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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF MYCOTOXIN-PRODUCING FUNGI IN TABLE GRAPES FROM TUNISIAN VINEYARDS

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ABSTRACT

Mycotoxins are secondary metabolites produced by fungal species that mainly belong to *Aspergillus, Fusarium, Penicillium* and *Alternaria* genus, which can grow in a variety of crops. *Aspergillus* spp. are fungal pathogens that infect the grape and are known for their production of mycotoxins, in particular ochratoxin A (OTA), which is considered to be nephrotoxic, immunotoxic, and carcinogenic. In this study, black rot symptomatic table grapes berries were collected from cultivars in several grapevine-growing regions of Tunisian grape production areas. One hundred eighteen *Aspergillus* spp. isolates were identified based on the morphological characteristics. The *Aspergillus* species were identified by molecular analysis, using both, Real Time quantitative PCR (qPCR) and qPCR High-Resolution Melting (qPCR-HRM) technologies. Specific primer pair for calmodulin, β-tubulin constitutive housekeeping genes, and polyketide synthase (PKS) genes, involved in OTA pathway, were used to discriminate within '*Aspergillus* section Nigri' species involved in OTA production. Overall, among *Aspergillus* spp. isolates investigated, *Aspergillus carbonarius* was the most predominant (66.1%), followed by *Aspergillus tubingensis* (20.3%) and *Aspergillus niger* (12.7%) with some difference related to the geographic areas and host cultivars. In all *A. carbonarius* isolates, the PKS genes involved in OTA production were amplified, which suggest that these isolates could produce OTA. These results demonstrate that the Tunisian table grapes suffers from contamination of the grape berry surface by '*Aspergillus* section Nigri' fungal producers of OTA. The findings of this study could facilitate better management of the risks posed by OTA to grape products.

RIASSUNTO

Le micotossine sono metaboliti secondari prodotti da specie fungine appartenenti principalmente ai generi *Aspergillus*, *Fusarium*, *Penicillium* e *Alternaria*, pericolose per l'uomo e per molti vertebrati. Le specie appartenenti al genere *Aspergillus* sono patogeni fungini che possono infettare l'uva e i suoi derivati, noti per la loro produzione di micotossine, in particolare ocratossina A (OTA), con attività essenzialmente nefrotossica, immunotossica e cancerogena. In questo lavoro di tesi, bacche affette da marciume nero sono state raccolte da diverse cultivar di vite allevate in diverse località della Tunisia. Dalle bacche, in base ai caratteri morfologici delle strutture fungine, sono stati caratterizzati 118 isolati appartenenti al gene *Aspergillus* spp. Gli isolati di *Aspergillus* sono stati identificati mediante analisi molecolare, utilizzando le tecnologie PCR quantitativa Real Time (qPCR) e qPCR High-Resolution Melting (qPCR-HRM). In particolare, per discriminare le specie all'interno del gruppo "*Aspergillus* section Nigri" coinvolte nella produzione di OTA, sono state utilizzate coppie di primers specifici per i geni costitutivi calmodulina, e β-tubulina e i geni della polichetide sintasi (PKS), coinvolti nella via metabolica dell'OTA. Complessivamente, tra gli isolati di *Aspergillus* spp., la specie *Aspergillus carbonarius* è risultata predominante (66,1%), seguita da *Aspergillus tubingensis* (20,3%) e *Aspergillus niger* (12,7%) con alcune differenze legate alle aree geografiche e alle cultivar ospiti. In tutti gli isolati di *A. carbonarius*, i geni PKS coinvolti nella produzione di OTA sono stati rilevati, il che suggerisce che questi isolati potrebbero produrre la tossina OTA. Questi risultati dimostrano che l'uva da tavola tunisina è colpita pesantemente della dalle specie fungine appartenenti a "*Aspergillus* sezione Nigri". I risultati di questo studio potrebbero facilitare una migliore gestione dei rischi posti dall'OTA per i prodotti a base di uva.

1. INTRODUCTION

1.1. SIGNIFICANCE OF GRAPE PRODUCTION IN THE WORLD AND TUNISIA

Grapevine (*Vitis vinifera* L.) is considered one of the most cultivated and economically important perennial fruit crops in the world. In addition, grapes and derived products, like wine, show high value of important nutrients. According to the statistical report of the International Organization of Vine and Wine (OIV, 2019), in 2018 the global area under vines was 7.4 million hectares and the world production of grapes was 77.8 million tons. The grapes production of Mediterranean countries represents more than 40% of the world grapes production in 2018 and Italy was the largest producer with 8.6 million tons (OIV, 2019). Indeed, viticulture has begun from the antiquity in the Mediterranean Basin countries and has been directly affected by the Phoenicians and Carthaginians. In Tunisia, the viticultural sector has an old tradition and occupies an important place in agricultural development sector. Currently, the Tunisian vineyards occupy 22,000 ha of which 12,000 ha are related to table grapes cultivars distributed mainly between the governorates of Ben Arous, Nabeul, Bizerte, Manouba and Sidi Bouzid. In year 2019, a total table grapes production in Tunisia was 135,000 tons [Interprofessional Grouping of Fruits (GIFRUIT) (2019)]. Despite the importance of the viticulture sector, the local production remains dependent on weather conditions, fungal diseases and pests that seem to be the main causes of the grapes yield and quality decline in the Tunisian vineyards.

1.2. MYCOTOXIGENIC FUNGI ON TABLE GRAPES

The term mycotoxin was only coined in 1962, after the sudden death of 100,000 young turkeys in England, who had ingested peanut meal contaminated with aflatoxins B1 produced by *Aspergillus flavus.* Bennett (1987), defined mycotoxins as “natural products produced by fungi that evoke a toxic response when introduced in low concentrations to higher vertebrates and other animals by a natural route”. They are secondary metabolites with a low molecular mass (MW ~700 Da) produced by many filamentous fungi belonging to the phylum Ascomycota (Alshannaq and Yu, 2017; Liew and Mohod-Redzwan, 2018). Table grapes are susceptible to infection by filamentous fungi that cause rots and can contaminate products with mycotoxins over the grape chain from field to postharvest.

In the field during maturation, the spoilage agent species, belong to *Botrytis, Aspergillus, Penicillium, Alternaria* and *Rhizopus,* genera increase their incidence. Gray mold caused by

Botrytis cinerea is the most important on grapes. However, when the temperature increases above 37°C, species related to *Aspergillus* section *Nigri*, usually called "black Aspergilli", was predominate (Valero et al., 2005; Somma et al., 2012). *Aspergillus, Penicillium*, and *Alternaria*.

Aspergillus section *Nigri*

Aspergillus section *Nigri* are the most mycotoxins producers', in particular *Aspergillus carbonarius*, *Aspergillus tubingensis* and *Aspergillus niger* (Serra et al., 2006, Rousseaux et al., 2014). They are *Deuteromycota* fungi (imperfect fungi, asexual reproduction) that belong to the Order *Hyphomycetes*, Family *Moniliaceae*, section *Nigri*. In addition to their ability to cause black rot diseases in the field, they can produce ochratoxin A (OTA) (**Table 1**), which is mycotoxin that is attracting increasing attention worldwide (Battilani and Pietri, 2002; Ayoub et al., 2010).

Guzev et al (2006) reported that *A. niger* and *A. carbonarius* are abundant in table grape vineyards in semi-arid warm regions of the Mediterranean basin. *A. carbonarius* is recognized by its large spores and its ability to produce OTA mycotoxin. In the vineyard, berries infected with *Aspergillus* spp. develop brown to black water-soaked lesions and are eventually covered by an abundance of black spores with powdery to charcoal appearance (**Figure 1**).

Figure 1. *Aspergillus carbonarius* rot on grapes berries (Hocking, 2014).

Table 1. *Aspergillus* species producing OTA in grape products in the Mediterranean Area (Covarelli et al., 2015).

Species	Grape Products	Distribution in the Mediterranean Area
A. carbonarius	Grapes, wine, raisins	France, Greece, Israel, Italy, Portugal, Tunisia
A. niger	Grapes, wine, raisins	France, Greece, Israel, Italy, Portugal, Spain
A. ochraceus	Grapes	Italy, Portugal, Spain
A. brasiliensis	Grapes	Portugal, Spain
A. awamori	Grapes	Greece, Israel, Italy, Portugal, Spain
A. tubingensis	Grapes	Italy, Spain
A. aculeatus	Grapes	Italy, Spain
A. japonicus	Grapes	France, Greece, Israel, Italy, Portugal, Spain
A. flavus,	Grapes, must	Lebanon, Tunisia

Penicillium expansum

The specie *Penicillium expansum*, causing blue mold is widely present on decaying organic substances including fruit and vegetables in cold storage (Errampalli, 2014). Blue mold is considered one of the most important postharvest diseases of pome fruits worldwide. Recently, several reports showed that *P. expansum* become one of the main fungi that causes grapes decay (Garcia et al., 2006; Kim et al., 2007; Neri et al., 2010; Sanzani, et al., 2013). Important economic losses related to *P. expansum* have been reported in South Africa and Chile on 'Red Globe' grapes during cold storage, due to injury on berries (Franck et al., 2005). Unlike other fungi, *Penicillium* spp. does not produce appressorium. Then, it is unable to penetrate the intact skin of fruit and vegetables. This fungus crosses the skin of fruit through wounds caused by birds or insects or via lenticels (Errampalli, 2014). *P. expansum* has been shown to produce several

toxic compounds including patulin, which has mutagenic, immunotoxic, and neurotoxic properties (Castoria et al., 2012). According to study of Sanzani et al. (2013) on the influence of the different host on *P. expansum* pathogenicity/virulence, focusing on the relationship with patulin production, the table grape strains were the most aggressive (81% disease incidence) and strongest patulin producers (up to 554 μg/g). However, *Penicillium verrucosum* is reported as main OTA-producing species in temperate and cold climates (Meulenberg, 2016).

