V.: Paris, France, 2, 476.

**Ojeda H., A. Deloire and A. Carbonneau (2001).** Influence of water deficits on grape berry growth. *Vitis*, **40(3)**:141-145.

**Oliva J, S Navarr, G Navarro, M.A Cámara and A Barba (1999).** Integrated control of grape berry moth (*Lobesia botrana*), powdery mildew (*Uncinula necator*), downy mildew (*Plasmopara viticola*) and grapevine sour rot (*Acetobacter* spp.). *Crop Protection*, **18(9)**:581-587

**Ollat N. and J. P. Gaudillère (1996).** Investigation of assimilate import mechanisms in berries of *Vitis vinifera* var. 'Cabernet Sauvignon'. *Acta Horticulture*, **427**:141-149.

**Ollat N., P. Diakou-Verdin, J. P Carde, F. Barrieu, J. P. Gaudillère and A. Moing (2002).** Grape berry development: a review. *Journal of International Science of Vigne and Vin*, **36**:109-131.

*References*

**Olsson J., T. Börjesson, T. Lundstedt and J. Schnürer (2002).** Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. *International Journal of Food Microbiology*, **72(3)**:203–214

**Ong B. Y. and C. W. Nagel (1978).** Hydroxycinnamic Acid-Tartaric Acid Ester Content in Mature Grapes and During the Maturation of White Riesling Grapes. *American Journal Enology Viticulture*, **29(4):**277-281

**Ono H., A. Kataoka, M. Koakutsu, K. Tanaka, S. Kawasugi, M. Wakazawa, Y. Ueno and M. Manabe (1995).** Ochratoxin A producibility by strains of *Aspergillus niger* group stored in IFO culture collection. *Mycotoxins,* **41**:47-51.

**Ospital M., J. M. Cazabeil, A. M. Betbeder, C. Tricard, E. E. Creppy and B. Medina (1998).** L'ochratoxin A dans les vins. *Revue Française d'Oenologie,* **169**:16-18.

**Ostry V., J. Skarkova, F.Malir, and S. Sycorova (2004).** Advances on the occurrence of toxigenic fungi and mycotoxins in the Czech Republic. *In:* Logrieco A. and A. Visconti (Eds.), An overview on toxigenic fungi and mycotoxins in Europe (pp. 67-81). Amsterdam: Kluwer Academic Publishers.

**Otteneder H. and P. Majerus (2001).** Ochratoxin A in coffee: nation-wide evaluation of data collected by German food control 1995-1999. *Food Additives and Contaminants,* **18**:431-435.

**Paillioti A., A. Cartechini and F. Ferranti (2000).** Morpho-anatomical and physiological characteristics of primary and lateral shoot leaves of Cabernet Franc and Trebbiano Toscano grapevines under two irradiance regimes. *American Journal of Enology and Viticulture,* **51**:122-130.

**Paldrok H. (1965).** Report on a case of subcutaneous dissemination of *Aspergillus niger* type awamori. *Acta Dermato-Venereologica*, **45**:275-282.

**Pandey K. D., N. N. Tripathi, D. R. Tripathi and S. N. Dixit (1982).** Fungitoxic and phytotoxic properties of essential oils of *Hyptis suaveolens*. *Zeits Pflanzenkrank Pflanzenschutz*, **89**:344-349.

**Paolesse R., A. Alimelli, E. Martinelli, C. Di Natale, A. D'Amico, M. G. D'Egidio, G. Aureli, A. Ricelli and C. Fanelli (2006)**. Detection of fungal contamination of cereal grain samples by an electronic nose. *Sensors and Actuators B: Chemical*, **119(2)**:425-430.

**Papachristou A. and P. Markaki (2004).** Determination of ochratoxin A in virgin olive oils of Greek origin by immunoaffinity column clean-up and high-performance liquid chromatography. *Food Additives and Contaminants,* **21**:85-92.

**Pardo E., S. Marín, A. J. Ramosand V. Sanchis (2004).** Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different origins. *Food Science and Technology International,* **10**:45-49.

**Parenicová L., P. Skouboe, J. C. Frisvad, R. A.Samson, L. Rossen, , M. ter Hoor-Suykerbuyk and J. Visser (2001).** Combined molecular and biochemical approach identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as two species. *Applied and Environmental Microbiology,* **67**:521-527.

**Patel S., C. M.Hazel, A. G. M. Winterton and A. E. Gleade (1996).** Survey of ethnic foods for mycotoxins. *Food Additives and Contaminants,* **13**:833-841.

**Pathan A. K., J. Bond and R. E. Gaskin (2008).** Sample preparation for scanning electron microscopy of plant surfaces-Horses for courses. *Micron*, **39**:1049-1061.

**Patiñoa B., A. González-Salgadob, M. T. González-Jaénb and C. Vázquez (2005).** PCR detection assays for the ochratoxin-producing *Aspergillus carbonarius* and *Aspergillus ochraceus* species. *International Journal of Food Microbiology*, **104(2)**:207-214.

**Pearson R. C. and Gadoury. D. M. (1992).** Powdery mildew of grape. In: Kumar J., H. S. Chaube, U. S. Singh and A. N. Mukhopadhyay, (Eds.). Plant diseases of international importance. Vol. 3. Diseases of fruit crops. Prentive Hall, Englewood Cliffs, N.J. pp. 129-146.

**Pedneault K., M. Dorais and P. Angers (2013).** Flavor of Cold-Hardy Grapes: Impact of Berry Maturity and Environmental Conditions. Journal of Agricultural and Food Chemistry (in press). DOI: 10.1021/jf402473u

**Pelegrinelli-Fungaro M. H.; P. C. Vissoto, D. Sartori, L. A. Vioas-Boas, M. C. Furlaneto, and M. H. Taniwaki (2004).** A molecular method for detection of *Aspergillus carbonarius* in coffee beans. *Current Microbiology*, **49**:123-127.

**Peraica M. and A. M. Dominjan (2001).** Contamination of food with mycotoxins and human health. *Arhiv za Higijenu Rada i Toksikologiu,* **52**:23-35.

**Pereira G. E., J.-P. Gaudillere, P. Pieri, G. Hilbert, M. Maucourt, C. Deborde, A. Moing and D. Rolin (2006).** Microclimate Influence on Mineral and Metabolic Profiles of Grape Berries. *Journal of Agricultural and Food Chemistry*, **54(18)**:6765–6775.

**Pérez de Obanos Castillo J. J. (2007).** Champignons: Oïdium (*Uncinula necator* burr.) Groupe de travail de la vigne, les parasites de la vigne. Dunod, Paris, 205-211.

**Pérez Marín J. L. (2007).** Champignons: Mildiou (*Plasmopara viticola* Berl. Et de Toni) Groupe de travail de la vigne, les parasites de la vigne. Dunod, Paris, 193-205.

*References*

**Perrone G., A. Susca, F. Epifani and G. Mulè (2006b).** AFLP characterization of Southern Europe population of *Aspergillus* sect. *Nigri* from grapes. *International Journal of Food Microbiology* **111**: S22–S27.

**Perrone G., A. Susca, G. Cozzi, K. Ehrlich, J. Varga, J .C. Frisvad, M. Meijer, P. Noonim, W. Mahakarnchanakul and R .A.Samson (2007).** Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology,* **59**:53-66.

**Perrone G., A. Susca, G. Stea and G. Mulè (2004).** PCR assay for identification of *Aspergillus carbonarius* and *Aspergillus japonicus*. *European Journal of Plant Pathology*, **110**:641-649.

**Perrone G., G. Mulè, A. Susca, P. Battilani, A. Pietri and A. Logrieco (2006a).** Ochratoxin A production and AFLP analysis of *Aspergillus carbonarius, Aspergillus tubingensis,* and *Aspergillus niger* strains isolated from grapes in Italy. *Applied and Environmental Microbiology* **72**: 680–685.

**Pestak J. J. and W. L. Casale (1990).** Mycotoxin in food chain: the role of ochratoxins. *Livestock Production Science*, **76**:245-250.

**Peterson S. W. (2000).** Phylogenetic relationship in *Aspergillus* based upon rDNA sequence analysis. In: *Integration of modern taxonomic methods for Penicillium and Aspergillus classification*. Samson, R.A. and Pitt, J.I. (Eds.). Harwood Academic Publishers, Amsterdam, The Netherlands, 323-355.

**Peterson S. W., Y. Ito, B. W. Horn and T. Goto (2001).** *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. *Mycologia,* **93:**689-703.

**Petzinger E. and K. Ziegler (2000).** Ochratoxin A from a toxicological perspective. *Journal of Veterinary Pharmacology and Therapeutics,* **23**:91-98.

**Pfohl-Leszkowicz A. and R. A. Manderville (2007).** Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Molecular Nutrition and Food Research,* **51**:61-99.

**Pietri A., T. Bertuzzi, L. Pallaroni and G. Piva (2001).** Occurrence of ochratoxin A in Italian wines. *Food Additives and Contaminants,* **18**:647-654.

**Pinelo M., A. Arnous and A. S. Meyer (2006).** Upgrading of grape skins: significance of plant cell-wall structural components and extraction techniques for phenol release.*Trends Food Science Technology*, **17**:579–590.

**Pitt J. I. (1987).** *Penicillium viridicatum*, *Penicillium verrucosum*, and production of ochratoxin A. *Applied and Environmental Microbiology,* **53**:266-269.

*References*

**Pitt J. I. and A. D. Hocking (1997).** Fungi and Food Spoilage, 2nd edn. Blackie Academic and Professional, London.

**Pitt J. I. and A. D. Hocking (2009).** Fungi and Food Spoilage. 3rd edition. Springer Dordtecht Heidelberg London New York. DOI: 10.1007/978-0-387-92207-2.

**Ponsone M. L., M. L. Chiotta, J. M. Palazzini, M. Combina and S. Chulze (2012).** Control of ochratoxin A production in grapes. *Toxins*, **4**:364-372; DOI: 10.3390/toxins4050364

**Ponsone M. L., M. L. Chiotta, J. M. Palazzini, M. Combina and S. Chulze (2012).** Control of ochratoxin A production in grapes. *Toxins*, **4**:364-372.

**Ponsone M. L., M. L. Chiotta, M. Combina, A. Dalcero and S. Chulze (2011).** Biocontrol as a strategy to reduce the impact of ochratoxin A and *Aspergillus* section Nigri in grapes. *International Journal of Food Microbiology,* **151**:70–77.

**Pool R. M., R. C. Pearson, M. J. Welser, A. N. Lakso and R. C. Seem (1984).** Influence of powdery mildew on yield and growth of Rosette grapevines. *Plant Disease*, **68**:590-593.

**Pozo-Bayón M. A., A. Ruíz-Rodriguez, K. Pernin and N. Cayot (2007)** Influence of eggs on the aroma composition of a sponge cake and on the aroma release in model studies on flavored sponge cakes. *Journal of Agricultural and Food Chemistry*, **55**:1418-1426.

**Prakitchaiwattana C. J., G. H. Fleet and G. M. Heard (2004).** Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Research*, **4**:865-877.

**Pratt C. (1971).** Reproductive anatomy in cultivated grapes - a review. *American Journal of Enology and Viticulture,* **22**:92-109.

**Pratt C. (1971).** Reproductive anatomy in cultivated grapes - a review. *American Journal of Enology and Viticulture,* **22**:92-109.

**Pratt C. (1974).** Vegetative anatomy of cultivated grapes - a review. *American Journal of Enology and Viticulture*, **25**:131-150.

**Prosser J. I. (1995).** Kinetics of filamentous growth and branching. In: *The growing fungus*. Gow, N.A.R. and Gadd, G.M. (Eds.). Chapman and Hall, London, UK, 301-318.

**Puisais J. (1984).** Sur les chemins des vignobles de France. Sélectiondu Reader's Digest, Paris.

**Quirce S., M. Fernandez-Nieto, B. Bartolomé, C. Bombin, M. Cuevas, and J. Sastre (2002).** Glucoamylase: another fungal enzyme associated with baker's asthma. *Annals of Allergy, Asthma and Immunology,* **89**:197-202.

**Ramamurthy V., S. Raveendran, S. Thirumeni and S. Krishnaveni (2012).** Antimicrobial activity of Heterocytic Cyanobacteria. *International Journal of Advanced Life Sciences*, **1**:32-39.

**Rapp A. and G. Versini (1996).** Influence of nitrogen compounds in grapes on aroma compounds of wines. *Wein-Wissenschaft*, **51(3-4)**:133-222.

**Reuveni M. and R. Reuveni (1995).** Efficacy of foliar applications of phosphates in controlling powdery mildew fungus on field-grown wine grapes: Effects on cluster yield and peroxidase activity in berries. *Journal of Phytopathology*, **143**:21-25.

**Reverberi M., A. Ricelli, S. Zjalic, A. A. Fabbri and C. Fanelli (2010).** Natural functions of mycotoxins and control of their biosynthesis in fungi. *Applied Microbiology and Biotechnology,* **87(3)**:899-911.

**Reynier A. (2003).** Manuel de Viticulture 9e édition. Lavoisier, Edition TEC &DOC, Paris.

**Rheeder J. P., W. F. Marasas and H. F. Vismer (2002).** Production of fumonisin analogs by *Fusarium* species. *Applied and Environmental Microbiology*, **68:**2102-2105.

**Ribeiro E. L., M. L. Scroferneker, M. S. Cavalhaes, C. C. Campos, G. M. Nagato, N. A. Souza, W. M. Ferreira, C. G. Cardoso, S. M. S. Dias, F. C. Pimenta and O. A. Toledo (2006).** Phenotypic aspects of oral strains of *Candida albicans* in children with Down's syndrome. *Brazilian Journal of Biolology*, **66(3)**:939-944.

**Rogiers S. Y., D. H. Greer, J. M. Hatfield, B. A. Orchard and M. Keller (2006).** Mineral sinks within ripening grape berries**.** *Vitis,* **45(3):**115-123.

**Rogiers S. Y., J. M. Hatfield, V. G. Jaudzems, R. G. White and M. Keller (2004).** Grape berry cv. Shiraz epicuticular wax and transpiration during ripening and preharvest weight loss. American *Journal of Enology and Viticulture*, **55**:121-127.

**Romero S.M., A. Patriarca, V. F. Pinto and G. Vaamonde (2007).** Effect of water activity and temperature on growth of ochratoxigenic strains of *Aspergillus carbonarius* isolated from Argentinean dried vine fruits. International Journal of Food Microbiology, **115(2)**:140-143.

**Rousseau J. (2004).** Ochratoxin A in wines: current knowledge – Mycotoxin and wine. *Vinidea.net Wine Internet Technical Journal*, 1-4.

**Ruiz-García Y. and E. Gómez-Plaza (2013).** Elicitors: A Tool for Improving Fruit Phenolic Content. *Agriculture,* **3**:33-52; DOI: 10.3390/agriculture3010033.

**Ruiz-Garcia Y., I. Romero Cascales, R. Gil-Muñoz, J. I. Fernandez-Fernandez, J. M. Lopez-Roca and Gómez-Plaza E. (2012).** Improving grape phenolic content and wine chromatic characteristics through the use of two different elicitors: Methyl Jasmonate *versus* Benzothiadiazole. *Journal of Agricultural and Food Chemistry*, **60**:1283-1290.

*References*

**Rumbou A., C. Gessler (2004).** Genetic dissection of a *Plasmopara viticola* population from a Greek vineyard in two consecutive years. *European Journal of Plant Pathology*, **110**:379-392.

**Saccani G., S. Gherardi, A. Trifirò, C. Soresi Bordini, M. Calza and C. Freddi (1995).** Use of ion chromatography for measurement of organic acids in fruit juices. *Journal of Chromatography A*, **706**:395-403.

**Sage L., D. Garon and F. Seigle-Murandi (2004).** Fungal microflora and ochratoxin A risk in french vineyards. *Journal of Agricultural and Food Chemistry*, **52**:5764-5768.

**Sage L., S. Krivobok, E. Delbos, F. Seigle-Murandi and E. E. Creppy (2002).** Fungal flora and ochratoxin A production in grapes and musts from France. *Journal of Agricultural and Food Chemistry*, **50**:1306-1311.

**Saito K and Z. Kasai (1968).** Accumulation of tartaric acid in the ripening process of grapes. *Plant Cell Physiology*, **9(3):**529-537.

**Samson R. A., E. S. Hoekstra, J. C. Frisvad and O. Filtenborg (1995).** Introduction to Food Borne fungi. 4th ed. Centraalnureau voor Schimmelcucutures, Netherlands.

**Samson R. A., J. A. M. P. Houbraken, A. F. A. Kuijpers, J. M. Frank and J. C. Frisvad (2004).** New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. *Studies in Mycology,* **50**:45-61.

**Samson R. A., P. Noonim, M. Meijer, J. Houbraken, J. C. Frisvad and J. Varga (2007).** Diagnostic tools to identify black aspergilli. Studies in Mycology, **59**:129-145. DOI: 10.3114/sim.2007.59.13

**Samuelian S. K., L. A. Greer, S. Savocchia, and C. C. Steel (2011).** Detection and monitoring of *Greeneria uvicola* and *Colletotrichum acutatum* development on grapevines by Real-Time PCR. *Plant Disease*, **95(3)**:298-303.

**Sánchez-Hervás M., J. V. Gil, F. Bisbal, D. Ramón, P. V. Martínez-Culebras (2008).** Mycobiota and mycotoxin producing fungi from cocoa beans. *International Journal of Food Microbiology*, **125**:336-340.

**Sánchez-Palomo E., M. C. Díaz-Maroto and M. S. Pérez-Coello (2005).** Rapid determination of volatile compounds in grapes by HS-SPME coupled with GC–MS. *Talanta*, **66**:1152-1157.

**Sarig P., Y. Zutkhi, A. Monjauze, N. Lisker and R. Ben-Arie (1997).** Phytoalexin elicitation in grape berries and their susceptibility to *Rhizopus stolonifer*. *Physiological and Molecular Plant Pathology,* **50**:337-347.

*References*

**Sauceda-Friebea J. C., X. Y.Z. Karsunkea, S. Vazaca, S. Bisellib, R. Niessnera and D. Knoppa (2011).** Regenerable immuno-biochip for screening ochratoxin A in green coffee extract using an automated microarray chip reader with chemiluminescence detection. *Analytica Chimica Acta*, **689(2)**:234-242.

**Saxena J., C. Munimbazi and L. B. Bullerman (2001).** Relationship of mould count, ergosterol and ochratoxin A production. *International Journal of Food Microbiology,* **69**:3996-3998.

**Schaller K., O. Löhnertz and V. Chikkasubbanna (1992).** Uptake of Potassium by the grape berries of different cultivars during growth and development. *Viticulture and Enology Science*, **47**:36-39.

**Schatzmayr G., D. Heidler, E. Fuchs, S. Nitsch, M. Mohnl, M. Täubel, A. P. Loibner, R. Braun and E. M. Binder (2003).** Investigation of different yeast strains for the detoxification of ochratoxin A. *Mycotoxin Research*, **19(2)**:124-128.

**Schatzmayr G., F. Zehner, M. Täubel, D. Schatzmayr, A. Klimitsch, A. P. Loibner and E. M. Binder (2006).** Microbiologicals for deactivating mycotoxins. *Molecular Nutrition and Food Research*, **50**,543-551. DOI: 10.1002/mnfr.200500181 543

**Schepers J. S. and E. Balázs (2007).** The mycotoxin threat to food safety. *International Journal of Food Microbiology*, **119**:1-2.

**Schmidt H., M. Ehrmann, R. F.Vogel, M. H. Taniwaki and L. Niessen (2003).** Molecular typing of *Aspergillus ochraceus* and construction of species specific SCAR-primers based on AFLP. *Systematic and Applied Microbiology*, **26**:434-438.

**Schmidt H., M. H.Taniwaki, R. F. Vogel and L. Niessen (2004).** Utilization of AFLP markers for PCR based identification of *Aspergillus carbonarius* and indication of its presence in green coffee samples. *Journal of Applied Microbiology,* **97**:899-909.

**Schmidt-Heydt M. and R. Geisen (2007).** A microarray for monitoring the production of mycotoxins in food. *International Journal of Food Microbiology*, **117(2)**:131-140.

**Schnürer J., J. Olsson and T. Börjesson (1999).** Fungal Volatiles as Indicators of Food and Feeds Spoilage. *Fungal Genetics and Biology*, **27(2-3)**:209-217.

**Schoch C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chenb and Fungal Barcoding Consortium (2012).** Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *PNAS*, **109(16)**: 6241-6246. DOI: 10.1073/pnas.1117018109.

**Schultz H. R., W. Kiefer and W. Gruppe (1996).** Photosynthetic duration, carboxylation efficiency and stomatal limitation of sun and shade leaves of different ages in field-grown grapevine (*Vitis vinifera* L.). *Vitis,* **35**:169-176.

**Scott P. M. and M. W. Trucksess (1997).** Application of immunoaffinity columns to mycotoxins analysis. *Journal of AOAC International,* **80**:941-949.

**Scott P. M. and S. R. Kanhere (1995).** Determination of ochratoxin A in beer. *Food Additives and Contaminants,* **12**:591-598.

**Scott P. M., W. van Walbeek, B. Kenedy and D. Anyeti (1972).** Mycotoxins and toxigenic fungi in grains and other agricultural products. *Journal of Agricultural and Food Chemistry,* **20**:1103-1109.

**Selma M. V., P. M. Freitas, L. Almela, R. González-Barrio, J. C. Espín, T. Suslow, F. Tomás-Barberán and M. I. Gil (2008a).** Ultraviolet-C and induced stilbenes control ochratoxigenic *Aspergillus* in grapes. *Journal of Agricultural and Food Chemistry*, **56(21)**:9990-9996. DOI: 10.1021/jf8018062

**Selma M. V., P. V. Martínez-Culebras and R. Aznar (2008b).** Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. *International Journal of Food Microbiology*, **122(1-2)**:126-134.

**Senyuva H. Z., J. Gilbert, S. Ozcan and U. Ulken (2005).** Survey of ochratoxin A and afaltoxin B1 in dried figs in Turkey using a single laboratory validated alkaline extraction method for ochratoxin A. *Journal of Food Protection*, **68**:1512-1515.

**Serra R., L. Abrunhosa, Z. Kozakiewicz and A. Venâncio (2003).** Black *Aspergillus* species as ochratoxin A producers in Portuguese wine. *International Journal of Food Microbiology*, **88(1)**:63-68.

**Serra-Bonvehí J. (2004).** Occurrence of ochratoxin A in cocoa products and chocolate. *Journal of Agricultural and Food Chemistry,* **52**:6347-6352.

**Sharma R. R., D. Singh and R. Singh (2009).** Biological Control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. *Biological Control,* **50**: 205-211.

**Sharma T. N., P. R.Gupta, A. K. Mehrota, S. D. Purohit and H. N. Mangal (1985).** Aspergilloma with ABPA due to *Aspergillus niger*. *Journal of Association of Physicians in India,* 33:748.

**Shina A. K. and K. S. Ranjan (1991).** A report of mycotoxin contamination in Bhutane cheese. *Journal of Food Science and Technology,* **28**:398-399.

**Simpson T. J. and R. J. Cox (2008).** Polyketide Biosynthesis, Fungi. Wiley Encyclopedia of Chemical Biology. John Wiley & Sons, Inc., 1-11.

**Singleton V. L., R. Orthofer and R. M. Lamuela-Raventos (1999).** Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology*, **299**:152-178.

**Skarkova J., V. Ostry, F. Malir and T. Roubal (2013).** Determination of ochratoxin A in food by high performance liquid chromatography. *Analytical Letters*, **46(10)**:1495-1504.

**Slayne M. A. (2001).** Ochratoxin A in food in the UK. In: *Mycotoxins and phycotoxins in perspective at the turn of the millennium*. Koe W. J., R. A. Samson, H. P.van Egmond, J. Gilbert and M. Sabino (Eds.). Ponsen and Looyen, Wageningen, The Netherlands, 143-149.

**Smith J. E. and M. O. Moss (1985).** The Toxigenic Fungi. *In:* Mycotoxins. Formation, analysis and significance. John Willey and Sons, Toronto, Canada, 27-29.

**Soleas G. J., J. Yan and D. M. Goldberg (2001).** Assay of ochratoxin A in wine and beer by high- pressure liquid chromatography photodiode array and gas chromatography mass selective detection. *Journal of Agricultural and Food Chemistry,* **49**: 2733-2740.

**Soto-Vaca A., A. Gutierrez, J. N. Losso, Z. Xu, and J. W. Finley (2012).** Evolution of phenolic compounds from color and flavor problems to health benefits. *Journal of Agricultural and Food Chemistry*, **60**:6658-6677.

**Soyer Y., N. Koca and F. Karadeniz (2003).** Organic acid profile of Turkish white grapes and grape juices. *Journal of Food Composition and Analysis*, **16**:629-636.

**Spadaro D., S. Patharajan, M. Kartikeyan, A. Lorè, A. Garibaldi, M. L. Gullino (2011).** Specific PCR primers for the detection of isolates of *Aspergillus carbonarius* producing ochratoxin A on grapevine. *Annals of Microbiology*, **61(2)**:267-272.

**Speijers G. J. A. and H. P. van Egmond (1993).** World-wide ochratoxin A levels in food and feeds. In: *Human ochratoxicosis and its pathologies*, 231. Creppy E., M. Castegnaro and G. Dirheimer (Eds.). John Libbey Eurotext Limited. Paris, France, 85-100.

**Speijers G. J. A. and M. H. M. Speijers (2004).** Combined toxic effects of mycotoxins. *Toxicology Letters,* **153(1)**:91-98.

**Sperry J. S., N. M. Holbrook, M. H. Zimmermann, and M. T. Tyree (1987).** Spring filling of xylem vessels in wild grapevine. *Plant Physiology*, **83**:414-417.

**Squire, R. A. (1981).** Ranking animal carcinogens: a proposed regulatory approach. *Science*, **214**:877-880.

**Stander M. A. and P. S. Steyn (2002).** Survey of ochratoxin A in South African wines. *South African Journal for Enology and Viticulture,* **23**:9-13.

**Staudt G. (1997).** Evaluation of resistance to grapevine powdery mildew (*Uncinula necator* [Scxw.] BURR., anamorph *Oidium tuckeri* BERK.) in accessions of *Vitis* species. *Vitis*, **36(3)**:1S1-1S4.

**Stegen V. D. G., U. Jorissen, A. Pittet, M. Saccon, W. Steiner, M. Vincenzi, M. Winkler, J. Zapp and C. Schlatter (1997).** Screening of European coffee final products for occurrence of ochratoxin A (OTA). *Food Additives and Contaminants*, **14**:211-216.

**Storari M., I. Pertot, C. Gessler, G. A. L. Broggini (2010).** Amplification of polyketide synthase gene fragments in ochratoxigenic and nonochratoxigenic black aspergilli in grapevine. *Phytopathologia Mediterranea*, **49**, 393-405.

**Suàrez-Quiroz M. L., B. De Louise, O. Gonzales-Rios, M. Barel, B. Guyot, S. Schorr-Galindo and J. P Guiraud (2005).** The impact of roasting on the Ochratoxin A content of coffee. *International journal of science and technology*, **40**:605-611.

**Suarez-Quiroz M. L., O. Gonzalez-Rios, M. Barel, B. Guyot, S. Schorr-Galindo and J. P. Guiraud (2004)**. Study of ochratoxinA-producing strains in coffee processing. *International Journal of Food Science and Technology*, **39**:501-507.

**Suttajit M. (1989).** Prevention and control of mycotoxins. In: *Mycotoxin prevention and control in food grains*. Semple R. I., S. A. Frio, P. A. Hicks and J. V. Lozare (Eds). FAO and the Information network on post-harvest operations. Bankok, Thailand.

**Sweeney M. J., and A. D. W. Dobson (1998).** Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *International Journal of Food Microbiology*, **43**:141-158.

**Sweeney M. J., S. White, and A. D. W. Dobson (2000).** Mycotoxins in agriculture and food safety. *Irish Journal of Agricultural and Food Research,* **39**:235–244.

**Tagni E. K., S. Ponchaut, M. Maudoux, R. Rozenberg and Y. Larondelle (2002).** Ochratoxin A in domestic and imported beers in Belgium: occurrence and exposure assessment. *Food Additives and Contaminants,* **19**:1169-1179.

**Taillandier P. and J. Bonnet (2005).** Le vin; Composition et transformations chimiques. Editions TEC & DOC, Paris.

**Taniwaki M.H., J. I. Pitt, A. A. Teixeira and B. T. Iamanaka (2003).** The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. *International Journal of Food Microbiology*, **82(2)**:173-179.

**Tawaha K., F. Q. Alali, M. Gharaibeh, M. Mohammad and T. El-Elimat (2007).** Antioxidant activity and total phenolic content of selected Jordanian plant species. *Food Chemistry*, **104(4)**:1372-1378.

**Teixeira A., J. Eiras-Dias, S. D. Castellarin and H. Gerós (2013).** Berry Phenolics of Grapevine under Challenging Environments. *International Journal of Molecular Science,* **14**:18711-18739. DOI: 10.3390/ijms140918711.

**Téren J., A. Palágyi and J. Varga (1997).** Isolation of ochratoxin A producing aspergilli from green coffee beans of different origin. *Cereal Research Communications;* **25**:303-304.

**Téren J., J. Varga, Z. Hamari, E. Rinyu and F. Kevei (1996).** Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathologia,* **134**:171-176.

**Terral J. F., E. Tabard, L. Bouby, S. Ivorra, T. Pastor, I. Figueiral, S. Picq, J. B. Chevance, C. Jung, L. Fabre, C. Tardy, M. Compan, R. Bacilieri, T. Lacombe and P. This (2010).** Evolution and history of grapevine (*Vitis vinifera*) under domestication: new morphometric perspectives to understand seed domestication syndrome and reveal origins of ancient European cultivars. *Annals of Botany*, **105**: 443-455.

**Tesniere C., L. Torregrosa, M. Pradal, J.M. Souquet, C. Gilles, K.D. Santos, P. Chatelet and Z. Gunata (2006).** Effects of genetic manipulation of alcohol dehydrogenase levels on the response to stress and the synthesis of secondary metabolites in grapevine leaves. *Journal of Experimental Botany*, **57(1)**:91–99.

**Thakur M. and B. S. Sohal (2013).** Role of elicitors in inducing resistance in plants against pathogen infection: a review. ISRN Biochemistry, DOI: 10.1155/2013/762412.

**Thirumala-Devi K., M. A. Mayo, G. Reddy, K. E. Emmanuel, Y. Larondelle and D. V. R. Reddy (2001).** Occurrence of ochratoxin A in black pepper, coriander, ginger and turmenic in India. *Food Additives and Contaminants,* **18**:830-835.

**Tiradoa M. C., R. Clarkeb, L. A. Jaykusc, A. McQuatters-Gollopd, and J. M. Franke (2010).** Climate change and food safety: A review. *Food Research International*, **43(7)**:1745-1765.

**Tjamos S. E., P. P. Antoniou, A. Kazantzidou, D. F. Antonopoulos, I. Papageorgiu, and E. C. Tjamos (2004).** *Aspergillus niger* and *A. carbonarius* in Corinth raisin and wine-producing vineyards in Greece: population composition, ochratoxin A production and chemical control. *Journal of Phytopathology*, **152**:250-255.

**Tognon G., A. Campagnoli, L. Pinotti, V. Dell'Orto and F. Cheli (2005).** Implementation of the Electronic Nose for the Identification of Mycotoxins in Durum Wheat (*Triticum durum*). *Veterinary Research Communications*, 29(S2):391-393.

**Torelli E., E. Gobbi, G. Firrao, and R. Locci, (2003).** Ochratoxin A producing strains of *Penicillium* spp. isolated from grapes. *Journal of Plant Pathology*, **85**:281.

**Tozlovanu M., V. Faucet-Marquis, A. Pfohl-Leszkowicz and R. Manderville (2006).** Genotoxicity of the hydroquinone metabolite of ochratoxin A: Structure-activity relationships for covalent DNA adduction. *Chemistry Research Toxicology,* **19**:1241-1247.