Alternaria alternata

Alternaria alternata, has been reported that it can be a causal agent of grapes berries rot in vineyard and in postharvest. Saprotrophic strains of *A. alternata* causes the most important problem, which colonizes the fruit and can produce reasonable amounts of mycotoxins, which exert poisonous effects after consumption by humans (Sanzani et al., 2016). This fungus has been related to food poisoning due to the production of mycotoxins which include alternariol, altenuene, alternariol monomethyl ether, altertoxins and L-tenuazonic acid (Scott, 2001). These toxins lead to problems during DNA replication, because they are potent inhibitors of the DNA-topoisomerase (Fehr et al., 2009) and may lead to DNA strand-breaking (Pfeiffer et al., 2007). In addition, the spores of *A. alternata* are considered the main aero-allergens associated with respiratory diseases and asthma in humans and animals (Downs et al., 2001; Breitenbach and Simon-Nobbe, 2002; Salo et al., 2006).

1.3.MAIN MYCOTOXINS ON TABLE GRAPES

Mycotoxins are secondary toxic metabolites toxic to humans, animals and plants, these toxins are synthesized by fungi and thought to be a kind of chemical defense system of these fungi (Atanda et al., 2013). *Aspergillus* spp., *Penicillium* spp., and *Alternaria* spp., are mycotoxin-producing fungi reported to causes mold on fruits, especially grapes and can produces harmful metabolites (Sanzani et al., 2019). It has been estimated that more than 50% of fruits and vegetables are lost all over the world mainly because of mycotoxin contamination (Gustavsson et al., 2011), which consequently are present in the diet of a large portion of the world's population (Wild and Gong, 2010). Especially in the developing countries, most of these losses occur in postharvest, when the product has a higher value due to the costs of handling (storage, processing) (Sanzani et al.,

2019). Ochratoxin A, patulin and *Alternaria* mycotoxins are considered the most important mycotoxins that contaminate grapes and grape derived products.

1.3.1. Ochratoxin A

Ochratoxin A (OTA) is the most common mycotoxin detected in grapes and grape derived products, such as grape juices, wines and dried vine fruits (Aksoy et al., 2007; Visconti et al., 2008). Several surveys reported that, grape products from the Mediterranean regions of Southern Europe and North Africa were the most contaminated by OTA (Battilani et al., 2003; Lasram et al., 2012). This contamination with OTA is mainly due to the growth of Black Aspergilli on grapes during maturation.

1.3.1.1.Characteristics

OTA is a secondary metabolite of certain filamentous fungi (*Aspergillus* spp. and *Penicillium* spp.) that may be present in food and feed. Consumption of contaminated food or feed may be harmful for humans and animals. OTA is the most toxic and frequently occurring member of ochratoxins group which has the most economical impact (Meulenberg, 2016). OTA is composed of an isocoumarin polyketide and the (modified) amino acid phenylalanine, which are linked via a peptide bond and its chemical name is L-phenylalanine-N - ((5-chloro-3, 4-dihydro-8-hydroxy-3-methyl-1-oxoH-2-benzopyran-7-yl) carbonyl) - (R) – isocoumarin (**Figure 2**).

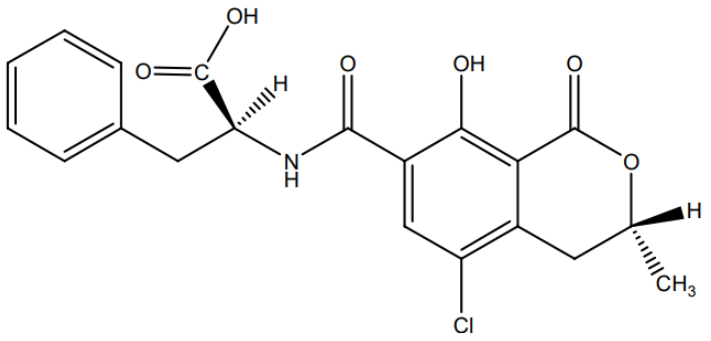

Figure 2. The chemical structure of ochratoxin A (Meulenberg, 2016).

1.3.1.2. Biosynthesis

Several studies have been carried out to predict OTA biosynthetic pathway, although, this latter has not yet been completely elucidated in detail and its regulatory mechanism remained unknown. However, this pathway involves some crucial steps, such as the biosynthesis of the isocoumarin group through the catalyzing action of a polyketide synthase (PKS), its ligation with the amino acid phenylalanine through the carboxyl group in a reaction catalyzed by a peptide synthase, and the chlorination step, but the order of the reactions is not yet well defined (Huff and Hamilton. 1979; Harris and Mantle. 2001; Gallo et al., 2012). In this regard, according to the literature data available, several schemes have been proposed (**Figure 3**). Indeed, the biosynthetic pathway of OTA involves phenylalanine and sodium acetate as starting material and several enzymes. Polyketide synthases (PKSs) have been shown to be the key enzyme involved in OTA biosynthesis (El Khoury and Atoui, 2010).

The production of OTA depends on the genetic traits of the fungi. Polyketide synthases (PKS) and nonribosomal peptide synthase (NRPS) genes are among the genes involved in the biosynthesis of OTA (Gallo et al., 2012). In addition, many ecophysiological studies reported that the biosynthesis of OTA by ochratoxigenic species belonging to *Aspergillus* section *Nigri* such as *A. niger* aggregate and *A. carbonarius* was influenced by the water activity aW, temperature and composition of the nutrient medium (Mitchell et al., 2003; Bellí et al., 2004). Indeed, the temperature and the aW range are more restrictive for OTA production than for fungal growth, both on natural substrates and culture media. Production of OTA in *Aspergillus* generally occurs at temperatures (20 - 30ºC) below the optimal for fungal growth between.

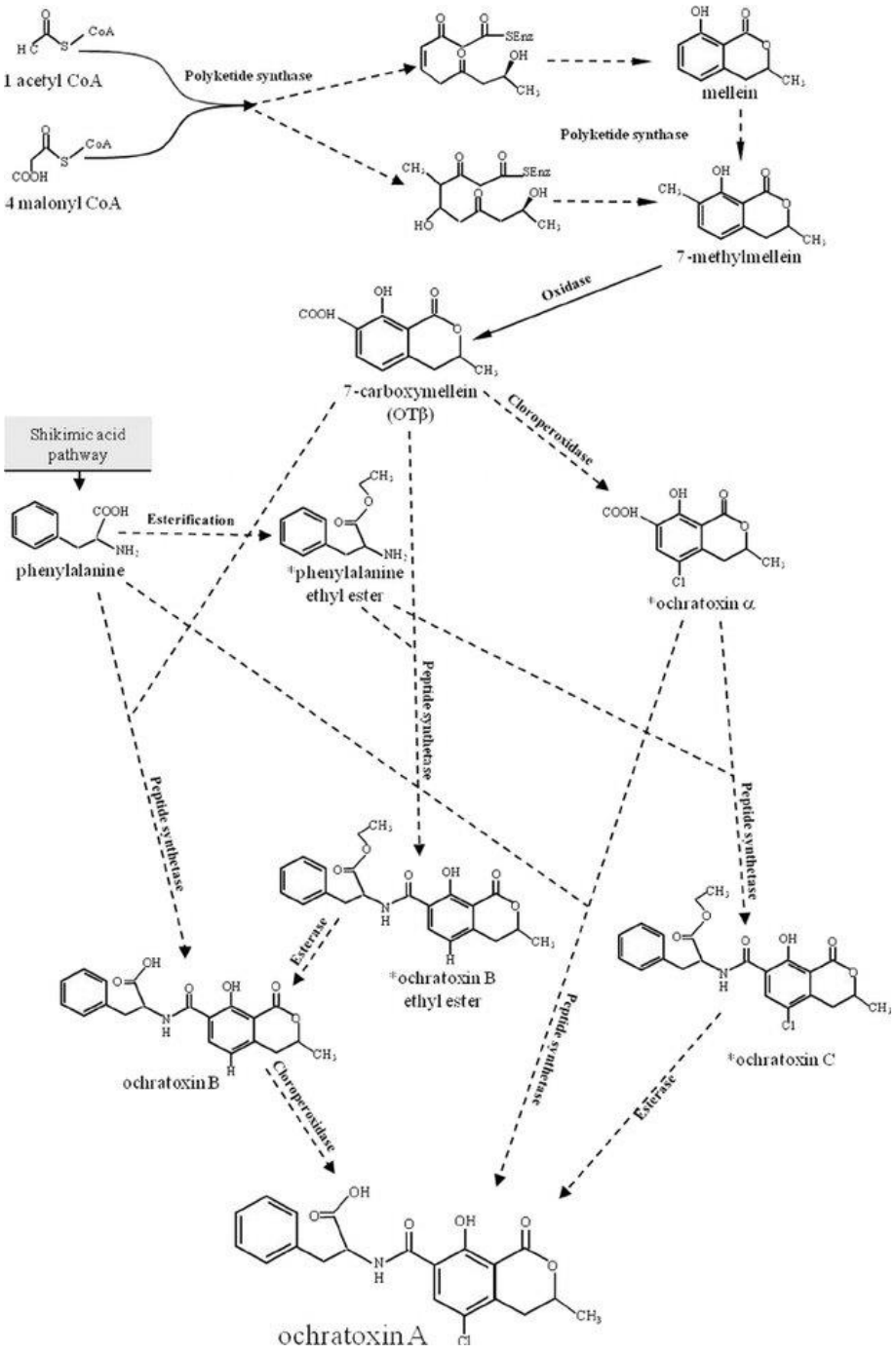

Figure 3. Scheme of the different hypotheses of the OTA biosynthesis pathway (Gallo *et al.*, 2012).