**Trivedi A. B., E. Doi and N. Kitabatake (1992).** Detoxification of ochratoxin A on heating under acidic and alkaline conditions. *Bioscience, Biotechnology, and Biochemistry*, **56**:741-745.

**Trotel-Aziz P., M. Couderchet, G. Vernet and A. Aziz (2006).** Chitosan stimulates defense reactions in grapevine leaves and inhibits development of *Botrytis cinerea*. *European Journal of Plant Pathology*, **114(4)**:405-413.

**Trucksess M. W. and Y. Tang. (2001).** Solid phase extraction method for patulin in apple juice and unfiltered apple juice, p. 205–213. *In* M. W. Trucksess and A. F. Pohland (Eds), Mycotoxin protocols. Humana Press, Totowa, N.J.

**Trucksess M. W., J. Giler, K. Young, K. D. White and S. W. Page (1999).** Determination and survey of ochratoxin A in wheat, barley and coffee-1997. *Journal of the AOAC International,* **82**:85-89.

**Turner N. W., S. Subrahmanyam and S. A. Piletsky (2009).** Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta*, **632(2)**:168-180.

**Turner W. B. and D. C. Aldridge (1983).** Fungal metabolites II. Academic Press, London, UK.

**Ueno Y. (1998).** Residue and risk of ochratoxin A in human plasma and beverages in Japan. *Mycotoxins,* **47**:25-32.

**Ueno Y., O. Kawamura, Y. Sugiura, K. Horiguchi, M. Nakajima, K. Yamamoto and S. Sato (1991).** Use of monoclonal antibodies enzyme-linked immunosorbent assay and immunoaffinity column chromatography to determine ochratoxin A in porcine sera, coffee products and toxin-producing fungi. In: *Mycotoxins, endemic nephropathy and urinary tract tumors*. Castegnaro M., R. Plestina, G. Dirheimer, I. N. Chernozemsky and H. Bartsch (Eds.). IARC Scientific Publications, Lyon, France, 71-75.

**Ullrich C. I., R. G. Kleespies, M. Enders, E. Koch (2009).** Biology of the black rot pathogen, *Guignardia bidwellii*, its development in susceptible leaves of grapevine *Vitis vinifera. Journal für Kulturpflanzen*, **61(3)**:82-90

**Utto W., A. J. Mawson and J. E. Bronlund (2008).** Hexanal reduces infection of tomatoes by *Botrytis cinearea* which maintain quality. *Postharvest biology and technology,* **47**:434-437.

*References*

**Valenta H. and M. Goll (1996).** Determination of ochratoxin A in regional samples of cow's milk from Germany. *Food Additives and Contaminants,* **13**:669-676.

**Valero A., S. Marín, A. J. Ramos, and V. Sanchis (2005).** Ochratoxin A-producing species in grapes and sun-dried grapes and their relation to ecophysiological factors. *Letters in Applied Microbiology,* **41**:196-201.

**Valero A., S. Oliván, S. Marín, V. Sanchis and A. J. Ramos (2007).** Effect of intra and interspecific interaction on OTA production by *A.* section Nigri in grapes during dehydration. *Food Microbiology*, **24**:254-259.

**van der Merwe K. J., P. S. Steyn and L. Fourie (1965).** Mycotoxins. Part II. The constitution of ochratoxin A, B and C, metabolites of *Aspergillus ochraceus* Wilh. *Journal of the Chemical Society,* **5**:7083-7088.

**van Egmond H. P. and G. J. A. Speijers (1994).** Survey of data on the incidence and levels of ochratoxin A in food and animal feed worldwide. *Journal of Natural Toxins,* **3**:125-144.

**Van Hannen E. J., G. Zwart, M. P. Van Agterveld, H. J. Gons, J. Ebert, and H. J. Laanbroek. (1999).** Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. *Applied and Environmental Microbiology*, **65**:795-801.

**Varga J. and Z. Kozakiewicz (2006).** Ochratoxin A in grapes and grape derived products. *Trends in Food Science and Technology*, **17**:72-81.

**Varga J., E. Kevei, E. Rinyu, J. Téren and Z. Kozakiewic (1996).** Ochratoxin production by *Aspergillus* species. *Applied and Environmental Microbiology,* **62**:4461-4464.

**Varga J., K. Rigo and J. Teren (2000).** Degradation of ochratoxin A by *Aspergillus* species. *International Journal of Food Microbiology*, **59**:1-7.

**Varga J., K. Rigó, J. Téren and Á. Mesterházy (2001).** Recent advances in ochratoxin research I. Production, detection and occurrence of ochratoxins. *Cereal Research Communications,* **29**:85-92.

**Varga J., R. Kiss, T. Mátrai, T. Mátrai, and J. Téren, (2005).** Detection of ochratoxin A in Hungarian wines and beers. *Acta Alimentaria*, **34**:381-392.

**Vezzulli S., S. Civardi, F. Ferrari and L. Bavaresco (2007).** Methyl jasmonate treatment as a trigger of resveratrol synthesis in cultivated grapevine. *American Journal of Enology and Viticulture,* **58**:530-533.

**Villa P. (2005).** La culture de la vigne: les variétés, les différents types de cultures, les engrais, l'irrigation, les traitements. Edition De Vecchi S. A., Paris.

**Visconti A., G. Perrone, G. Cozzi, and M. Solfrizzo (2008).** Managing ochratoxin A risk in the grape-wine food chain. Food Additives and Contaminants, **25(2)**:193-202. DOI: 10.1080/02652030701744546.

**Visconti A., M. Pascale and G. Centonze (1999).** Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. *Journal of Chromatography A,* **864**: 89-101.

**Vos P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J.Peleman, M. Kuiper and M .Zabeau (1995).** AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**:4407-4414.

**Waldorf A. R. and A. Polak (1983).** Mechanisms of action of 5-fluorocytosine. *Antimicrob Agents Chemother*, **23**:79-85.

**Walter B. and G. P. Martelli (1998).** Considerations on grapevine selection and certification. *Vitis,* **37(2)**:87-90.

**Walters W. D., A. Newton and G. Lyon (2005)** Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. *Phytopathology*, **95**:1368-1373.

**Wanga Y., N. Liu, B. Ning, M. Liu, Z. Lv, Z. Sun, Y. Peng, C. Chen, J. Li and Z. Gao (2012).** Simultaneous and rapid detection of six different mycotoxins using an immunochip. *Biosensors and Bioelectronics*, **34(1)**:44-50.

**Wataru G., K. Takahito, U. Takahiko, O. Fumitoshi, and M. Yasufumi (2005).** Improvement of condition for hybridization to obtain high sensitivity in oligonucleotide microarray systems. *Mycotoxins*, **55(2)**:117-128.

**Wickow D. T., P. F. Dowd, A. A. Alfatafta and J. B. Gloer (1996).** Ochratoxin A: an antiinsectan metabolite from the sclerotia of *Aspergillus carbonarius* NRRL 369. *Canadian Journal of Microbiology,* **42**:1100-1103.

**Wiebe M. G. and A. P. J. Trinci (1990).** Dilution rate as a determninat of mycelial morphology in continuous culture. *Biotechnology and Bioengineering,* **38**:75-81.

**Williams L. E. (1987).** Growth of Thompson Seedless grapevines: II. Nitrogen distribution. *Journal of the American Society for Horticultural Science*, **112**:330-333.

**Williams L. E. and M. A. Matthews (1990).** Grapevine In: B.A. Stewart and N.R. Nielson, (Eds), Irrigation of Agricultural Crops. Agronomy Monograph No. 30. ASA-CSSA-SSSA Madison, WI.

**Williams L. E. and P. J. Biscay (1991).** Partitioning of dry weight, nitrogen, and potassium in Cabernet Sauvignon grapevines from anthesis until harvest. *American Journal of Enology and Viticulture*, **42**:113-117.

**Winkler A. J., J. A. Cook, W. M. Kliewer and L. A. Lider (1974).** The grape flower and berry set. In: General Viticulture. University of California Press. Los Angeles, CA. pp. 117-137.

**Wolff J. (2000).** Ochratoxin A in cereals and cereal products. *Archiv für Lebensmittelhygiene,* **51**:85-88.

**Wood G. M., S. Patel, A. C. Entwistle and A. Boenke (1996).** Ochratoxin A in wheat; a second inter-comparison of procedures. *Food Additives and Contaminants,* **13**:519-539.

**Wu Q., V.Dohnal, L. Huang, K. Ku, X. Wang, G. Chen and Z. Yuan (2011).** Metabolic Pathways of Ochratoxin A. *Current Drug Metabolism,* **12**:1-10.

**Xiao H., R. R. Marquardt, A. A. Frohlich and Y. Z. Ling (1995).** Synthesis and structural elucidation of analogs of ochatoxin A. *Journal of Agricultural and Food Chemistry,* **43**:524-530.

**Yokotsuka K. and M. Fukui (2002).** Changes in Nitrogen Compounds in Berries of Six Grape Cultivars during Ripening over Two Years. *American Journal of Enology Viticulture*, **53**:69-77.

**Yokoyama K., L. Wang, M. Miyaji and K. Nishimura (2001).** Identification, classification and phylogeny of the *Aspergillus* section *Nigri* inferred from mitochondrial cytocrome b gene. *FEMS Microbiology Letters,* **200**:241-246.

**Zimmerli B. and R. Dick (1995).** Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column clean-up: methodology and Swiss data. *Journal of Chromatography B,* **666**:85-99.

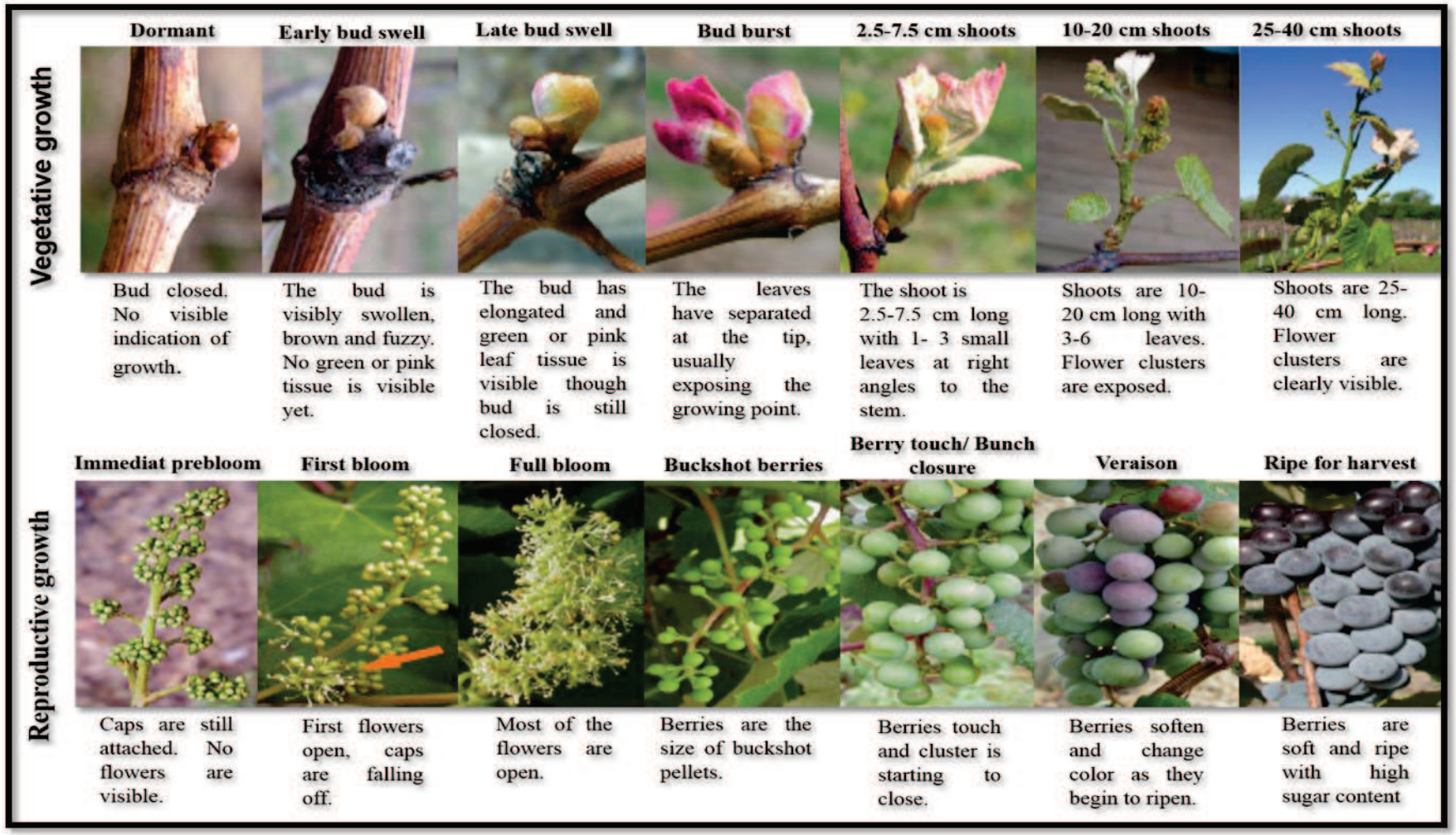
**Zimmerli B. and R. Dick (1996).** Ochratoxin A in table wines and grape-juice: occurrence and risk assessment. *Food Additives and Contaminants,* **13**:655-668.

**Zohri A. A. and K. M. Abdelgawad (1993).** Survey of mycoflora and mycotoxins of some dried fruits in Egypt. *Journal of Basic Microbiology,* **33**:279-288.

# Appendix

# Appendix 1:

## Grapevine growth stages



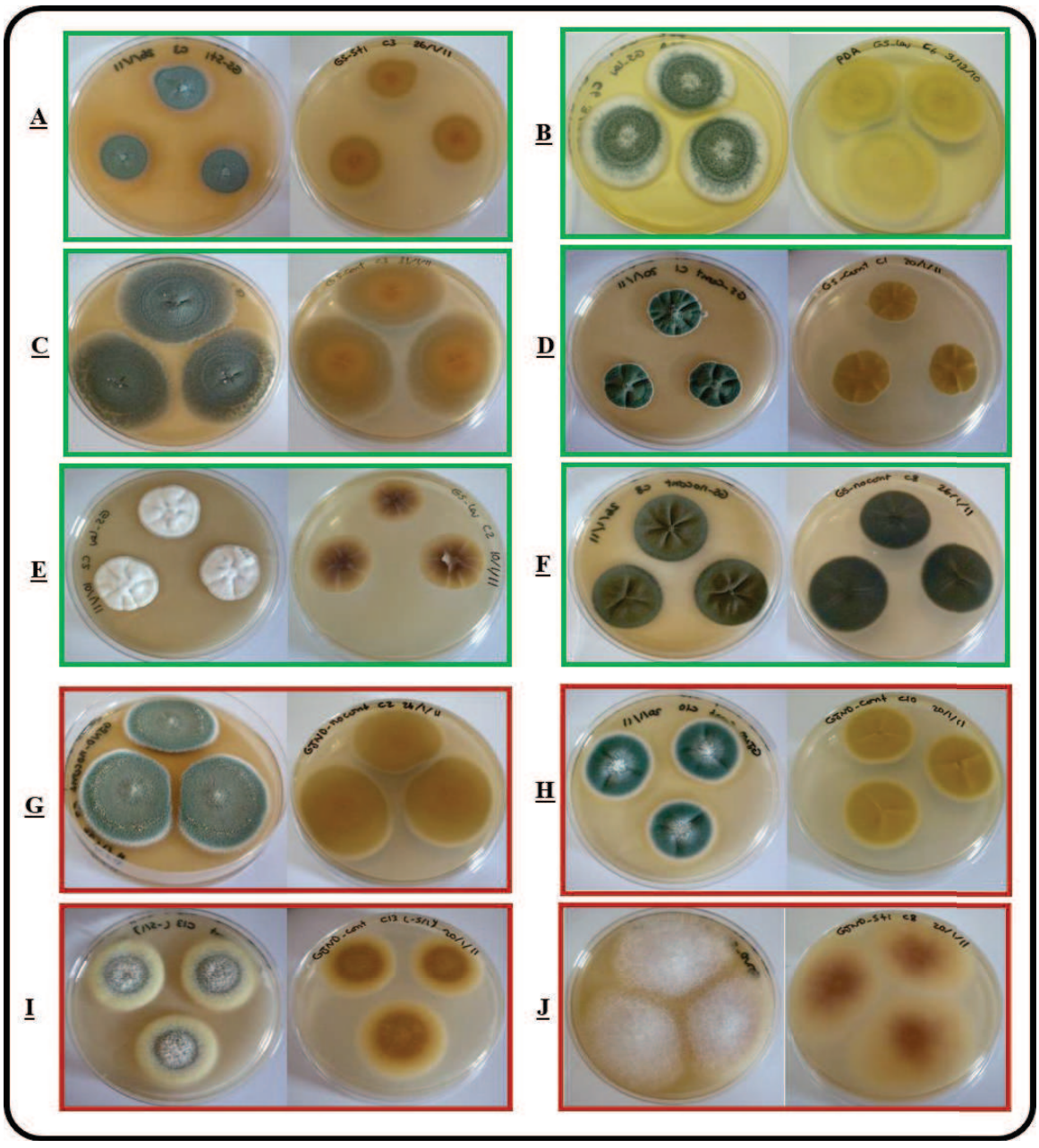
## Appendix 2

## Field sampling from different preharvest treatments


**A:** uncontaminated and untreated modality, **B:** artificially contaminated by *Aspergillus carbonarius* (OTA-PF) but not treated modality, **C:** contaminated by OTA-PF and treated with Scala®, **D:** contaminated by OTA-PF and treated with *Trichoderma atroviride* , **E:** contaminated by OTA-PF and treated with *Saccharomyces cevericea*, **F:** contaminated by OTA-PF and treated with Stifénia®,

# Appendix 3

**Growth of some non-black *Aspergillus* strains isolated from different treatments on PDA culture media**

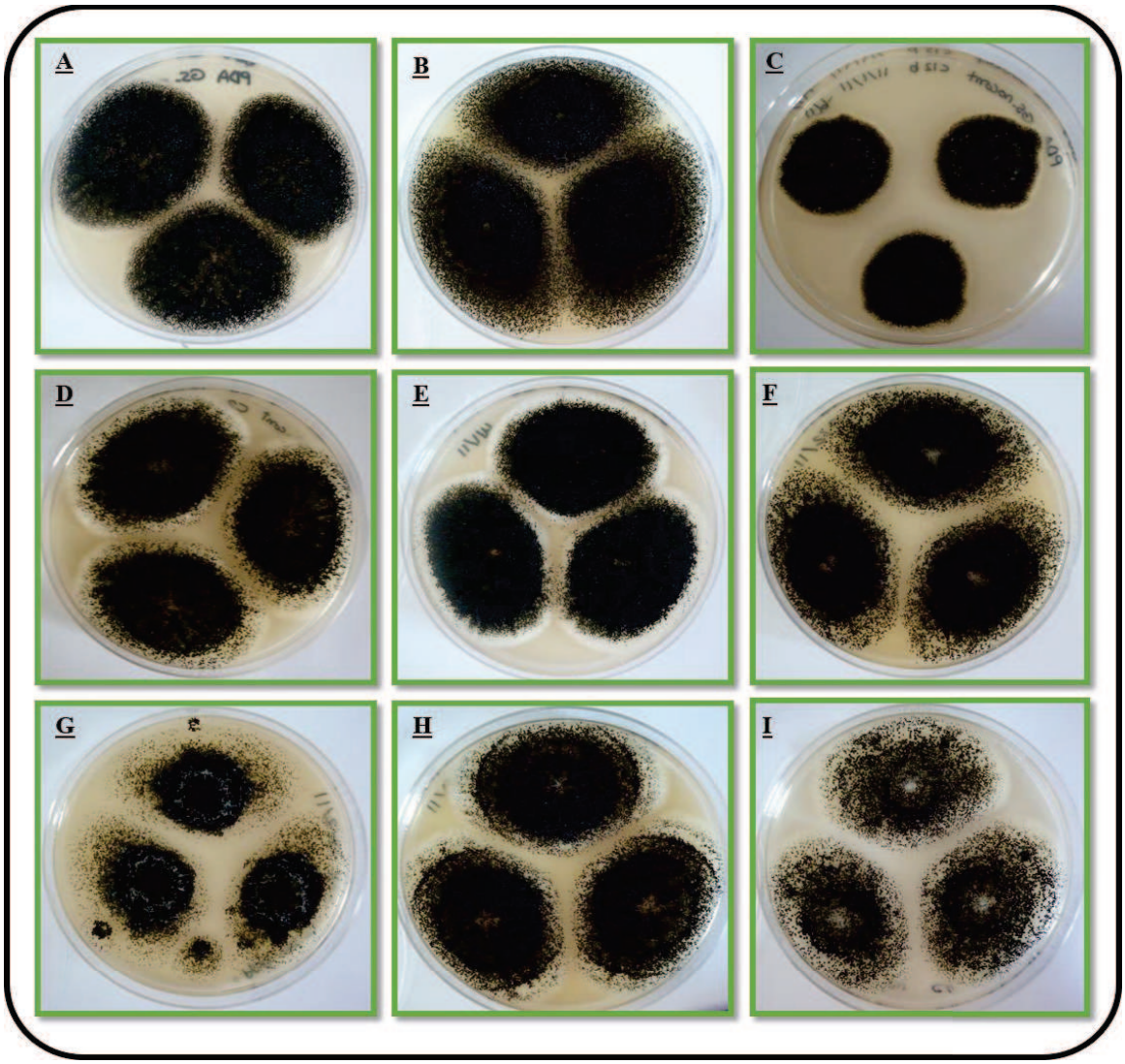



**Strains isolated from grape juice (G - J) and stems (A - F); A:** Sti 3, **B:** Saccharo. 6, **C:** Cont 3, **D:** Cont 1, **E:** Saccharo. 2, **F:** Nocont 8, **G:** Nocont 2, **H:** Cont 10, **I:** Cont 13 , **J:** Sti 8

***Nocont:*** *uncontaminated-nor-treated modality,* ***Cont:*** *contaminated-untreated modality,* ***Sti:*** *contaminated-&-Stiénia®-treated treatment,* ***Tri:*** *contaminated-&-Trichoderma-treated treatment,* ***Saccharo:*** *contaminated-&-Saccharomyces –treated treatment.*

# Appendix 4

**Growth of some black Aspergillus species isolated from grape stalks on PDA**

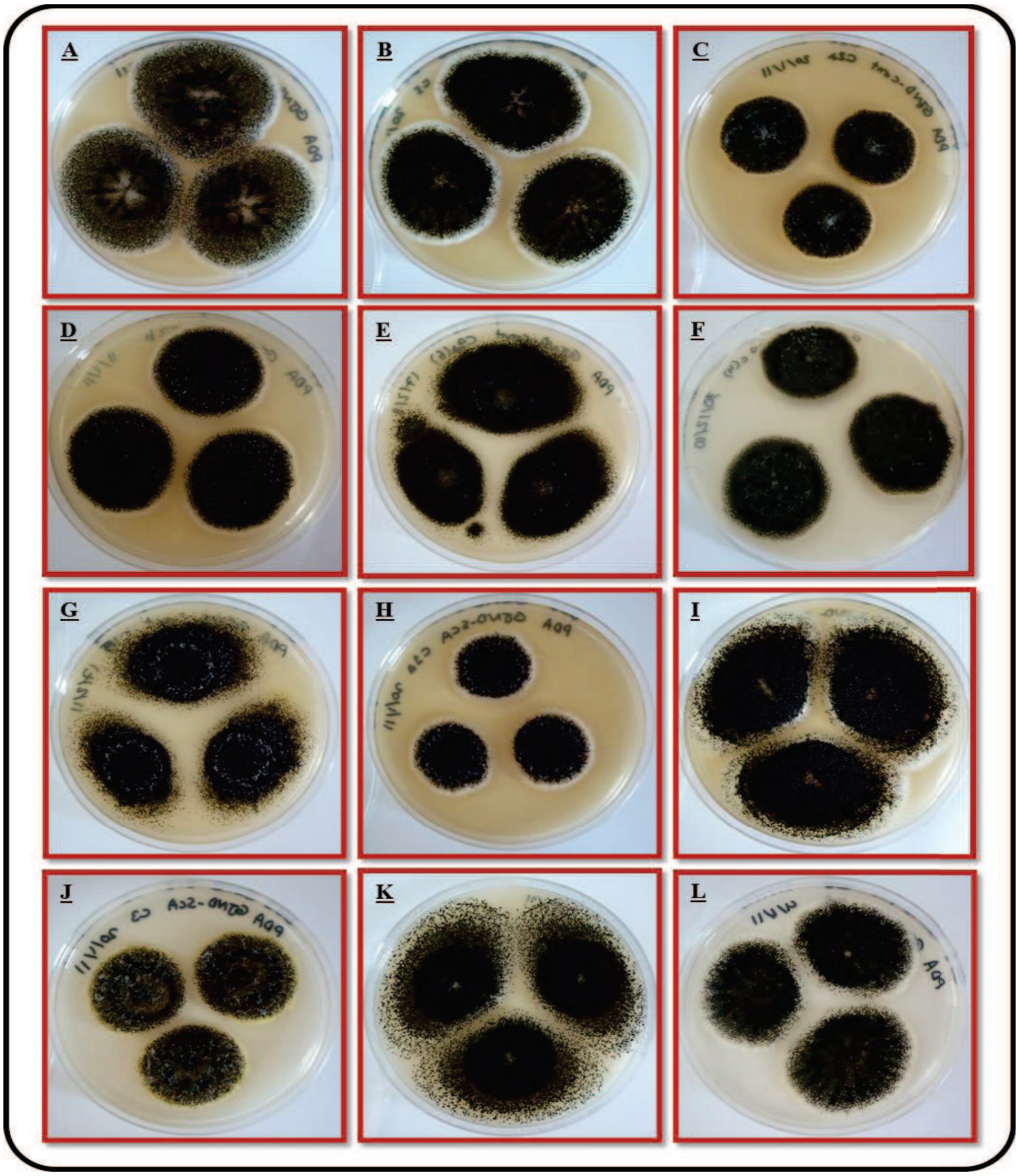



**A:** Nocont 3, **B:** Nocont 7, **C:** Nocont 12b, **D:** Cont 2, **E:** Cont C5, **F:** Scala 5, **G:** Tri 2b, **H:** Tri 10, **I:** Saccharo. 4

***Nocont:*** *uncontaminated-nor-treated modality,* ***Cont:*** *contaminated-untreated modality,* ***Sca:*** *contaminated-&-Scala®-treated treatment,* ***Tri:*** *contaminated-&-Trichoderma-treated treatment,* ***Saccharo****: contaminated-&-Saccharomyces –treated treatment.*

# Appendix 5

**Growth of some black Aspergillus species isolated from grape juices on PDA**

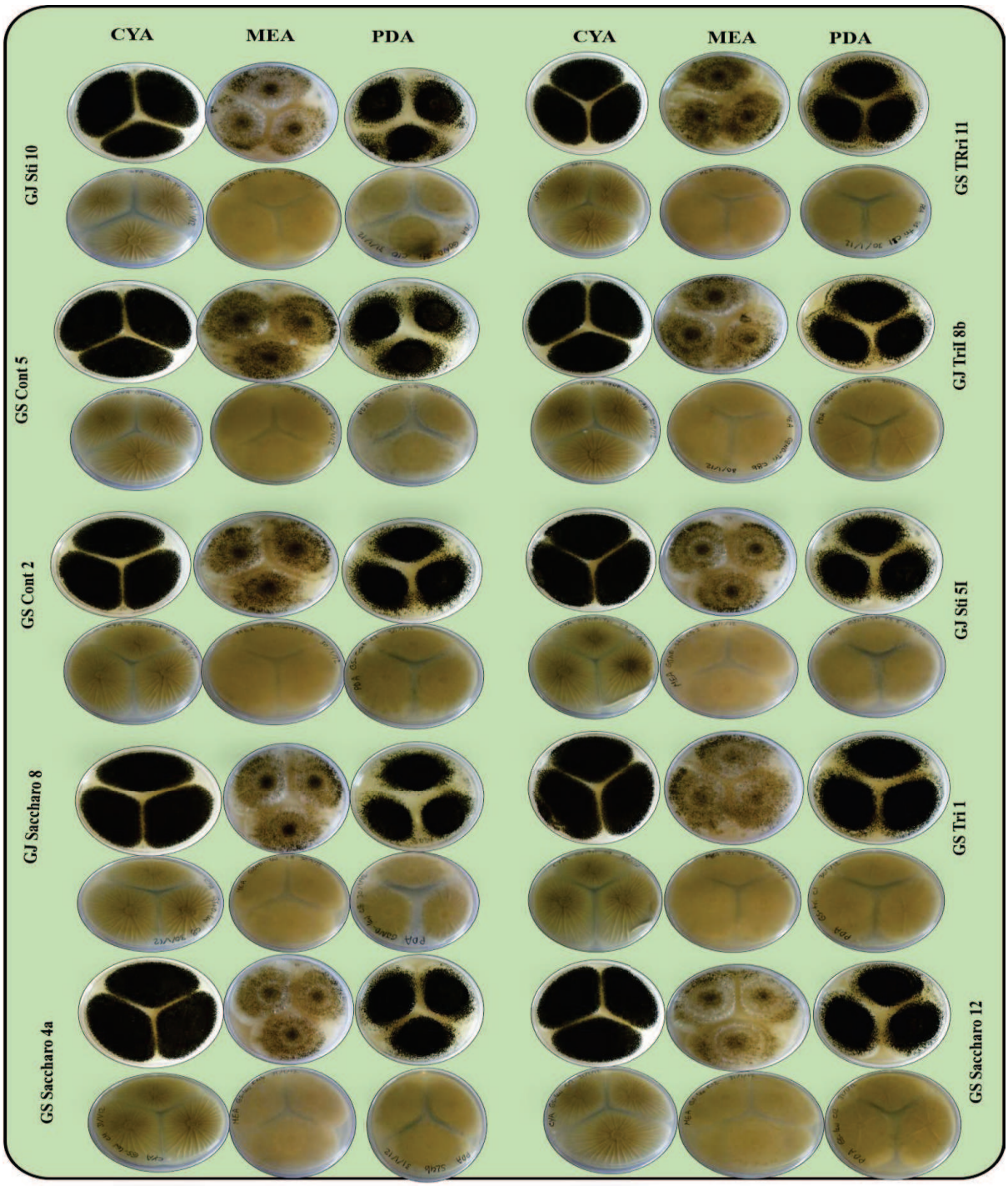



**A:** Nocont 1, **B:** Nocont 5, **C:** Cont 2a, **D:** Cont 2b, **E:** Cont 9a, **F:** Cont 9c, **G:** Cont 15, **H:** Sca 1a, **I:** Sca 2, **J:** Sca 3, **K**: Tri 8, **L**: Saccharo 9.