1.3.1.3. Toxicity

The presence of OTA in grape products results in a health risk to consumers since this toxin is classified as a possible carcinogen to humans (group 2B) by the International Agency for Research on Cancer (IARC, 1993). OTA can also associate to cause a fatal human kidney disease referred to Balkan Endemic Nephropathy and urinary tract cancer (Battilani and Camardo, 2015). Moreover, it has demonstrated a number of toxic effects against various experimental animals: neurotoxic, genotoxic, carcinogenic, mutagenic, teratogenic and immunosuppressive effects (Sava et al., 2006; Tozlovanu et al., 2006; Brown et al., 2007; Palma et al., 2007; Rossiello et al., 2008). In humans, other symptoms were recorded include anemia, anorexia, discolored skin, fatigue, headache, and a range of abnormal clinical parameters such as increased clotting time, leukocytes, neutrophils, eosinophils.

Based on the available scientific toxicological and exposure data and on the opinion of the Scientific Panel on Contaminants in Food of the European Food Safety Authority, the European Union has established the maximum tolerable levels of OTA at 2 μg/kg maximum level permitted for OTA in wines, musts, grape juice and 10 μg/kg for raisins but no limits exist for fresh table grapes (European Commission, 2006).

1.3.2. Patulin

Patulin is a mycotoxin which is most often associated with *Penicillium expansum* responsible of soft rot on grape berries. Patulin is a water soluble lactone produced via the polyketide metabolic pathway mainly by *P. expansum*. In terms of carcinogenicity, patulin is classified as being of 'no evidence in humans' (group 3). This toxin exhibits mutagenic properties and have a strong antibiotic activity against many human disease-causing bacteria, including *Mycobacterium tuberculosis*. However, it was found very toxic to humans and animals and it has been demonstrated to induce gastrointestinal disorders including ulceration, distension and bleeding. In addition, it has been reported to cause pulmonary edema with dilatation of blood vessels and excess of fluid into the tissues, hepatic and gastrointestinal congestion (Puel et al., 2010).

1.3.3. *Alternaria* mycotoxins

A. alternata mycotoxins, including alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxins (ATX), tenuazonic acid (TeA) has been reported to contaminate

several food commodities. Indeed, mainly the AOH, AME, TeA, and, in some cases, ALT and ATX-I mycotoxins were associated with fruits, vegetables and oilseeds visibly infected by *Alternaria* rot, including grapes (Ostry et al., 2007). Scott et al. (2006) reported that AOH and AME have been detected in red and white wines and grape juice. Indeed, AOH has been identified as a topoisomerase I and II poison which might contribute to the impairment of DNA integrity in human colon carcinoma cells. In addition, TeA is toxic to several animal species, e.g. mice, chicken, dogs. AOH and AME have been reported to be mutagenic and genotoxic (Ostry, 2008). The European Food Safety Authority (EFSA, 2011) suggested that *Alternaria* toxins are of high concern for public health, although regulatory limits do not exist until today.

1.4. MYCOTOXINS ANALYSIS

Since the low levels at which mycotoxins are usually present in food and the general restrictive guidelines concerning the maximum acceptable levels, robust, selective, sensitive and accurate methods are required for the analysis of mycotoxins. Generally, mycotoxins are determined by chromatographic techniques such as TLC (thin layer chromatography), HPLC (high performance liquid chromatography) and gas chromatography (GC) singly or coupled with mass spectrometry (MS) (Sanzani et al., 2019).

These analytical methods required some essential steps i) sampling, ii) homogenization, iii) sample extraction and clean-up step to avoid or eliminate unwanted matrix components, which may include sample concentration, and iv) the separation and detection steps, usually accomplished either by a chromatographic technique in combination with a variety of detectors (fluorescence, mass spectrometry, diode array detectors, etc.) or by immunochemical methods. These latter it is only used for one mycotoxin or a small group of structural related compounds at a time. However, chromatographic techniques can separate a great number of analytes, including some with a very different chemical structure (Turner et al., 2009). The International Organization of Vine and Wine reported a normative analytical procedure to quantify OTA in wines (OIV, 2020). Indeed, OTA analysis is generally performed by HPLC-FD due to its natural fluorescence, coupled or not with a sample preparation based on dispersive liquid liquid microextraction (DLLME), solid phase extraction (SPE), molecular imprinted polymer extraction (MIP) or immunoaffinity (IA) purification. In some cases, the fluorescence detection (FD) enabled to quantify OTA traces in wines without any sample preparation due to the high

sensitivity of such detector (Roland et al., 2014). Analytical methods using HPLC–MS/MS procedures coupled with SPE or IA sample cleanup were also reported.

1.5. CONTROL OF MYCOTOXIGENIC FUNGI OF TABLE GRAPES

1.5.1. Conventional means

The control of mycotoxin accumulation is not only necessary to ensure consumers' safety but useful to prevent pathogen infection and rot development. The use of chemical fungicides is crucial in conventional agriculture to control fungal diseases, there is a long list of active ingredients approved on table grapes to control decays including those caused by mycotoxigenic fungi. The application of fungicides containing active ingredients including fludioxonil, pyrimethanil, fosetyl-Al and folpet have been reported to be effective for reducing black Aspergilli infection on grapes and also OTA content in wines (Molot and Solanet, 2003; Tjamos et al., 2004). In postharvest, the use of fungicides is not allowed for table grapes. However, the use of sulfur dioxide during storage of table grapes is allowed because it is considered as an adjuvant and not as a fungicide. This treatment can be carried out by repeated fumigation with SO_2 or by sulfurization in the individual packages using SO_2-generating pads placed inside polyethylene-lined grape containers (Romanazzi and Feliziani, 2014). In addition, it has been reported that *Aspergillus* spp. infection does not occur after cold storage of table grapes, and the incidence of contamination was reduced during cold storage (Guzev et al., 2008). However, the intensive use of fungicides over years has led to the development of resistant populations and even increase mycotoxin biosynthesis (Li and Xiao, 2008; Sanzani et al., 2016). Recently, the public concern and the recent legislation over pesticide residues in food, the environmental impact and the resistance to fungicides of plant pathogens has increased the interest in alternative methods for disease control. Moreover, the prevention of the mycotoxin-producing fungi growth is the most effective strategy for controlling the contamination of food with mycotoxin.

1.5.2. Alternatives means

The approach of biological control agents (BCAs) has been reported to be an ecofriendly alternative to chemical treatments in vineyards to control grapes mold and growth and sporulation of mycotoxins-producing fungi (Sonker et al., 2016). Several yeast, lactic acid bacteria (LAB) and non-toxic fungi as antagonistic microorganisms have been shown to be able

to inhibit the growth of mold and mycotoxin-producing fungi. A strain of *Aureobasidium pullulans* has been demonstrated that it was effective in reducing sour rot infection, *A. carbonarius* presence on berries at harvest and OTA contamination in must (Dimakopoulou et al., 2008). In addition, volatile organic compounds released by yeasts (*Cyberlindnera jadinii*, *Candida friedrichii*, *Candida intermedia* and *Lachancea thermotolerans*) have been reported to affect gene expression in *A. carbonarius* and *Aspergillus ochraceus*, as confirmed by downregulation of polyketide synthase, non-ribosomal peptide synthase, monooxygenase, and the regulatory genes *laeA* and *veA*, which are involved in OTA biosynthesis (Farbo et al., 2018). Taroub et al. (2019), reported that LAB (*Pediococcus pentosaceus* and *Lactobacillus plantarum*) isolated from 'Cardinal' and 'Red Globe' grape cultivars cultivated in Tunisia, showed antifungal activities against ochratoxinogenic *A. niger* and *A. carbonarius.*

In the recent years the essential oils and their major bioactive compounds have been extensively studied throughout the world for their antifungal activity against many fruit rot including mycotoxigenic ones. A study of An et al. (2019) showed the efficacy of α-terpineol and terpene-4-ol, the main components of tea tree essential oil (*Melaleuca alternifolia*), against *A. niger* in grapes by inducing morphological damage and metabolic changes of fungus. In other study the essential oils extracted from medical and aromatic plants such as thyme, caraway and carnation are effective in the suppression of mycotoxigenic fungi such as *Aspergillus niger*, *Alternaria alternata Penicillium digitatum* from stored table grapes (Arik and Arik, 2017). In addition, the application of chitosan and *Origanum vulgare* L. essential oil *in vitro* at sub-inhibitory concentrations inhibited spore germination and caused morphological changes in fungal spores and mycelia of *Rhizopus stolonifera* and *Aspergillus niger*, furthermore the same treatment *in vivo* inhibited the growth of the assayed fungi strains in artificially infected grapes as well as the autochthonous mycoflora of grapes (dos Santos et al., 2012). Furthermore, several decontaminating agents classified as GRAS (generally recognized as safe) including acetic acid, electrolyzed oxidizing water, ethanol and ozone, have been applied, alone or combined with physical means, to control postharvest fungi causing mold in table grapes including mycotoxigenic ones. (Romanazzi et al., 2012; Torlak, 2019).