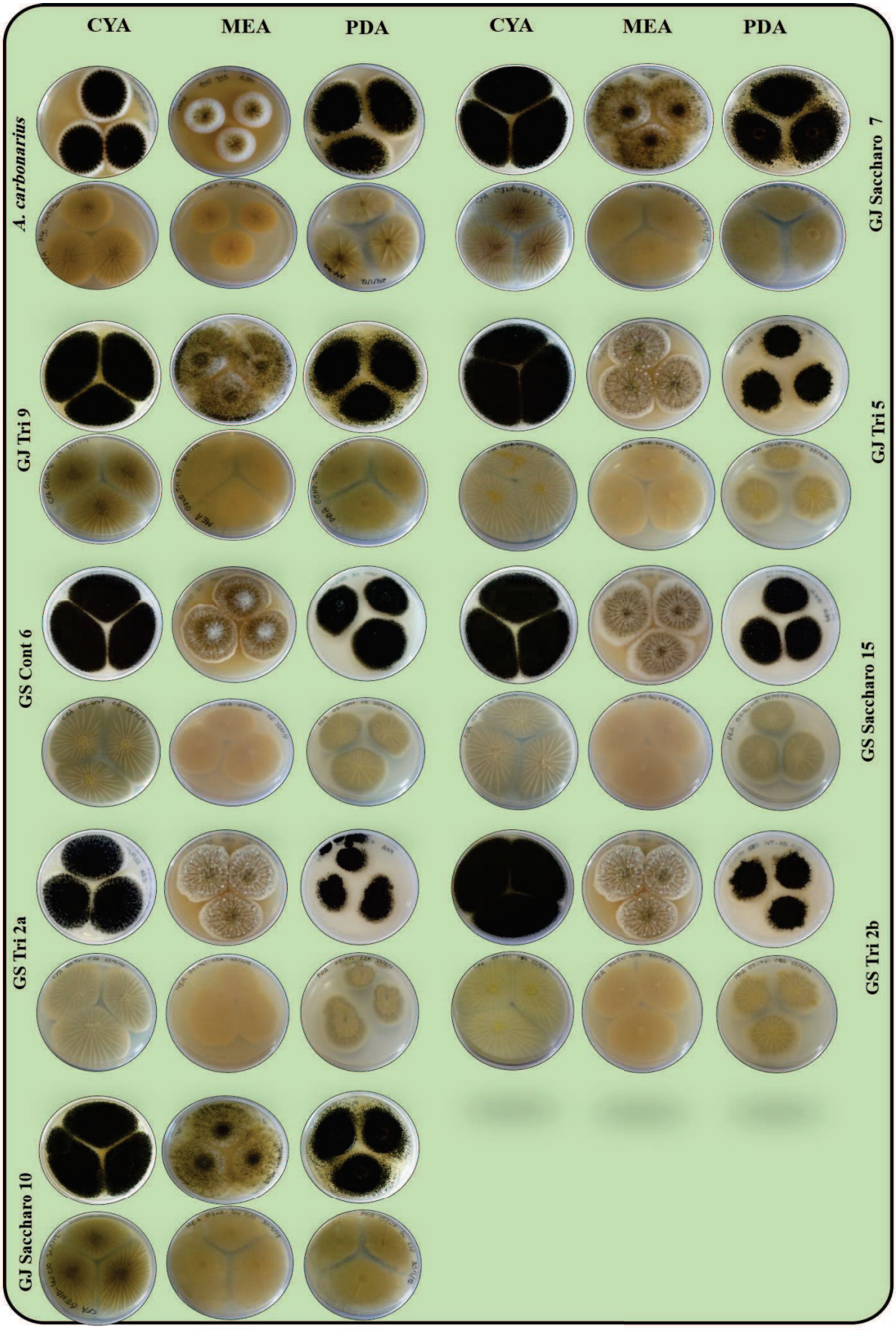
***Nocont:*** *uncontaminated-nor-treated modality,* ***Cont:*** *contaminated-untreated modality,* ***Sca:*** *contaminated-&-Scala®-treated treatment,* ***Tri:*** *contaminated-&- Trichoderma –treated treatment,* ***Saccharo:*** *contaminated-&-Saccharomyces –treated treatment.*

***Appendix 6***

***Moorpholigical on different media (PDA, CYA and MEA) followed by microscopic identification of black aspergilli strains***



## Appendix 6 continued



**Appendix 7**

**The reactions used for the DNA**

1- **Tris-EDTA(TE) buffer (pH 8.0)**

The Tris-HCl provides the nessissary pH for the cellular lysis enzyme's activity, and EDTA provides the nessissary iones for the activity of the bacterial DNAse.

It consists of 10mM Tris-HCl pH 8.0 (Promiga, France), 1.0 mM EDTA pH 8.0. (Sterilized at 121 °C/15 min).

2- **Solution of Lysozyme 25mg/mL**

25 mg of Lysozyme were soulabilizes in 1 mL of pure water and conserved at 4 °C.

3- **Solution of Protinase K 20mg/mL**

20 mg of Proteinase K were soulabilizes in 1 mL of pure water and conserved at 4 °C.

4- **Solution of SDS (Sosium dodecyl sulfate) 20%**

20 g of SDS were soulabilizes in 100 mL of sterilized pure water and conserved at room temperature.

5- **Solution of NaCl 3M**

175.32 g (3M) of NaCl were soulabilizes in 1 L of pure water then sterilized at 121 °C/15 min and conserved at 4 °C.

6- **Solution of MATAB (Mixed Alkyl Trimethyl Ammonium Bromide) 2%**

2 g/mL of MATAB were soulabilizes in 100 mL of NaCl (3M) and conserved at 4 °C.

7- **Solution of sodium acetate (3M, pH 5.2)**

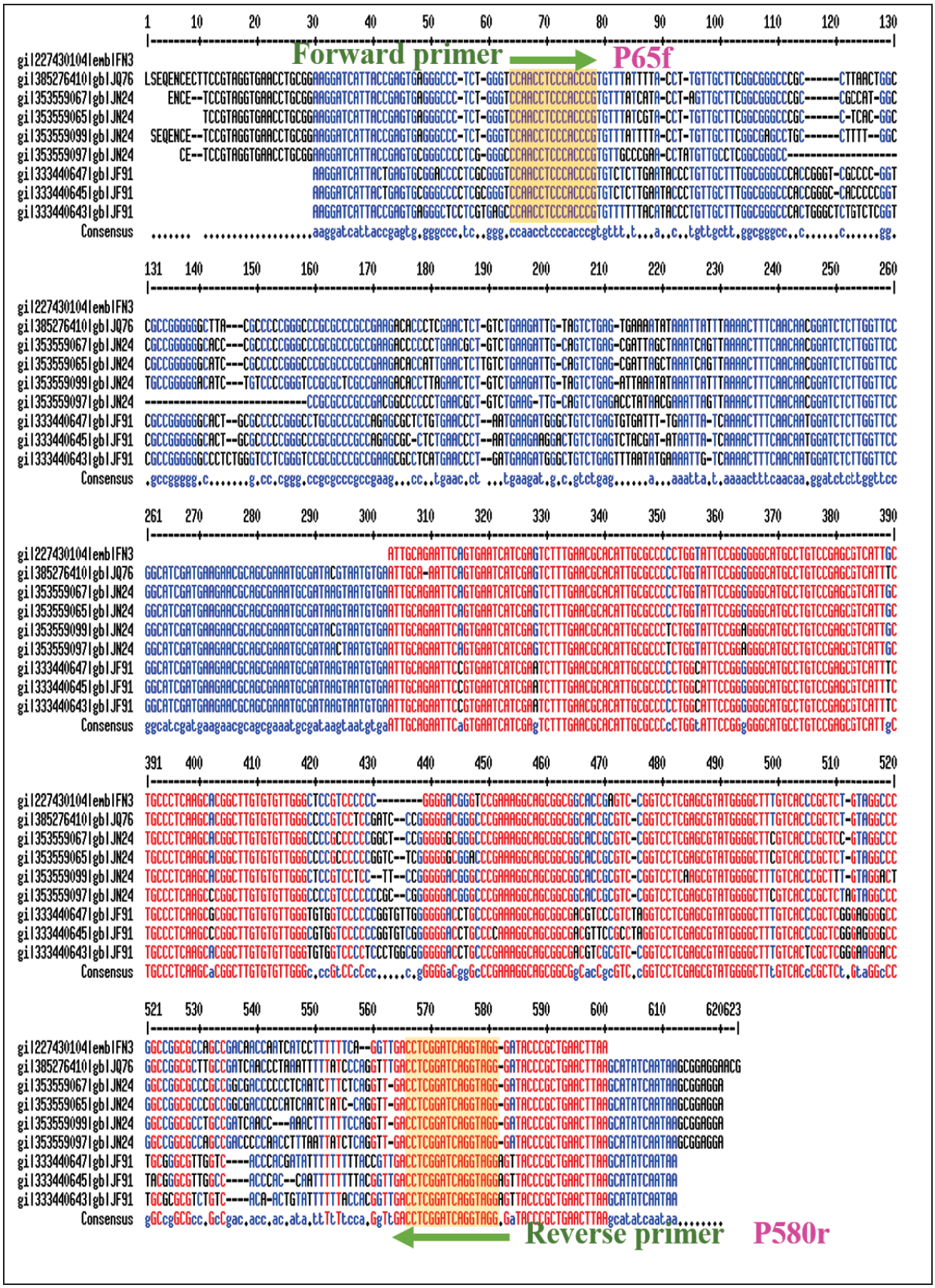
246.09 g (3M) of $CH_3COONa$ were soulabilizes in 1 L of pure water and the pH was adjusted to 5.2 using acetic acid. The solution was sterilized at 121 °C/15 min and conserved at 4 °C.

8- **Peptone water**

10 g peptone, 5 g NaCl, 9 g $Na_2HPO_4$ and 1.5 g $KH_2PO_4$ were soulabilizes in 1 L of pure water and the pH was adjusted to 7.0 using acetic acid. The solution was sterilized at 121 °C/15 min and conserved at 4 °C.

**Appendix 8**

**Primer design 1; alignment of different fungal straind at ITS region**




Using Multalin web site and NBCI data bases.

**Appendix 9**

**Primer design 2; designed primers self complementarity check**

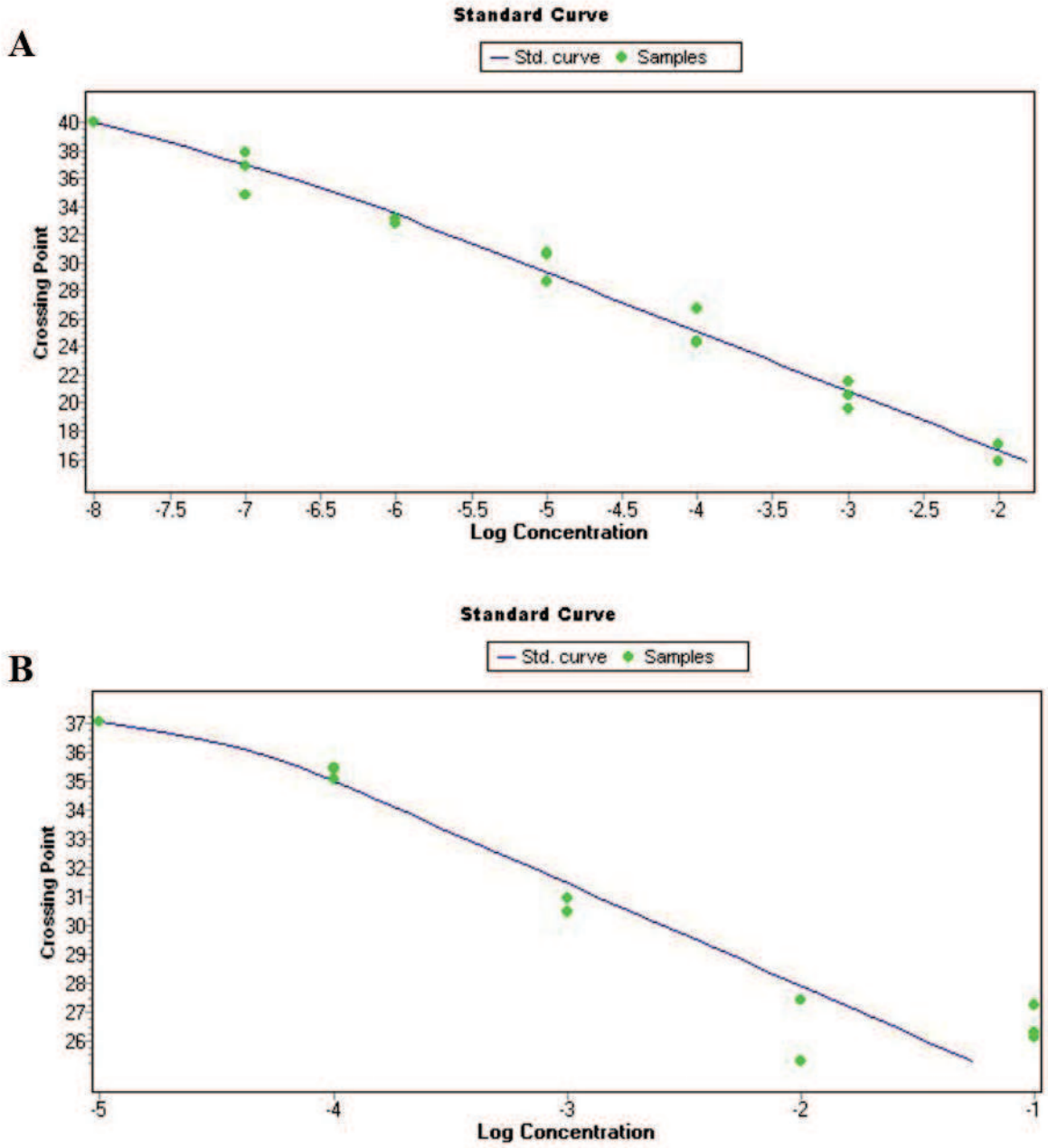
| Primer | | Length | Molecular weight | GC content | single primer self-dimerization | hairpin |
|---|---|---|---|---|---|---|
| **P65f** | 5'-caa cct ccc acc cg-3' | 14 bp | 4113.7 | 71 % | None | None |
| **P580r** | 5'-cct cgg atc agg tag-3' | 15 bp | 4553 | 60 % | None | None |

Using Oligo Calculator version 3.26 software

http://www.basic.northwestern.edu/biotools/OligoCalc.html

# Appendix 10

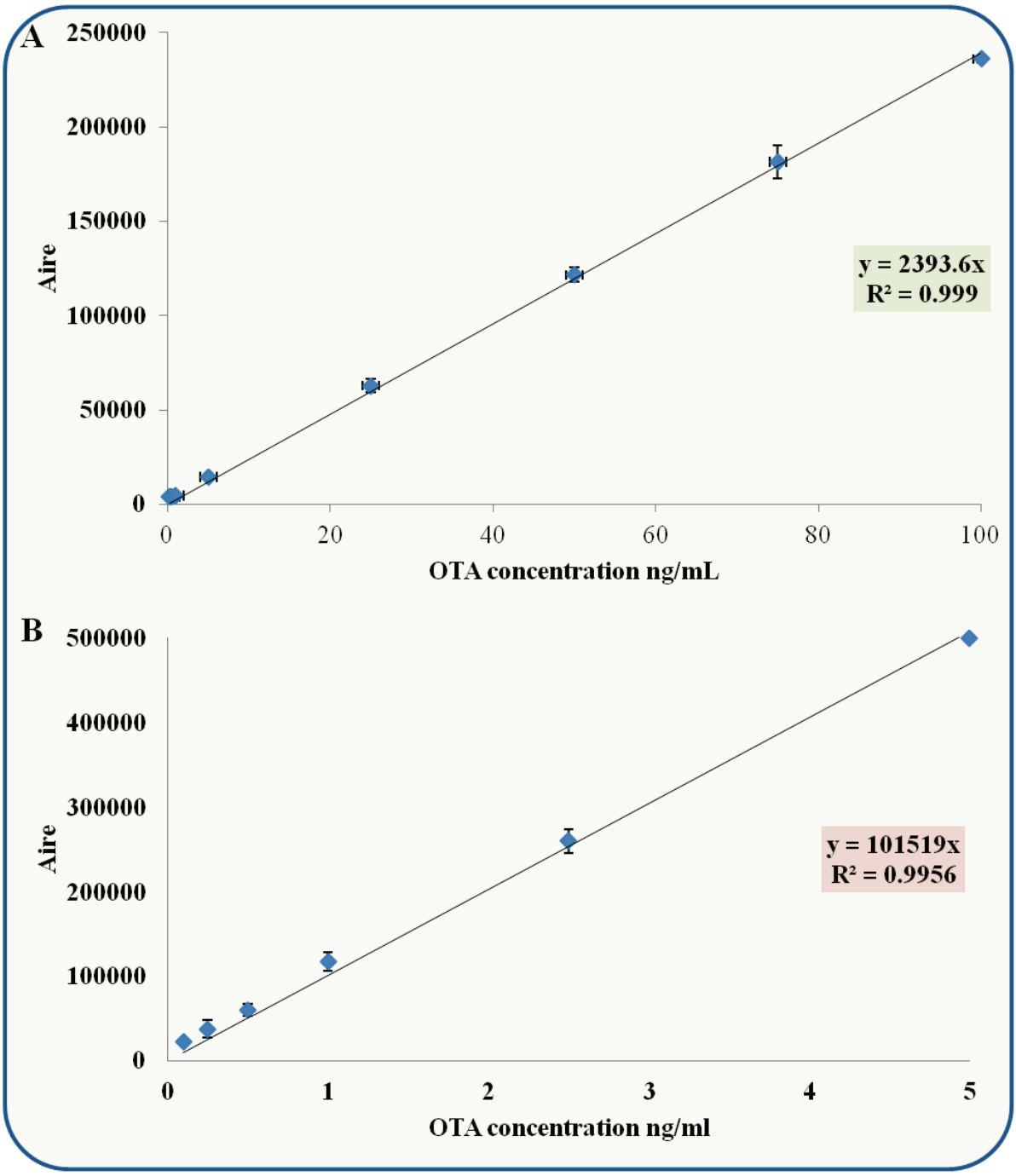
**Standard cutves of qPCR**




**A:** for ITS (Error: 0.0562, Efficiency: 1.728, Slope: -4.212, YIntercept: 9.239, Link: 0.00000119), **B:** for atoui (Error: 0.146, Efficiency: 1.909, Slope: -3.561, YIntercept: 21.96, Link: 0.0000730)

# Appendix 11

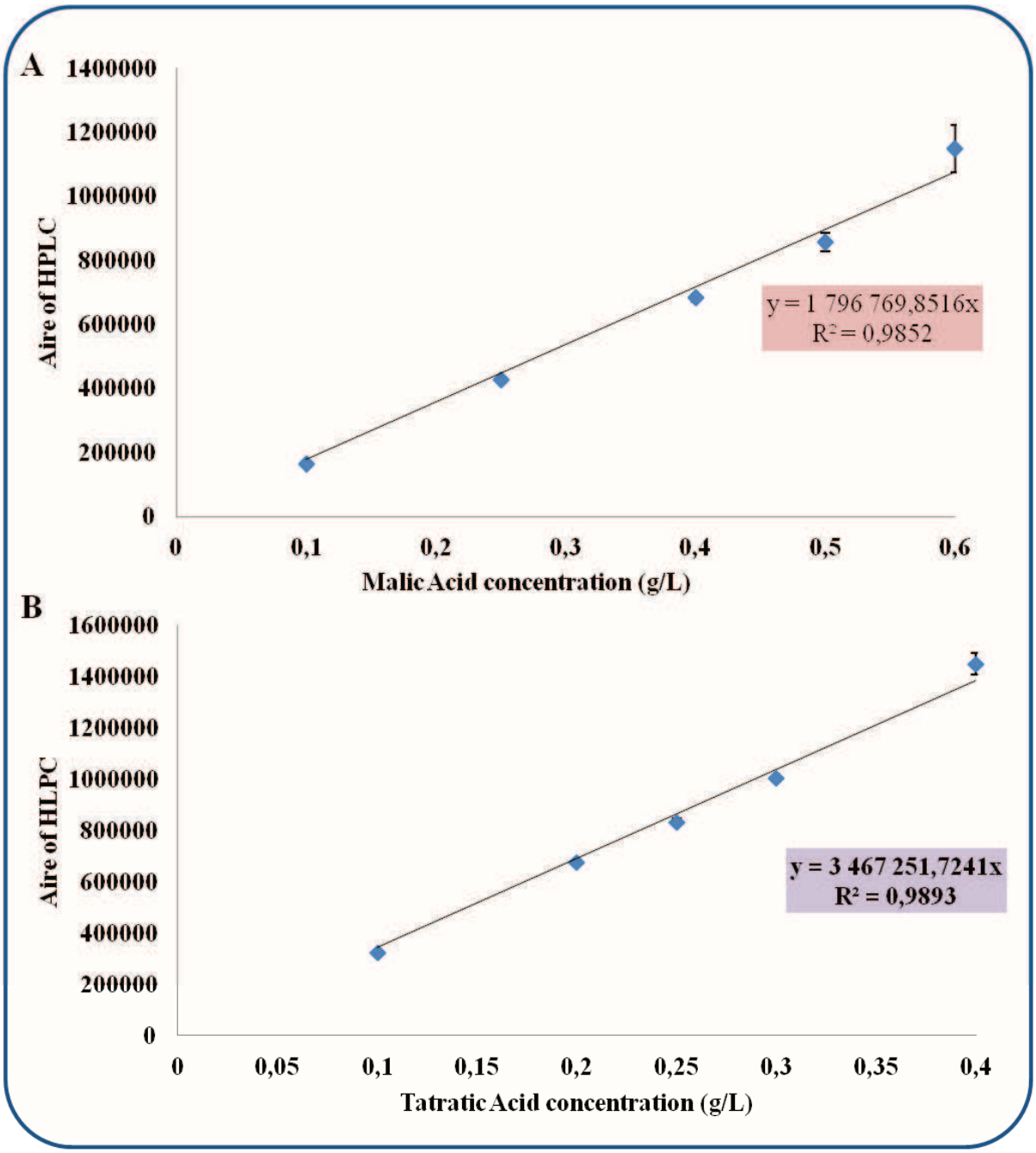
**Standard curvesof OTA**




**A:** for the extracted OTA from cultural media, **B:** for the OTA extracted from grape juices.

# Appendix 12

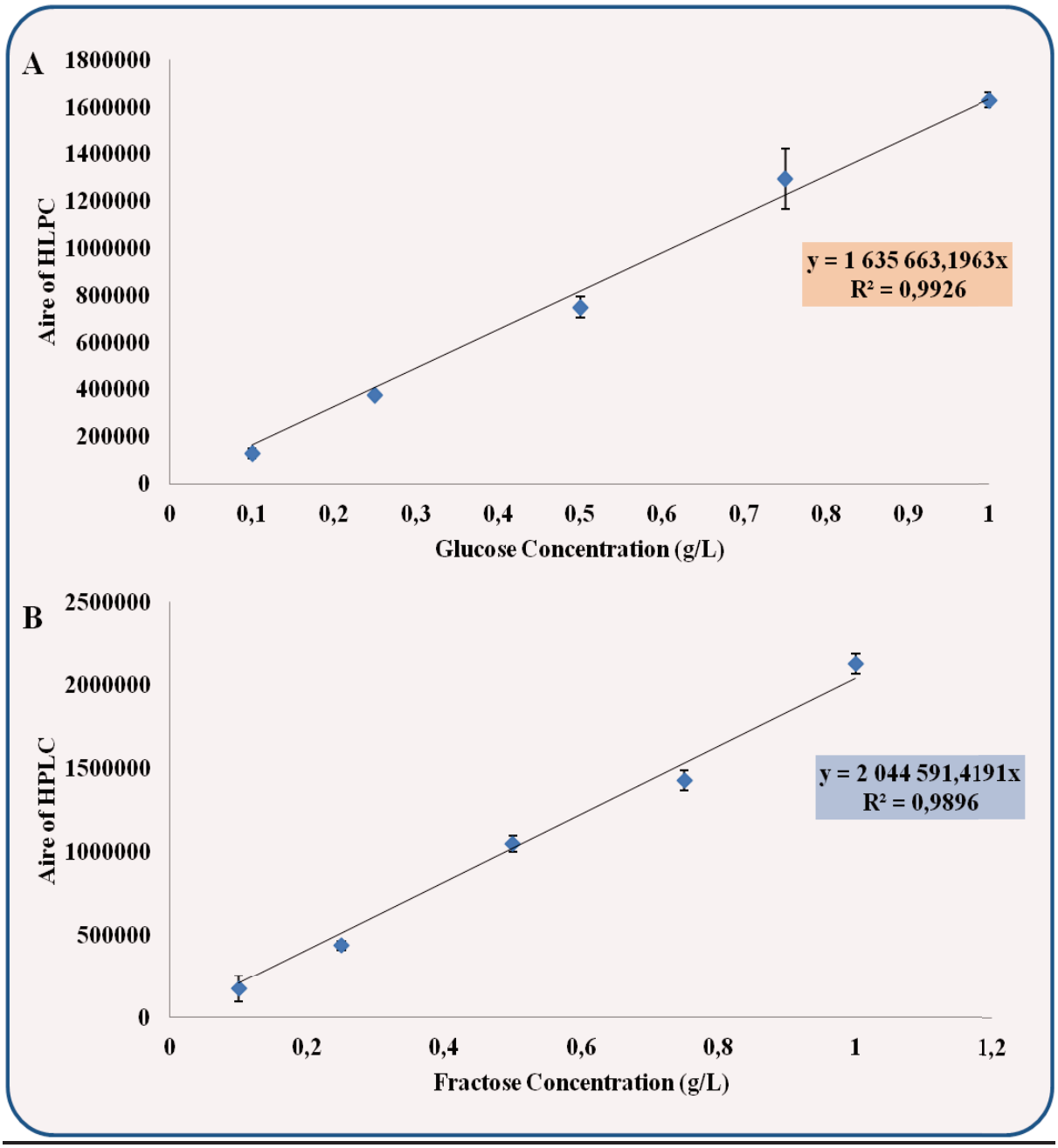
**Standard curvesof organic acids**




**A:** for malic acid, **B:** for fractose

# Appendix 13

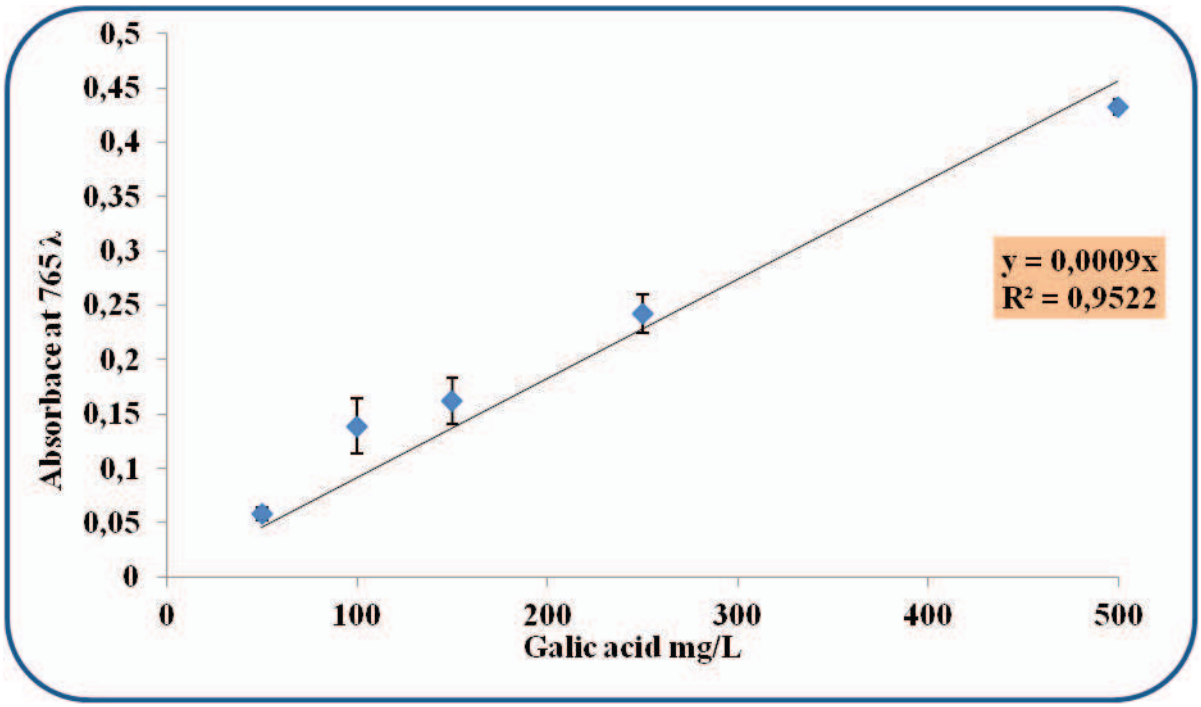
**Standard curvesof sugars**




**A:** for glucose, **B:** for fractose

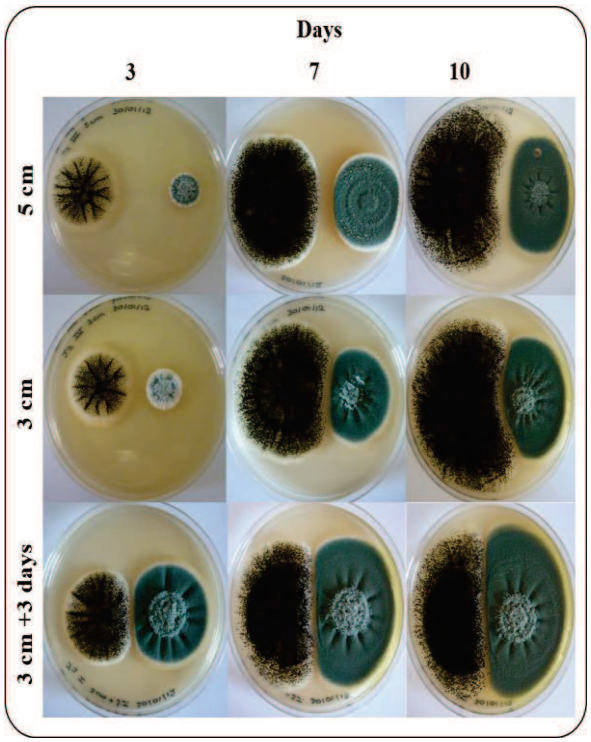
# Appendix 14

**Standard curvesof polyphenols (galic acid)**

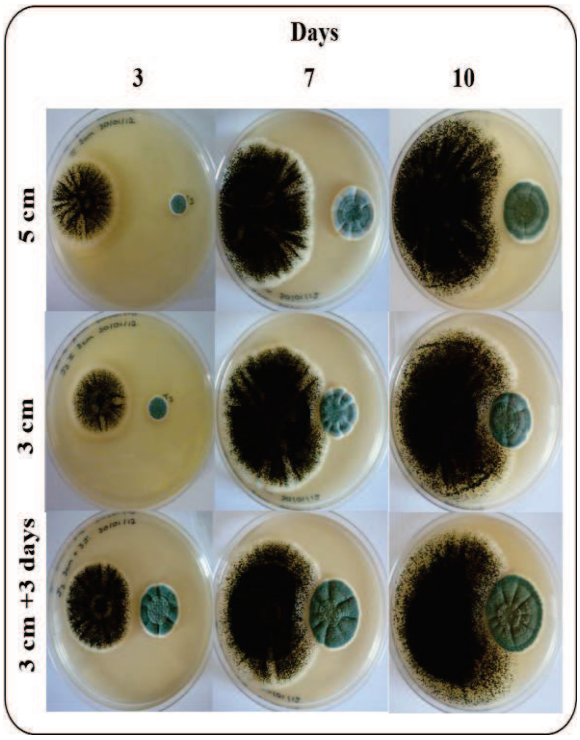


## Appendix 15

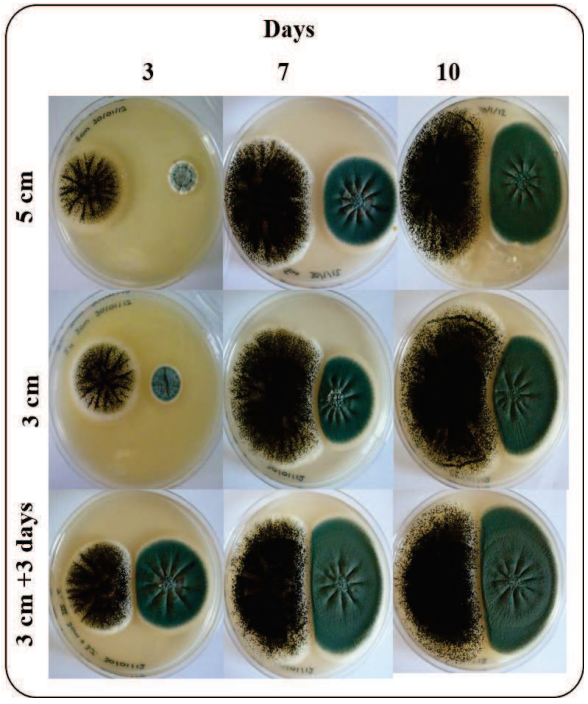
**Direct challenge between *A. carbonarius* and strains isolated from grape juice treated with Stifénia on PDA media with different distances and inoculation times**