1.6. AIM OF THE WORK

Grapes and their derived products, wine in particular, are among the key components of the Mediterranean diet. The Tunisian population consumes high amounts of both, fresh and dried fruits. This consumption becomes very high in special celebrations such as Ramadan month and weddings. Many biotic and abiotic factors cause quantitative and qualitative losses of grape every year. Fungal diseases represent the most important threats to grapes and grape-derived products, often resulting in yield losses and severe quality reductions due to the accumulation of mycotoxins, secondary metabolites biosynthesized by some fungal species. Therefore, the contamination of mycotoxins should be minimized by designing a series of measures of prevention and control. A number of mycotoxigenic fungal species that are able to biosynthesize mycotoxins can be found in grape berries in the Mediterranean area as *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp.. However, the *A. carbonarius* and *A. niger* are the main agents of *Aspergillus* brunch rot of grape, and they, especially *A. carbonarius*, are responsible of OTA contamination. A deeper understanding of the species diversity of black *Aspergilli*, together with the specific knowledge of their ecology and epidemiology, can help to predict their occurrence. For these reasons was important to validate the methods able to determine both the fugal specie and OTA occurrence on grape. The aim of this work was to identify the mycotoxigenic fungi causing black rot on tables grapes from different Tunisian vineyards, using morphological and molecular methods, and examine their ability for OTA production.

2. MATERIALS AND METHODS

2.1. SAMPLE COLLECTION

In this study, commercial table grapes vineyards were chosen from four different regions, located in the north of Tunisia (**Figure 4**). The grape sampling was conducted from July to August 2019 from different farms and cultivars (**Table 2**). From each vineyard, the berries showing symptoms of *Aspergillus* rot were collected and placed separately in paper bags, and kept in a cooler until arrival at the laboratory, then stored at 4 °C until use.

Figure 4. Location of vineyards surveyed in Tunisia

Table 2. Field sites and Number of berries samples collected from Tunisia

Province	Locality	Berry samples collected (No)	Cultivar	Maturity
Nabeul	Takelsa (36°47′N 10°38′E)	12	Michele Palieri	Mature
		12	Red Globe	Mature
		8	Victoria	Mature
	Douala (36°81′N 10°56′E)	4	Italia	Mature
		6	Michele Palieri	Mature
Béja	Medjez el Bab (36°35′04.92″N 10°03′09″E)	13	ARRA 34	No mature
		10	ARRA32	No Mature
		8	ARRA 4	Mature
		6	ARRA27	Mature
		6	ARRA32	Mature
Ben Arous	Mornag (36°41′5″N 10°1′37″E)	9	Victoria	Mature
		9	Italia	Mature
		10	Victoria	Mature
		5	Michele Palieri	Mature

2.2. ISOLATION

Isolation of the fungi was carried out aseptically under a laminar flow hood from small pieces of berries skin. From each skin berries sample, small pieces (~2 mm) were placed on Petri plates (90 mm ø) with potato dextrose agar (PDA) and incubated at 25 ± 2 °C for 8 days. The plates were examined daily, and the colonies of *Aspergillus* spp. were transferred to PDA plates and incubated under the same conditions to obtain pure cultures. Purified fungal isolates were stored at 4 °C.

2.3. MORPHOLOGICAL IDENTIFICATION

Fungus identification was carried out first by examination of colonies of pure cultures under a stereomicroscope (M125; Leica Microsystems CMS, Wetzlar, Germany), then examined under a

microscope (DM 2500; Leica). The morphological identification was carried out according to the colors and shapes of the colonies, with measurements of the fungal structures (i.e., conidiophores, conidial head and conidia) using the LAS V3.8 software (Leica DFC 295), which was applied to 50 conidia and 10 conidiophores and conidial head for each isolate. The fungal species identification was based on the keys of Raper and Fennell (1965).

2.4. MOLECULAR IDENTIFICATION

2.4.1. DNA extraction

From each fungal isolate the DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1990) modified by Landi et al. (2019). In detail, from each sample, grown for 14 days on PDA media, 100 mg of mycelium was collected and put in the 2-mL micro centrifuge tubes. The liquid nitrogen was used with the aim to grind the mycelium. After this phase, 1 mL extraction buffer (3% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% [w/v] soluble PVP-40), and 1% (w/v) metabisulphite, was added. The tubes were mixed and incubated at 65°C for 30 min. After incubation one volume of chloroform/isoamyl alcohol (24:1), was added and mixed for 2 min. The DNA was separated from the chloroform solution using the centrifuge at 8,000 rpm for 10 min. The supernatant was collected and put in the new tube. After this, the precipitation phase was performed and 0.6% isopropanol was added to the solutions and centrifuge at 14,800 rpm for 20 min. The DNA pellet obtained was washed adding 70% of cold ethanol and centrifuged for 5 min. The DNA was drayed at room temperature for eliminate the alcohol, and it was dissolved in 50 µL of ultrapure water. The DNA purity and quantity were determined (BioPhotometer plus; Eppendorf Inc., Westbury, NY, USA). For the samples the quantity ranged from 50 to 100 ng/µL DNA while the absorption ratios at 280/260 ranged from 1.6-1.8, and at 260/230 from 1.3-2.0. The DNA was extracted two times from two sub-cultured.

2.4.2. Primer selections and validation

For the *Aspergillus* spp. identification, both, specie specific and universal primers were selected according to β-tubulin and calmodulin genes and the Polyketide Synthase (PKS) gene included in the OTA metabolic pathway (table 3). The sequences related to these genes were selected from the National Center for Biotechnology Information (NCBI). The primers were designated

using Primer3web version 4.1.0. (http://bioinfo.ut.ee/primer3-0.4.0/). The species specificity was verified *in silico* using primer BLAST software (http://www.ncbi.nlm.nih.gov/Blast.cgi). Primer-BLAST was designed to make primers that are specific to an input PCR.

Table 3. Primers selected for *Aspergillus* spp. identification.

NCBI code	**Gene**	**Primer**	**Sequence (5'-3') (F, forward; R, reverse)**	**Amplicon Size (bp)**
Aspergillus niger				
LC573660.1	β -tubulin	*AN-tub*	F-ATGATTCCAGACCCAGCACA R-GGAACCATGTTGACAGCCAG	229
HM011537.1	polyketide synthase (pks5)	*AnPKS*	F-TCGTCAACAGCATCAACAGC R-AGGACATATCAGATGCCGCA	165
Aspergillus carbonarius				
FR775314.1	beta-tubulin	*AC-tub*	F-GATTGTGAGTTGTTCCCCGG R-R-CTTACACACTGGCCGGTTTG	165
MG701890.1	polyketide synthase (pks5)	*AC. PKS2*	F-TCACCAATCGACTGTTCCCA R-R-GAATCTTCACTTCGTCGGGC	188
Aspergillus tubingensis				
MN220258.1	Calmodulin (CaM)	*AT-cal/ AT-cal/*	F-CAGCTATTTCCCCCTTCGAT R-GAAGTCGATCGTTCCGTTGT	173
Aspergillus welwitschiae				
MN220492.1	Calmodulin (CaM)	*WE-cal*	F-CTCAGAATTATTTGGATCATA R-CAATCCAGCCCGCATTTCCG	187
Aspergillus spp.				
MN220231.1	Calmodulin (CaM)	*NT-cal*	F-AGGCCTTCTCCCTCTTTGTG R-GGATCGAGCGCTACAGGTAT	175

2.4.3. qPCR-High Resolution Melting (HRM)

The best quantitative Real time PCR (qPCR) conditions were set-up. The primers were validated to their ability to amplify fungal DNA species using qPCR. Two µL of DNA from each sample was mixed and a serial dilution from 100 ng/µL to 1 ng/µL of total DNA were analyzed according the thermal conditions described below. Both melting profile and sequencing analysis were performed for the positive samples. In addition, a blank control sample, obtained by inserting the water instead of the DNA, was added. The final step included the melting curve analyses (0.2 °C step increments; 10s hold before each acquisition), which were analyzed from 70 °C to 95 °C. The qPCR-HRM and nested-qPCR-HRM amplifications were both performed using the CFX real-time PCR detection system and analyzed using the 'High-Resolution Melting analysis software' (Bio-Rad Laboratories). This software automatically clusters the samples according to their melting profiles and assigns confidence scores to each of the samples. The confidence level threshold for a sample to be included in a cluster was 99.0%. All experiments were performed in duplicate and repeated three times.