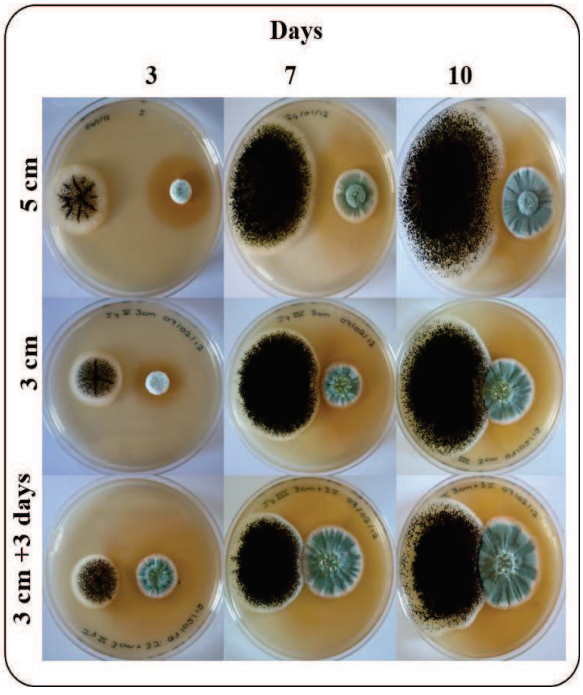

J2




J3



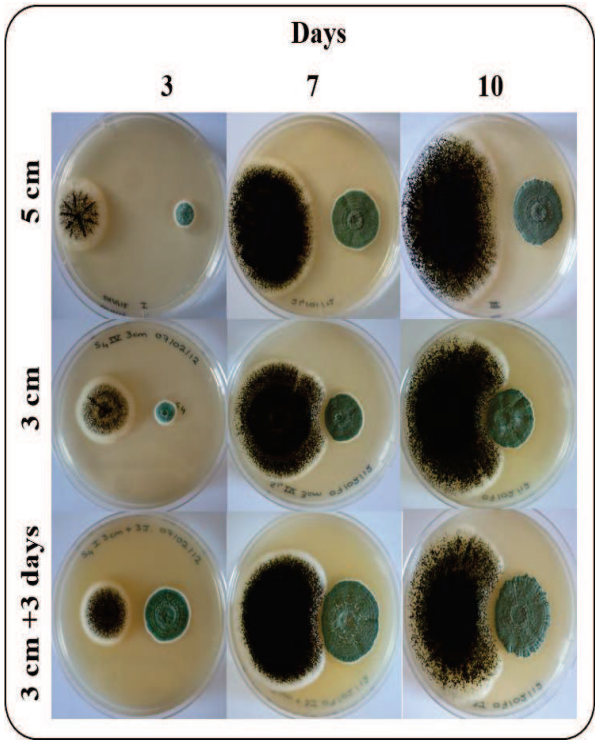

J4



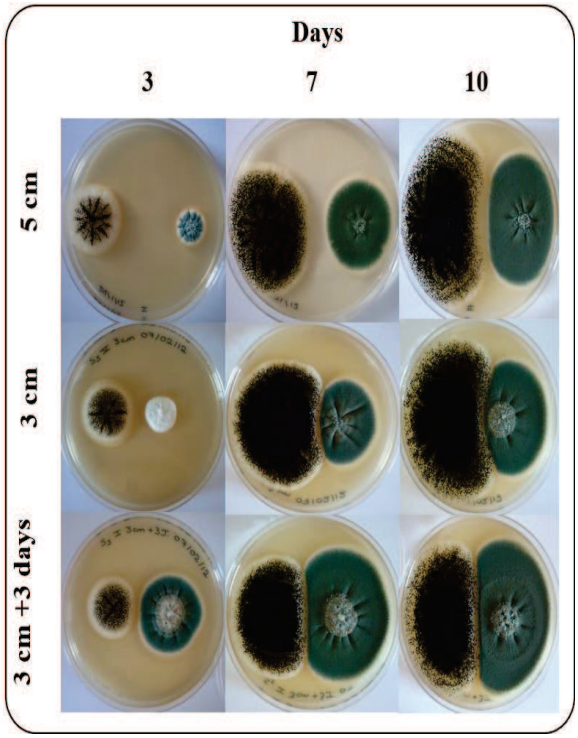

J7

## Appendix 16

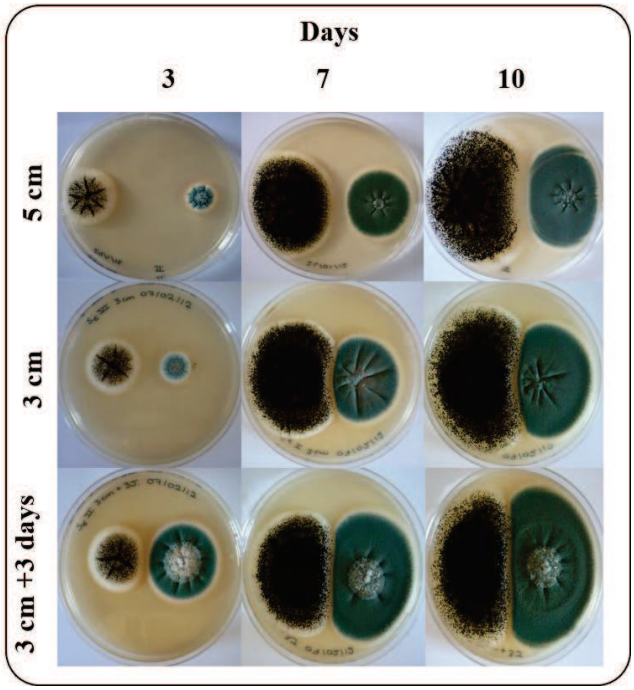
**Direct challenge between *A. carbonarius* and strains isolated from grape stalk treated with Stifénia on PDA media with different distances and inoculation times**




S4




S5




S6

# Appendix 17

**Equation for Kovacs Index**

$$I^T = 100 \left( \frac{t^T_{Ri} - t^T_{Rz}}{t^T_{R(z+1)} - t^T_{Rz}} + Z \right)$$

Where:

| | | |
|---|---|---|
| $I^T$ | = | Index for retention of GC analyses at certain temperature |
| $t^T_{Ri}$ | = | Retention Time of sample peak. |
| $t^T_{Rz}$ | = | Retention Time of peak of n-alkane eluted immediately before sample peak. |
| $t^T_{R\,(z+1)}$ | = | Retention Time of peak of n-alkane eluted immediately after sample peak |
| **Z** | = | Carbones Number peak of n-alkane eluted immediately before sample peak |

# Appendix 18

**Curve hydrocarbons by GC-MS used for experimental calculations Kovacs Index**

Analysis program (55 min, total time): the Initial Temperature (IT) 40°C was increased from 3°C to 250°C using isotherm of 5 min at the final temperature.

| Carbon number | RT (min) | Carbon number | RT (min) |
|---|---|---|---|
| 9 | 9.86 | 19 | 36.66 |
| 10 | 13.363 | 20 | 38.637 |
| 11 | 16.644 | 21 | 40.497 |
| 12 | 19.711 | 22 | 42.293 |
| 13 | 22.577 | 23 | 44.015 |
| 14 | 25.262 | 24 | 45.708 |
| 15 | 27.811 | 25 | 47.713 |
| 16 | 30.186 | 26 | 50.186 |
| 17 | 32.452 | 27 | 53.361 |
| 18 | 34.61 | | |

# Appendix 19

**Experimental calculations of Kovacs index**

| RT | Identified compounds by CPG-MS | $t^T_{Rz}$ | $t^T_{R(z+1)}$ | z | $I^T$ | IK Lit |
|---|---|---|---|---|---|---|
| 10.266 | Trnas, trans, 2,4-Hexadienal | 9.86 | 13.363 | 9 | 912 | 909 |
| 12.092 | Benzaldehyde | 9.86 | 13.363 | 9 | 964 | 936; 970 |
| 14.303 | Limonene | 13.363 | 16.644 | 10 | 1029 | 1028;1074 |
| 14.523 | Benzyl alcohol | 13.363 | 16.644 | 10 | 1035 | 1032 |
| 14.976 | 3-Carene | 13.363 | 16.644 | 10 | 1049 | 1011 |
| 15.293 | Trnas, 2-Octenal | 13.363 | 16.644 | 10 | 1059 | 1062 |
| 15.717 | Cyclopropane. pentyl- | 13.363 | 16.644 | 10 | 1072 | |
| 16.304 | 4-Nonanol | 13.363 | 16.644 | 10 | 1090 | IS |
| 16.635 | 1.6-Octadien-3-ol. 3.7-diméthyl- | 13.363 | 16.644 | 10 | 1100 | 1101; 1098 |
| 16.793 | Trans-2-undecen-1-ol | 16.644 | 19.711 | 11 | 1105 | |
| 17.046 | Phenylethyl alcohol | 16.644 | 19.711 | 11 | 1113 | 1120; 1117 |
| 18.406 | 5-méthy. Cyclohexanone | 16.644 | 19.711 | 11 | 1157 | |
| 18.502 | Trans, 6-Nonenal | 16.644 | 19.711 | 11 | 1161 | |
| 19.487 | Methyl Salicylate | 16.644 | 19.711 | 11 | 1193 | 1198; 1193 |
| 19.887 | Cyclodecanol | 19.711 | 22.577 | 12 | 1206 | |
| 20.291 | 1-Cyclohexene-1-carboxaldehyde. 2.6.6-trimethyl- | 19.711 | 22.577 | 12 | 1220 | 1224 |
| 20.575 | Propanoic acid.4-hexen-1-yl ester | 19.711 | 22.577 | 12 | 1230 | |
| 21.157 | 1.6-Octadien-3-ol.3.7-dimethyl | 19.711 | 22.577 | 12 | 1250 | |
| 21.510 | 13-tetradecenal | 19.711 | 22.577 | 12 | 1263 | 1608 |
| 21.659 | 2.6-Octadienal. 3.7-dimethyl | 19.711 | 22.577 | 12 | 1268 | |
| 23.980 | Eugenol | 22.577 | 25.262 | 13 | 1352 | 1356; 1358 |
| 24.226 | Cis-9-Octadecenal | 22.577 | 25.262 | 13 | 1361 | |
| 25.828 | Caryophyllene | 25.262 | 27.811 | 14 | 1422 | 1418; 1420 |
| 26.424 | Cis 5.9-Undecadien-2-one. 6.10-dimethyl | 25.262 | 27.811 | 14 | 1446 | 1438 |
| 27.167 | Butanedioic acid. bis (2-methylpropyl) | 25.262 | 27.811 | 14 | 1475 | |
| 27.265 | ß-Ionone | 25.262 | 27.811 | 14 | 1479 | 1456 |
| 29.860 | Diethyl phthalate | 27.811 | 30.186 | 15 | 1586 | 1603; 1585 |
| 40.653 | Phytol | 40.497 | 42.293 | 21 | 2109 | 2114; 2128 |

**RT:** Retention Time; **IS:** Internal Standard

## Appendix 20

**The volatile compounds of Moldévre grape cultiver leaves treated by Stifénia**

| No. | Identified compounds by CPG-MS | Retention time |
|---|---|---|
| 1 | Trans, trans, 2.4-Hexadienal | 10.266 |
| 2 | Benzaldehyde | 12.092 |
| 3 | Limonene | 14.303 |
| 4 | Benzyl alcohol | 14.523 |
| 5 | 3-Carene | 14.976 |
| 6 | Trans 2-Octenal | 15.293 |
| 7 | Cyclopropane. pentyl- | 15.717 |
| **8** | **4-Nonanol** | **(IS) 16.304** |
| 9 | 1.6-Octadien-3-ol. 3.7-diméthyl- | 16.635 |
| 10 | Trans-2-undecen-1-ol | 16.793 |
| 11 | Phenylethyl alcohol | 17.046 |
| 12 | 5-méthy. Cyclohexanone | 18.406 |
| 13 | Trans 6-Nonenal | 18.502 |
| 14 | Methyl Salicylate | 19.487 |
| 15 | Cyclodecanol | 19.887 |
| 16 | 1-Cyclohexene-1-carboxaldehyde. 2.6.6-trimethyl- | 20.291 |
| 17 | Propanoic acid.4-hexen-1-yl ester | 20.575 |
| 18 | 1.6-Octadien-3-ol.3.7-dimethyl | 21.157 |
| 19 | 13-tetradecenal | 21.510 |
| 20 | 2.6-Octadienal. 3.7-dimethyl | 21.659 |
| 21 | Eugenol | 23.980 |
| 22 | Cis 9-Octadecenal | 24.226 |
| 23 | Caryophyllene | 25.828 |
| 24 | Cis 5.9-Undecadien-2-one. 6.10-dimethyl | 26.424 |
| 25 | Butanedioic acid. bis (2-methylpropyl) | 27.167 |
| 26 | ß-Ionone | 27.265 |
| 27 | Diethyl phthalate | 29.860 |
| 28 | Phytol | 40.653 |

**IS:** Internal Standard

## Appendix 21

**Grape *Molvédre* straine Leaves (Sti); leaves treated by Stifénia (Sca); leaves treated by Stifénia (T)**

| N° | Sti µg/kg | Standard error | Sca µg/kg | Standard error | FEN 560 µg/kg | Standard error |
|---|---|---|---|---|---|---|
| 1 | 96.90 | 56.58 | 232.71 | 25.71 | 0 | 0 |
| 2 | 396.55 | 28.73 | 276.99 | 9.11 | 2.80 | 0.34 |
| 3 | 28.89 | 14.67 | 87.81 | 17.83 | 0.00 | 0.00 |
| 4 | 55.75 | 18.04 | 22.33 | 0.80 | 0.00 | 0.00 |
| 5 | 5.14 | 1.74 | 4.84 | 0.88 | 0.00 | 0.00 |
| **6** | **121.27** | **13.92** | **49.36** | **5.09** | **3.32** | **0.55** |
| 7 | 25.67 | 56.77 | 20.90 | 1.29 | 0.00 | 0.00 |
| 8 | 184.00 | 0.00 | 184.00 | 0.00 | 46.00 | 0.00 |
| 9 | 26.91 | 6.79 | 18.47 | 4.61 | 0.00 | 0.00 |
| 10 | 196.08 | 142.97 | 182.24 | 31.48 | 12.63 | 1.22 |
| 11 | 20.58 | 5.31 | 18.86 | 26.28 | 0.00 | 0.00 |
| 12 | 5.82 | 3.63 | 12.79 | 1.07 | 0.00 | 0.00 |
| **13** | **74.07** | **4.84** | **13.34** | **2.59** | **1.04** | **0.22** |
| 14 | 11.95 | 7.56 | 23.43 | 2.94 | 0.00 | 0.00 |
| 15 | 16.68 | 4.65 | 33.97 | 4.50 | 2.10 | 0.13 |
| 16 | 22.51 | 12.22 | 37.70 | 10.03 | 0.00 | 0.00 |
| 17 | 26.87 | 16.68 | 70.68 | 11.01 | 5.22 | 2.44 |
| 18 | 100.54 | 41.23 | 186.37 | 2.23 | 0.00 | 0.00 |
| 19 | 19.10 | 12.75 | 25.69 | 1.40 | 3.64 | 0.51 |
| 20 | 12.60 | 5.83 | 13.01 | 1.64 | 0.00 | 0.00 |
| 21 | 31.22 | 3.57 | 5.24 | 0.45 | 0.00 | 0.00 |
| 22 | 19.38 | 1.30 | 6.62 | 0.93 | 0.90 | 0.07 |
| 23 | 15.55 | 8.94 | 56.47 | 7.93 | 0.00 | 0.00 |
| 24 | 40.09 | 20.89 | 86.74 | 7.66 | 1.20 | 0.36 |
| 25 | 20.03 | 9.57 | 19.52 | 2.54 | 0.00 | 0.00 |
| 26 | 3.86 | 1.86 | 7.94 | 1.59 | 0.00 | 0.00 |
| 27 | 3.12 | 2.36 | 2.51 | 0.64 | 0.00 | 0.00 |
| 28 | 18.73 | 7.58 | 17.61 | 0.59 | 0.00 | 0.00 |