2.4.4. Sequencing

The specific amplicons obtained according to *AC-PKS* primers, related to the fungal isolates B3B4F1, VB9F3, WB4F1, MB1F3, B2B2F1, the amplicons obtained according to *AT-cal* primers related to fungal isolates W2B12F1, B3B1F1, B3B4F1, B1B1F1, W2B10F1, and the amplicons obtained according to the primers *NT-cal* linked to fungal isolates RB2F2_116, WB10F1_85, W2B2F1_22, W2B13F1_13, RB3F2_69, WB7F1_82, W2B5F1_16, B3B3F1_36, B3B6F1_39, IB7F3_49, IB10F3_52, VB5F3_60, RB7F2_73, RB10F2_74 were sequenced by Genewiz (Hope End, Takeley, UK) and subjected to bioinformatic analysis. Sequence similarity searches were performed using BLAST analysis in NCBI.

2.4.5. Phylogenetic Analysis of qPCR amplicons

The sequences with the highest homology with qPCR amplicons were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were achieved using Clustal X (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the molecular evolutionary genetics analysis (MEGA) program, version 5.2 (Tamura *et al.,* 2011), according to the neighbor-joining method (Saitou and Nei, 1987), with

pairwise deletion with 1,000 bootstrap replicates. The sequence pairs within and among the *Aspergillus* spp. profiles were carried out using the maximum composite likelihood model for the β-tubulin sequences. The average genetic distances among the clades inferred by the phylogenetic analyses were computed according, to the NCBI Blast analysis of the sequences.

3. RESULTS

3.1. TABLE GRAPES BERRIES SYMPTOM EVALUATION

In all the vineyards from four different locations and seven cultivars surveyed, *Aspergillus* black rot symptoms occurred on table grapes berries collected. Soft and tan to brown infected berries covered with masses of brown and black fungal sporulation were observed (**Figure 5**). In addition, typical symptoms on grapes berries were examined, including cracking and breakdown of berry tissue (**Figure 6**). Other berries that seem completely empty and dry (**Figure 7).**

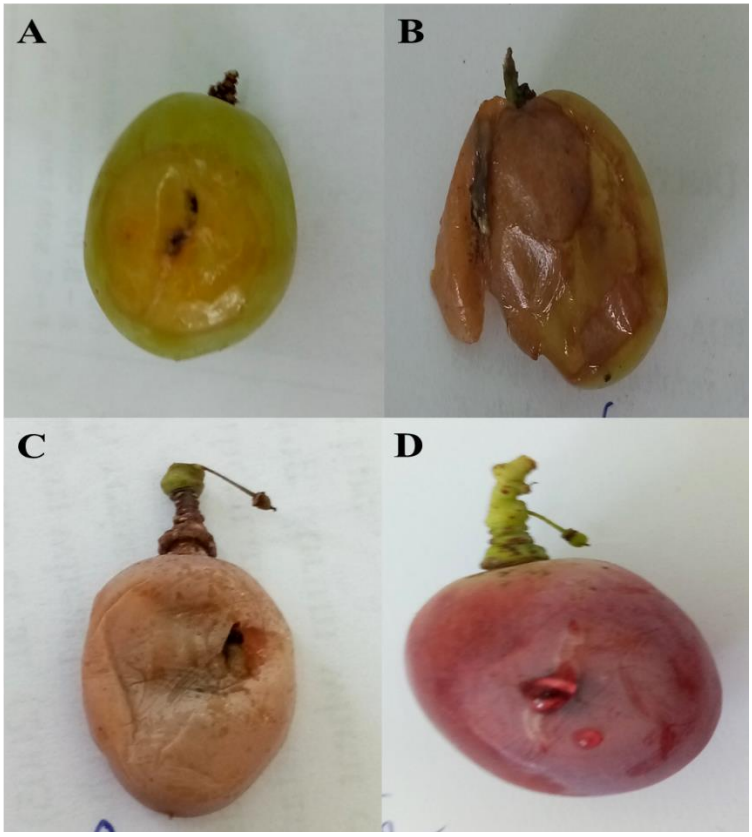

Figure 5. Soft and tan to brown infected grapes berries caused by black *Aspergilli*. **A**) Cv. Victoria; **B**) Cv. Italia; **C)** and **D)** Cv. Red Globe.

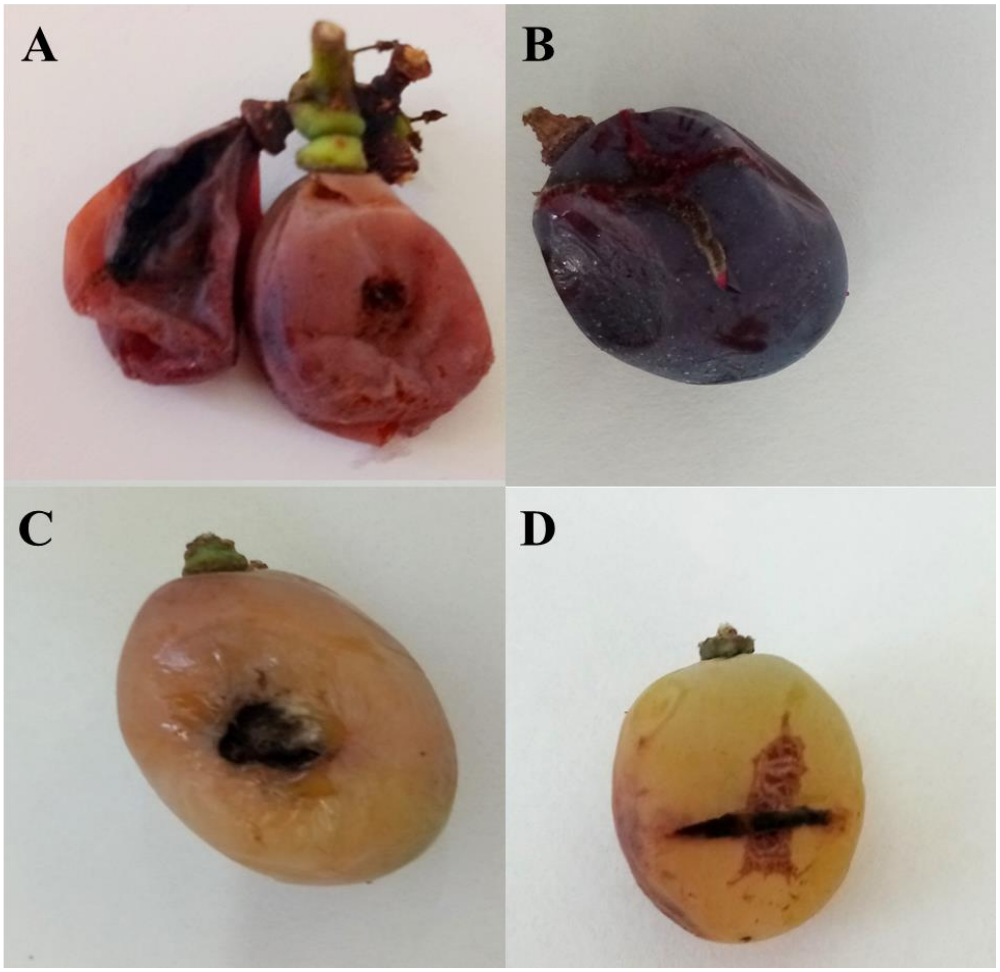

Figure 6. Cracked grape berries and berry tissue breakdown caused by black *Aspergilli* on grapes. **A**). Cv. Red Globe; **B**) Cv. Michele Palieri; **C**) Cv. Victoria; **D)** Cv. Italia.