## ***Published researches:***

| | |
|---|---|
| **1** | **Ahmed-Hoda M.H., M. Digiaro and G.P. Martelli (2004).** Viruses and virus diseases of grapevine in Egypt. EPPO Bulletin, 34(3):395-398. |

## Submitted articles

| | |
|---|---|
| **1** | **Hoda AHMED, A. STEMMELEN, N. DURAND, C. STRUB and S. SCHORR-GALINDO (2013).** Influence of preharvest treatments (chemical, fungal and yeast bioagents, and elicitor) on the OTA juice content and physical and chemical characteristics of the *Mourvèdre* grape cultivar. ***Journal of Agricultural and Food Chemistry***. Manuscript ID, jf-2013-044327. |
| **2** | **Hoda AHMED, F. HILAIRE, G. BASTARD, C. STRUB and S. SCHORR-GALINDO (2013).** First report: *Penicillium adametzioides*, an emerging biocontrol agent to Ochratoxin producing fungus in grapes as a result of natural product pre-harvest treatment***. Food Control.*** |
| **3** | **Hoda AHMED, N. SALAH, G. BASTARD, C. STRUB and S. SCHORR-GALINDO (2013).** Study of vine preharvest treatments to control *Aspergillus* and their effects on the fungal ecosystem and OTA contamination. **Journal of Crop Protection** |
| **4** | **Hoda AHMED, C. DACHOUPAKAN, C. STRUB and S. SCHORR- GALINDO (2013).** Plant defense stimulation against OTA producing fungus (*Aspergillus carbonarius*) on grapes. **Crop Protection** |

# Conferences

## Oral presentations

| | |
|---|---|
| 1 | **Ahmed-Hoda M.H., M. Digiaro and G.P. Martelli (2003).** A preliminary survey for grapevine viruses in Egypt. Proceeding of the 14th ICVG conference, Locorotondo 12-17th September, 2003. |
| 2 | **Hoda Ahmed, R. Ratomahenina, J. C. Baccou et S. Schorr-Galindo (2010).** Effect of Pre-Harvest Treatments on the OTA Production and Fungal Flora Isolated from Grape « RAFLES ». **International workshop " Mycotoxicological risks in Mediterranean countries: economic impact, prevention, management and control". 25-27 October 2010 *Cairo, Egypt*** |
| 3 | **Hoda Ahmed, F. Hilaire, J.C. Baccou, C. Strub and S. Schorr-Galindo (2012).** Impact of Vine Biocontrol and Plant Defense Stimulation on the Fungal Ecosystems and Ochratoxin A Contamination of Grapes. **International congress of Natural Products and Biocontrol. 19-21th September 2012, *Perpignan FRANCE***. |
| 4 | **Hoda Ahmed' F. Hilaire, N. Salah, G. Bastard, C. Strub and S. Schorr-Galindo (2013).** First report: *Penicillium adametzioides* an emerging biocontrol agent to ochtatoxin producing fungi in grapes as a result of natural product pre-harvest treatment. **10th International Conference 'Mycotoxins and moulds'. 27-28 June 2013, *Bydgoszcz, Poland.*** |
| 5 | **Hoda Ahmed, N. Salah, G. Bastard, C. Strub and S. Schorr-Galindo (2013).** Impact of vine biocontrol and elicitor on the fungal ecosystems and the Ochratoxin A contamination of grapes using DGGE and q-PCR. **2013 EFFoST Annual Meeting: Bio-based Technologies in the Context of European Food Innovation Systems. 12-15th November 2013, *Bologna, Italy*** |

## ***Conferences** (continued)*

### ***Posters***

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|---|---|
| **1** | **Hoda Ahmed, F. Hilaire, C. Strub, S. Schorr-Galindo (2012).** Impact of alternative preharvest treatments on fungal ecosystems of grapes in order to reduce the Ochratoxin A contamination. **EFFoST annual Meeting, 20-23 November 2012, *Montpellier, France*.**<br><br>http://elsevier.conference-services.net/programme.asp?conferenceID=2939&action=prog_presenters |
| **2** | **Hoda Ahmed, C. Strub, R. Ratomahenina, J. C. Baccou et S. Schorr-Galindo (2012).** Impact de traitements pré-récolte avec antagonistes ou éliciteur sur l'occurrence et la toxinogenèse des contaminants fongiques ochratoxinogènes de raisin. **Journée Mycotoxines 2012. 19 Janiver 2012, *Bordeaux, France***<br><br>*www7.bordeaux-aquitaine.inra.fr/var/.../P10_Hoda%20AHMED.pdf* |

### ***Book Chapters***

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|---|---|
| **1** | **Hoda AHMED, C. STRUB and S. SCHORR- GALINDO** (In progress). Rapid Molecular Techniques as Safe Control Method for Mycotoxins Molds: Application on Grapes. *In*: El Sheikha A. F. (Ed), Molecular Techniques in Food Microbiology. 6000 Broken Sound Parkway NW Suite 300, Boca Raton, FL 33487, USA: Science Publishers Inc., CRC Press. |

# RESUME

L'industrie viti et vinicole est affectée par la présence d'Ochratoxine A (OTA) dans ses produits en raison de la contamination des raisins par des souches fongiques d'Aspergilli section Nigri. Le vin et le jus du raisin sont considérés comme les deuxièmes contributeurs en Europe à l'ingestion par la consommateus de cette mycotoxine ayant des effets néphrotoxiques, neurotoxiques et tératogènes. La Communauté Européenne a établi une limite maximale acceptable d'OTA dans le vin et le jus de raisin à 2 mg/kg. *A. carbonarius*, suivi par *A. niger*, joue un rôle majeur dans la contamination par l'OTA des raisins français. Les régions du sud de la France, proches de la Méditerranée, en particulier le Languedoc- Roussillon, ont les plus concernées par cette contamination des productions viticoles par l'OTA. L'objectif principal de ce travail est de proposer des méthodes alternatives aux traitements phytosanitaires chimiques pour contrôler la contamination en OTA dans les raisins et le vin, dans le respect de l'environnement et la santé des principaux intervenants de la filière (producteurs et consommateurs). Différents traitements ont été comparés dans le vignoble expérimental de Pech-Rouge de l'INRA à Narbonne avec appui de l'IFV après contamination artificielle du cépage Mourvèdre par une souche d'*A. carbonarius* fortement productrice d'OTA (champignon produisant l'OTA: CP-OTA) précédement isolée de raisin: un fongicide chimique (Scala®) servant de témoin conventionnel et 3 traitement alternatifs, un extrait de plante agissant comme éliciteur (Stifénia®), et *Saccharomyces cerevisiae* et *Trichoderma atroviride* utilisés comme agents biologiques antagonistes. Deux parcelles non traitées ont servi de témoins, l'une a été artificiellement contaminée. Des études microbiologiques, chromatographiques, physico-chimiques et moléculaires ont été réalisées afin d'évaluer l'impact des différents traitements sur la teneur en OTA dans le jus de raisin ainsi que l'effet sur les écosystèmes microbiens, en particulier la biodiversité des champignons Aspergilli noirs. Les études microbiologiques ont montré 66,6% et 71,4 % de réduction de l'occurrence des aspergilli noirs dans les jus de raisin et les rafles respectivement après traitement avec l'éliciteur ainsi qu'une réduction de la présence des souches aspergilli noires de 99%. La Q-PCR utilisant des amorces spécifiques d'*A. carbonarius* a permis d'estimer l'effet des différents traitements sur la présence du CP-OTA. La présence la plus faible de souches d'*Aspergillus carbonarius* a été obtenue pour le traitement par élicitation. La PCR-DGGE a montré un aperçu de l'effet des différents traitements sur l'écosystème fongique. Ainsi, une grande similarité entre les modalités non contaminée et contaminée + traitée par éliciteur (76 %), suivie par la modalité contaminée et traitée par la levure et la modalité la plus faible était celle contaminée. L'analyse des résultats de PCR-DGGE a confirmé les résultats obtenus à l'aide des méthodes microbiologiques classiques. En effet, une proportion élevée de microorganismes fongiques a été isolée des baies traitées par le Stifénia® alors que ces moisissures n'ont pas été mises en évidence sur les vignobles ayant subi les autres traitements. Ces résultats pourraient rapidement évaluer l'effet du traitement différent sur l'écosystème fongique sur les raisins. La qualité du jus de raisin issus des baies du cépage Mourvèdre a été caractérisée par l'étude de leur morphologie (diamètre et épaisseur de la peau), la mesure de la couleur et l'analyse des principaux composés chimiques solubles des jus de raisin (sucres, acides et polyphénols totaux). La teneur en OTA dans les jus de raisins a été caractérisée par HPLC couplé a un détecteur fluorimétrique. Une réduction significative de la teneur en OTA dans les jus (de 38 à 42 %) a été observée pour les traitements avec le fongicide chimique, avec la levure en tant qu'agent biologique et avec l'éliciteur permettant, une amélioration notable de la qualité sanitaire des jus. Les deux traitements biologiques et le traitement par éliciteur ont considérablement augmenté l'épaisseur des peaux de baies (quantités de cire, et de cuticule et épaisseur de la peau de la baie). Cette augmentation pourrait être due à des mécanismes de résistance des baies de raisin à certains agents pathogènes et pourraient expliquer simultanément la réduction OTA ainsi que l'amélioration de la qualité globale du jus de raisin, avec . Une étude plus approfondie a été menée afin de comprendre le mode d'action du Stifénia® concernant la réduction de l'occurrence des aspergilli noirs et de la contamination par l'OTA, alors que les souches isolées de ces mêmes baies ont encore une capacité élevée de production d'OTA. Le profil des composés organiques volatils (COV) des feuilles des traitements chimique et éliciteur ont été déterminés. Certains COV ont augmenté de façon significative dans le traitement Stifénia® par rapport au traitement chimique. Le trans-6-nonenal et trans-2-octenal, rencontrés à plus haute concentration dans les feuilles traitées au Stifénia® que dans celles traitées chimiquement ont montré une activité antifongique sur la croissance du CP-OTA et sur la production d'OTA àde faibles concentrations (0,5 et 0,05 $mg.L^{-1}$, respectivement). D'autre part, aucune activité antifongique de la poudre Stifénia® sur la croissance du mycélium du CP-OTA ou sur la production d'OTA n'a été mesurée. Cela pourrait expliquer en partie

le mode d'action de défense de la plante qui produit des COV au niveau des feuilles, ces molécules induisant des modification métaboliques sur le CP-OTA engendrant les teneurs d'OTA résiduites dans les raisins. D'autres tests d'antagonisme ont été menés en utilisant des moisissures non aspergilli isolées du traitement éliciteur, à la place du Stifénia®, contre le CP-OTA. L'autre possibilité envisagée de défense induite par le Stifénia® serait la promotion de souches microbiennes antagonistes des souches fongiques mycotoxinogènes, lors des études de diversité ce traitment étant en effet celui qiu permet une modification plus nette de l'écosystème fongique. Certaines souches isolées de raisin et rafles de parcelles traitées au Stifénia®, *Penicillium adametzioides* (S3) et un *Penicillium sp.* (J2) ont eu un effet positif sur la croissance du mycélium du CP-OTA et aussi un effet de réduction de sa production d'OTA. Ces souches pourraient être utilisées comme agents de lutte biologique.. L'effet éliciteur du Stifénia® pourrait se résumer par l'amélioration de la teneur en polyphénols des baies et en modifiant le profil des COV des feuilles de vigne, en augmentant la proportion de composés ayant un effet antifongique sur le CP-OTA. De plus, le traitement Stifénia a amélioré les caractéristiques physiques des baies avec l'augmentation des couches de cire et de l'épaisseur cuticule qui pourraient augmenter la résistance des baies aux blessures et aux perforations par parasites qui sont des voient d'entrée des champignons toxingènes. Le traitement par le Stifénia® permet donc par tous ces mécanismes de diminuer la contamination des jus de raisins par l'OTA.

# ABSTRACT

The grape and wine industry is affected by the presence of Ochratoxin A (OTA) in its products because of contamination of grapes by strains of Aspergillus section Nigri. Wine is considered as the second contributor in Europe to the ingestion of this mycotoxin with nephrotoxic, neurotoxic and teratogenic effects. The EC has set the max. limit for OTA in wine and grape juice to 2 mg/Kg. A. carbonarius, followed by A. niger aggregate, plays a major role in the OTA contamination of French grapes. Southern Mediterranean regions, particularly Languedoc-Roussillon had the greatest amount of OTA contamination. The main objective of this work is to provide non-chemical alternative methods to control OTA contamination in grapes and wine, in respect with environment and stakeholder health (producers and consumers). Different treatments were compared in experimental vineyard PECH-ROUGE of INRA and IFV, Narbonne, France on near parcels after artificial contamination of the Mourvèdre grape cultivar by A. carbonarius: (OTA producing fungus; OTA-PF) a chemical fungicide (Scala®); Saccharomyces cerevisiae and Trichoderma atroviride as antagonists; and a plant extract as elicitor (Stifénia®). Two untreated parcels served as controls, one was artificially contaminated. Microbiological, chromatographically, physic-chemical and molecular studies were conducted in order to evaluate the impact of different treatment on the OTA content in grape juice and the impact on the fungal ecosystems and black aspergilli biodiversity. The microbiological studies showed 66.6% and 71.4% reduction percent of the black aspergilli incidence at grape juice and grape stalk respectively within the elicitor treatment and reduced the CFU of the black aspergilli strains to 99%. Q-PCR using universal and specific primers for A. carbonarius had estimated the effect of the different treatments on the presence of OTA-PF .The lowest occurrence of black aspergilli strains was obtained for treatment used elicitation. While, the DGGE gave an overview on their effect on the fungal ecosystem, that showed higher similarity between the non-contaminated and elicitor treatment (76%) followed by yeast one and the lowest treatment was the contaminated one. Whereas the DGGE couldn't be used as a single method for evaluating the ecosystem that depending on the band's number not on the to the different treatments that was confirmed with the results obtained from traditional methods of isolation that showed the elicitor treatment had a higher proportion of fungal species not isolated in the other treatments. These results could rapidly evaluate the effect of different treatment on the fungal ecosystem on grapes. The OTA content and quality of grape juice in grape berries of the cultivar Mourvèdre was characterized by studying their morphology (diameter and skin thickness), measuring the color and main soluble chemical compound contents in grape juices (sugars, acids and total polyphenols). A significant reduction (38 - 42%) was observed in the OTA juice content by the chemical, yeast bioagent and elicitor treatments with juice safety improvement. The two biological treatments and one elicitor treatment significantly increased the thickness of the berry skins in general (regarding the wax, cuticle layers and skin thickness), which could be related to the enhancement of the disease resistance of the grape berries to certain pathogens and could also simultaneously explain the OTA reduction and the grape juice quality improvement. Further study was conducted in order to understand the

Stifénia® mode of action because the reduction effect of the black aspergilli incidence and the OTA contamination while the isolated strains still have the high ability of producing OTA. The leaf volatile organic compound (VOC) profiles of chemical and elicitor pre-harvest treatments were assessed. Certain VOCs had significantly increased in the plant extract treatment regarding the chemical one. Trans6nonenal and trans2octenal, which recognized in Stifénia® treatment leaves with the highest significant concentration regarding to their concentration with the chemical treatment, have antifungal activity against the OTA-PF growth and OTA production with low concentrations (0.5 and 0.05 mg/L, respectively). No antifungal activity of the Stifénia® powder against the OTA-PF mycelial growth or its OTA produced amounts were measured. That may partially explain the mode of action of plant defence by producing leaf VOCs that induce positive changes on the OTA-PF and its OTA contents in grapes. Another In vitro antagonistic test was performed with Stifénia® non-Aspergillus isolates. Certain strains had a positive mycelial growth effect on OTA-PF colonies and have also a reduction effect on OTA production of OTA-PF, *Penicillium adametzioides* (S3) and another Penicillium strain (J2). This could be accomplished by applying as the elicitor one of the tested fungi with an antagonistic effect on OTA production, such as *P. adametzioides*. The elicitor effect of Stifénia could be summarized in improving the polyphenol content and modifying the volatile compound profile of the grape leaves that performed compounds with antifungal effect on OTA-PF. In addition, Stifénia treatment has improved the berry physical characteristics as the wax and cuticle masses that could affect the wounding susceptibility. Last not least positively affecting the OTA contamination