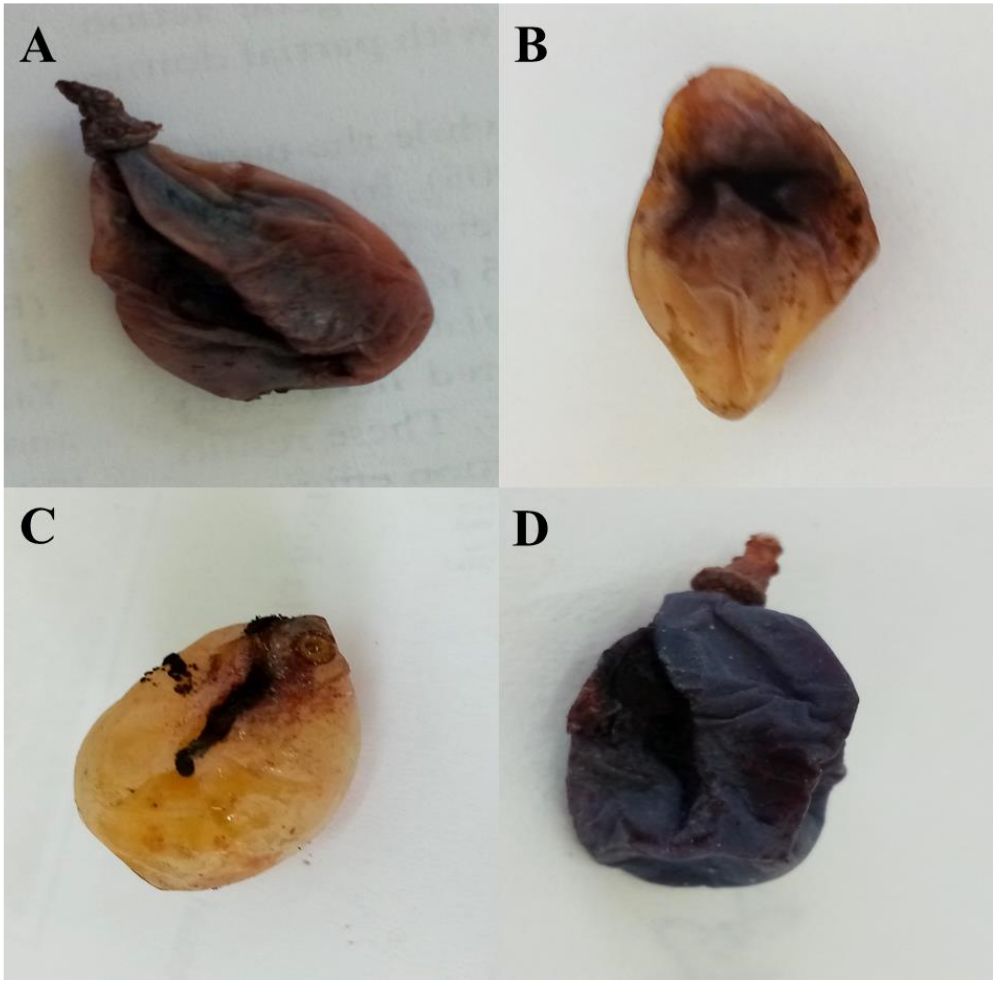

Figure 7. Dried grape berries covered by masses of black fungal sporulation. **A)** Cv. Red Globe, **B)** Cv. Italia, **C)** Cv. Victoria, **D)** Cv. Michele Palieri.

3.2.MORPHOLOGICAL IDENTIFICATION

After the sampling, the berries of grapes were examined for fungal disease symptoms caused by black Aspergilli under the stereomicroscope (**Figure 8**). The fungal structures (i.e., conidia, conidiophore) were analyzed using microscopy, to determine shape and sizes (**Table 4,5,6**). The main species of *Aspergillus* identified according to morphological feature were *Aspergillus niger* aggregate and *A. carbonarius*, whose descriptions are reported in the following subsections.

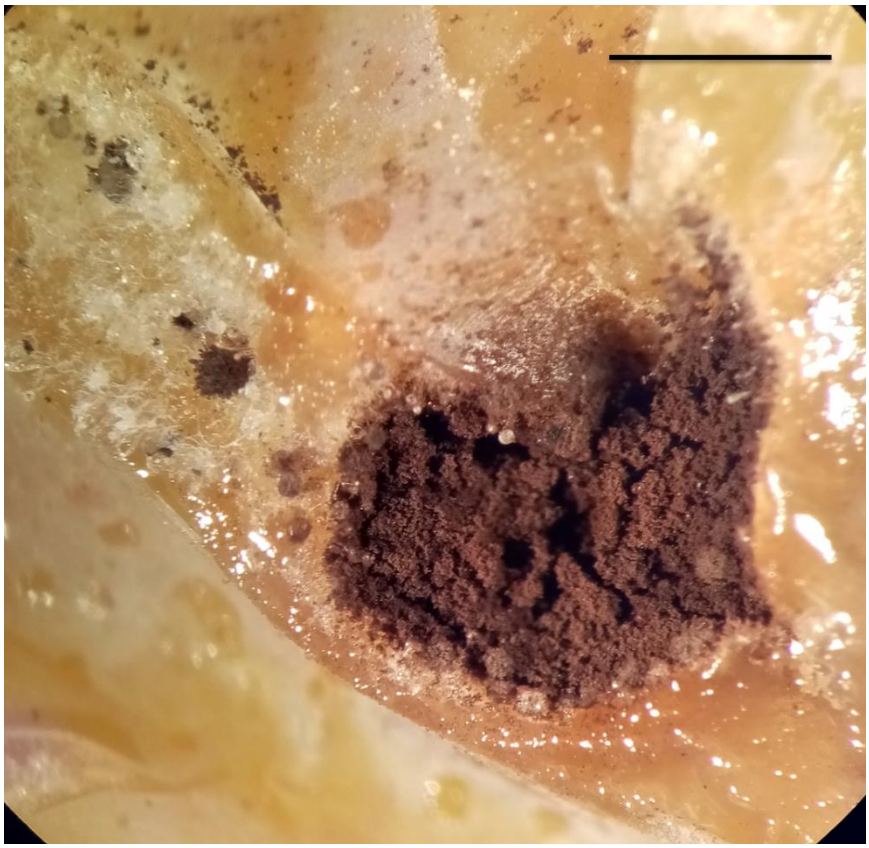

Figure 8. Aspergillus black rot symptoms on berries of grape under the stereomicroscope (bar=500µm)

***Aspergillus carbonarius* (Bainier) Thom**

After 7 days of incubation on PDA at 22±2°C, the mycelia of the colonies were black on the top and colorless on the bottom (**Figure 9 A, B**). Long conidiophores 614.5 to 1164.5 µm hyaline and darker at the apex forming a globular head 72.8 to 254.6 µm were observed for this species of *Aspergillus* (**Figure 9 C**). The conidia were wrinkled and large with diameters ranging from 4.8 to 6.16 µm (**Figure 9 D**). These morphological traits were consistent with *A. carbonarius*.

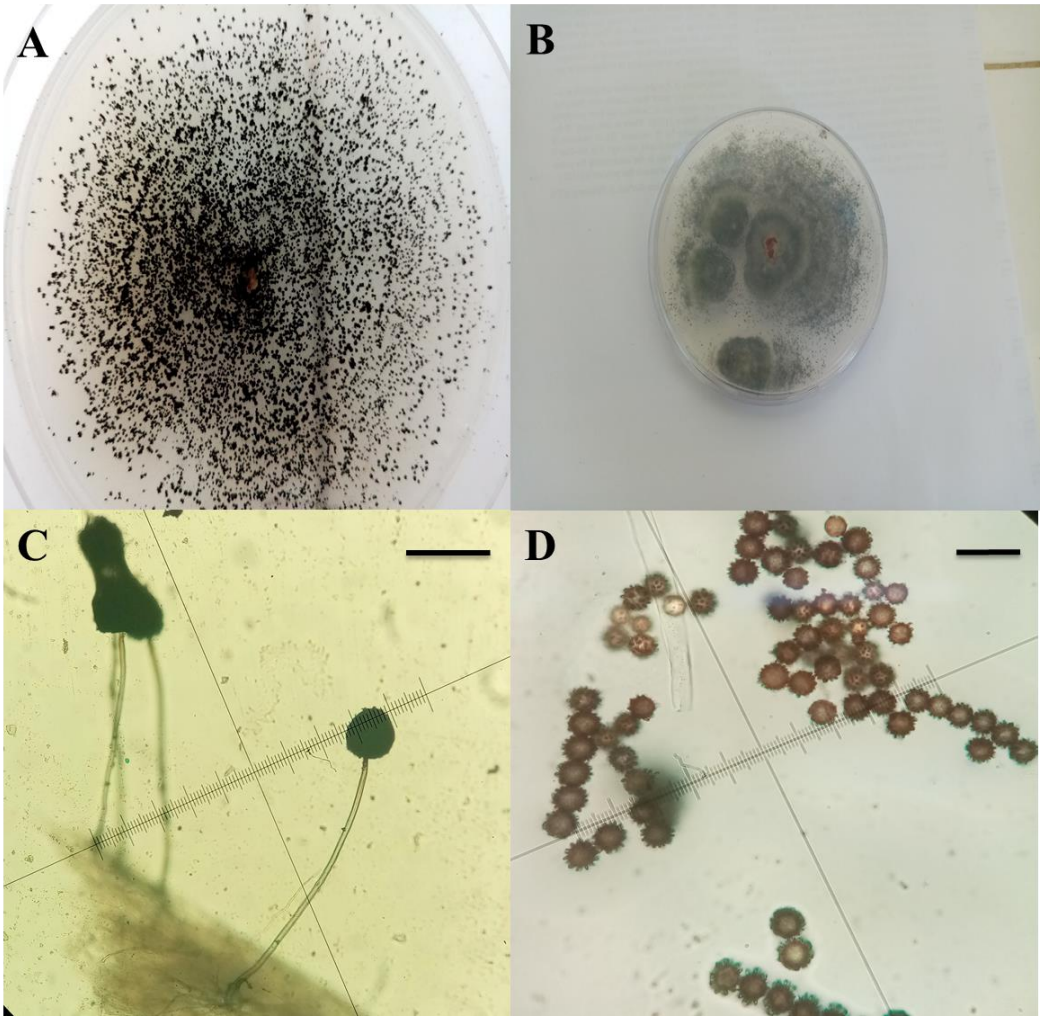

Figure 9. *Aspergillus carbonarius*. **A)** the top and **B)** the bottom of colony 7 days old on PDA at 22 ± 2. **C**) Conidiophores and Conidial heads (bar=400 µm). **D)** Conidia (bar=10 µm).

***Aspergillus niger* Tiegh.**

After 7 days of incubation on PDA at 22±2°C, on PDA this fungus produced black colonies (**Figure 10 A**). Conidia more or less globose, dark brown often rough and finely wrinkled with diameter measuring 2.17 to 3.13 µm (**Figure 10 B**). Brown to black conidial heads 59.9 to 100.6 µm of diameter, on erect, hyaline and the length of conidiophore was 289.2 to 514.1 µm (**Figure 10 C**). These morphological traits were consistent with *A. niger*.

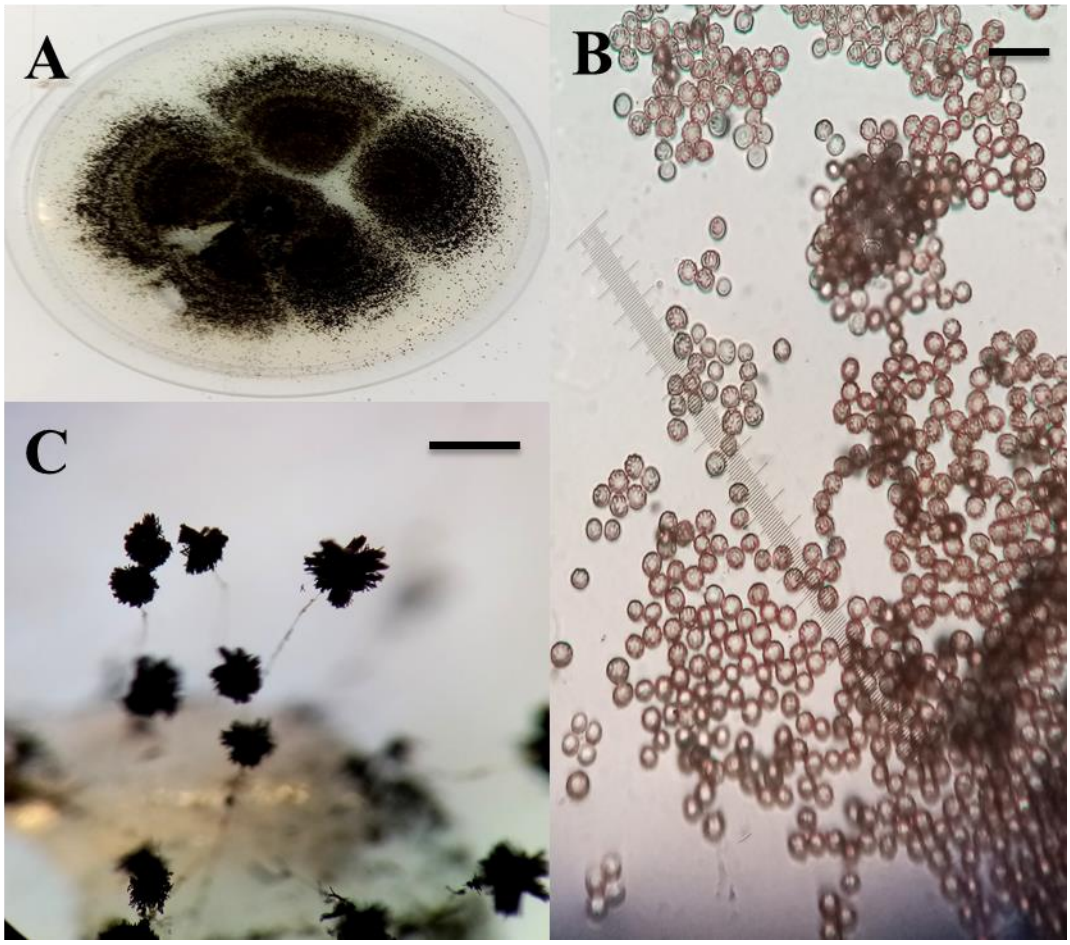

Figure 10. *Aspergillus niger*. **A**) seven-day-old colony on PDA at 22 ± 2 °C. **B**) Conidia (bar=10 µm). **C**) Conidiophores and Conidial heads (bar=200 µm)

3.3.MOLECULAR IDENTIFICATION

3.3.1. qPCR and HRM melt curve validation

Several of the primer's pairs tested **(table 3)** shown a specific single peak according the melting curves analysis which confirmed their homogeneity and specificity of the amplicons produced in the qPCR analysis. No amplification was observed in any of the control assays, which confirmed that the samples were free of contamination with other genomic DNA (data not shown). The thermic cycle established the specific melt curve (TM) for the primer's pairs specific for *A. carbonarius* species *AC-tub* and *ACPKS* at 83.0 °C, and 87.5 °C respectively (**Figure 11A** and **B**). About the primers specific for *A. niger*, species the TM temperature was verified for AN-tub at 78.4°C and for AN-PKS at 83.5 °C (**Figure 11C** and **D**). The primers pair *AT-cal*, selected for *A. tubingensis* identification, shown the TM peak at 84.5 °C while the primers *WE-cal* used to

detect *A. welwitschiae* species, at 84.2 °C (**Figure 11E** and **F**). The primers pair *NT-cal* used for qPCR-HRM analysis, able to identify DNA calmodulin sequences related to both, *A. tubingensis* and *A. niger* species, shown the TM peak at 80.6 °C for *A. tubingensis* specie while at 81.20 specie (**Figure 11G**).

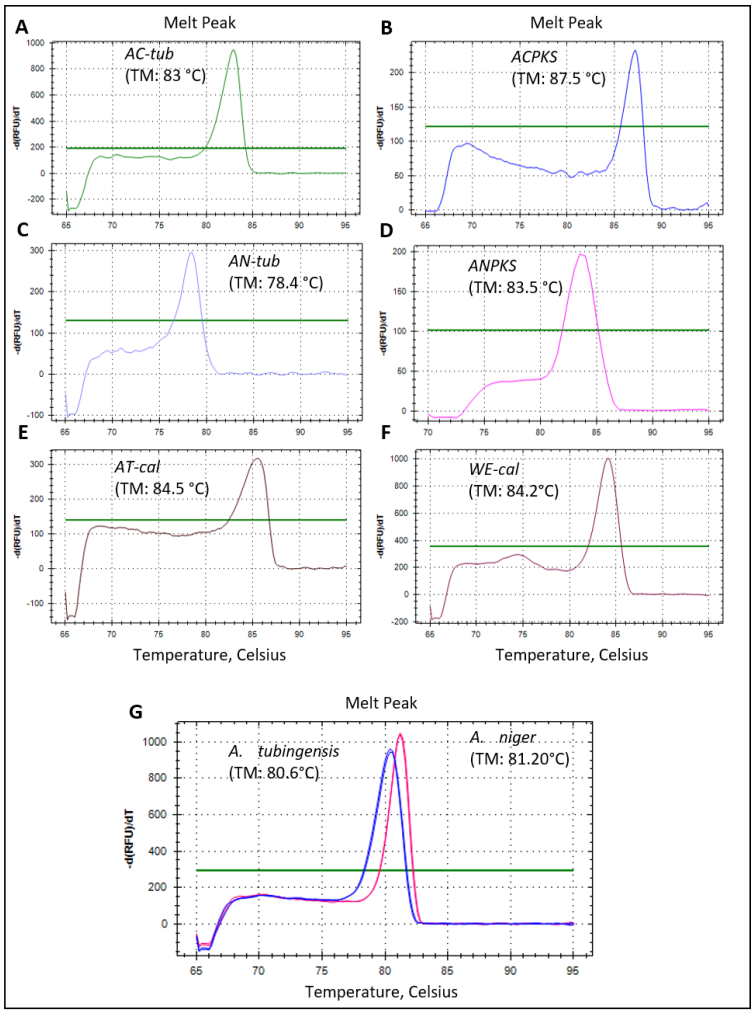

Figure 11. Specificity of the primers *AC-tub* (**A**) and *ACPKS* (**B**) used for *A. carbonarius* detection; AN-tub (**C**) and *ANPKS* (**D**) specific for *A. niger* species; (**E**) *AT-cal* specific for *A. tubingensis* species, (**F**) WE-cal specific for *A. welwitschiae* species; (**G**) *NT-cal* used in HRM-qPCR amplification able to identify both, *A. tubingensis* and *A. niger* species. All melting curves have shown a single peak specific for each primers pair. For more detail see text.

3.3.2. Sequencing and phylogeny analysis of isolates

Sequence similarity searches performed using the BLAST software was able to identify the primers specificity used for *Aspergillus* spp. identification. The sequence analysis of 188 pb PCR amplicons obtained using the *ACPKS* primers shown the homology of 100% with the PKS gene for polyketide synthase (OTA-A) of *A. carbonarius* species while the homology of 71.01% with the PKS gene of *A. niger* species (**Figure 12A**). A phylogenetic analysis of the amplicons obtained with *AC. PKS2* primers was shown in **Figure 12B**.

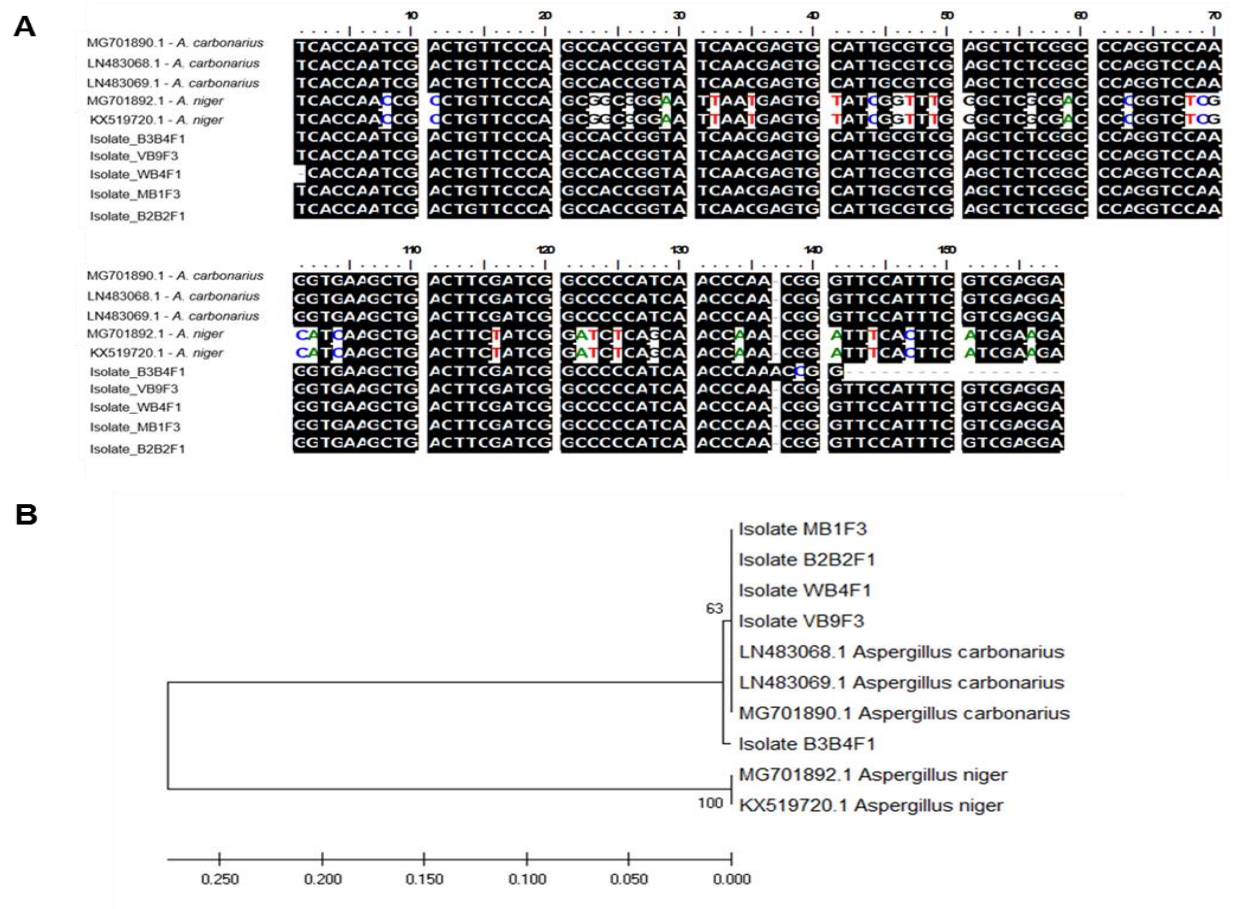

Figure 12 Sequence alignment of the qPCR products amplified with primers ACPKS from representative isolates of *Aspergillus* spp. collected from grapes, with the NCBI *Aspergillus* spp. showing highest homology **A**) BLAST alignment of part of Polyketide synthases (PKS) gene sequence related to *A. niger*, *A. carbonarius* with the qPCR amplicons sequence from isolates **B)** Phylogenetic tree based on PKS sequence of *Aspergillus* spp. Numbers next to nodes are bootstrap values. The *Aspergillus* spp. isolates collected from grapes, show the relationships with the NCBI PKS sequences selected as references.

BLAST sequence analysis performed for 173 bp amplicons related to *AT-cal* primers clustered with 411 *A. tubingensis* isolates related to calmodulin genes. The homology of the PCR sequences was of the 100% (**Figure 13A**). A phylogenetic tree of the amplicons obtained with *AT-cal* primers was shown in **figure 13B**.

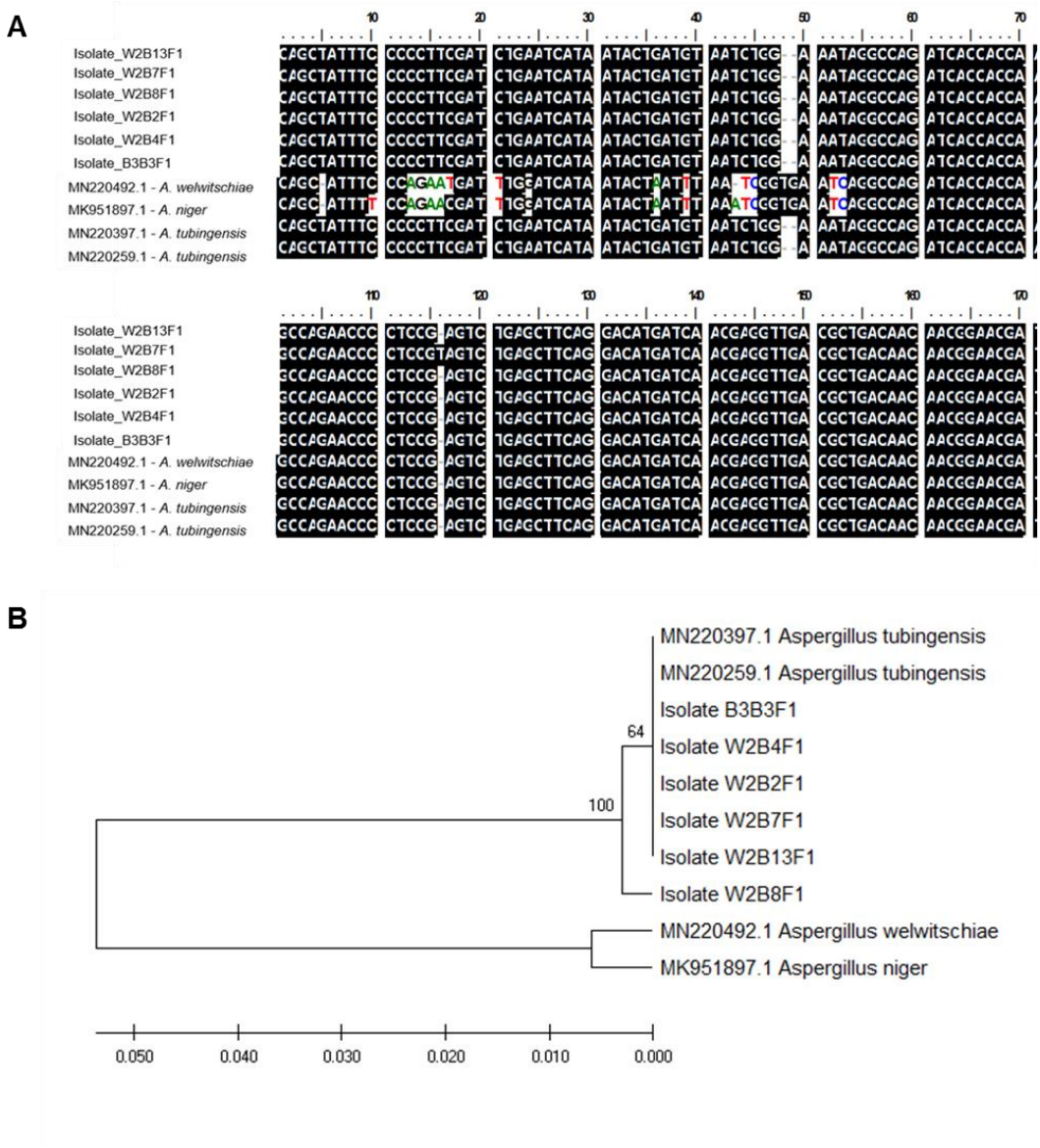

Figure 13 Sequence alignment of the qPCR products amplified with primers *AT-cal* from representative isolates of *Aspergillus* spp. collected from grapes, with the NCBI *Aspergillus* spp. showing highest homology **A**) BLAST alignment of part of calmodulin gene sequence related to *A. niger*, *A. tubingensis* and *A. welwitschiae* with the qPCR amplicons sequence from isolates **B)** Phylogenetic tree based on calmodulin sequence of *Aspergillus* spp. Numbers next to nodes are

bootstrap values. The *Aspergillus* spp. isolates collected from grapes, show the relationships with the NCBI calmodulin sequences selected as references.

Sequence similarity searches achieved using the BLAST analysis of the 175 pb amplicons obtained with *NT-cal* primers for the isolates B3B6F1, VB5F3, W2B5F1, WB7F1, W2B13F1, W2B2F1, RB2F2, and IB7F3, clustered with 202 *A. tubingensis* strains, according to calmodulin genes, showing the highest homology of 98.82 %. While the isolates RB3F2, IB10F3, RB7F2, and RB10F2, grouped with 198 *A. niger* isolates showing the highest homology of 100 % (**Figure 14 A**). A phylogenetic analysis of the amplicons obtained with NT-cal primers was shown in figure 14B.

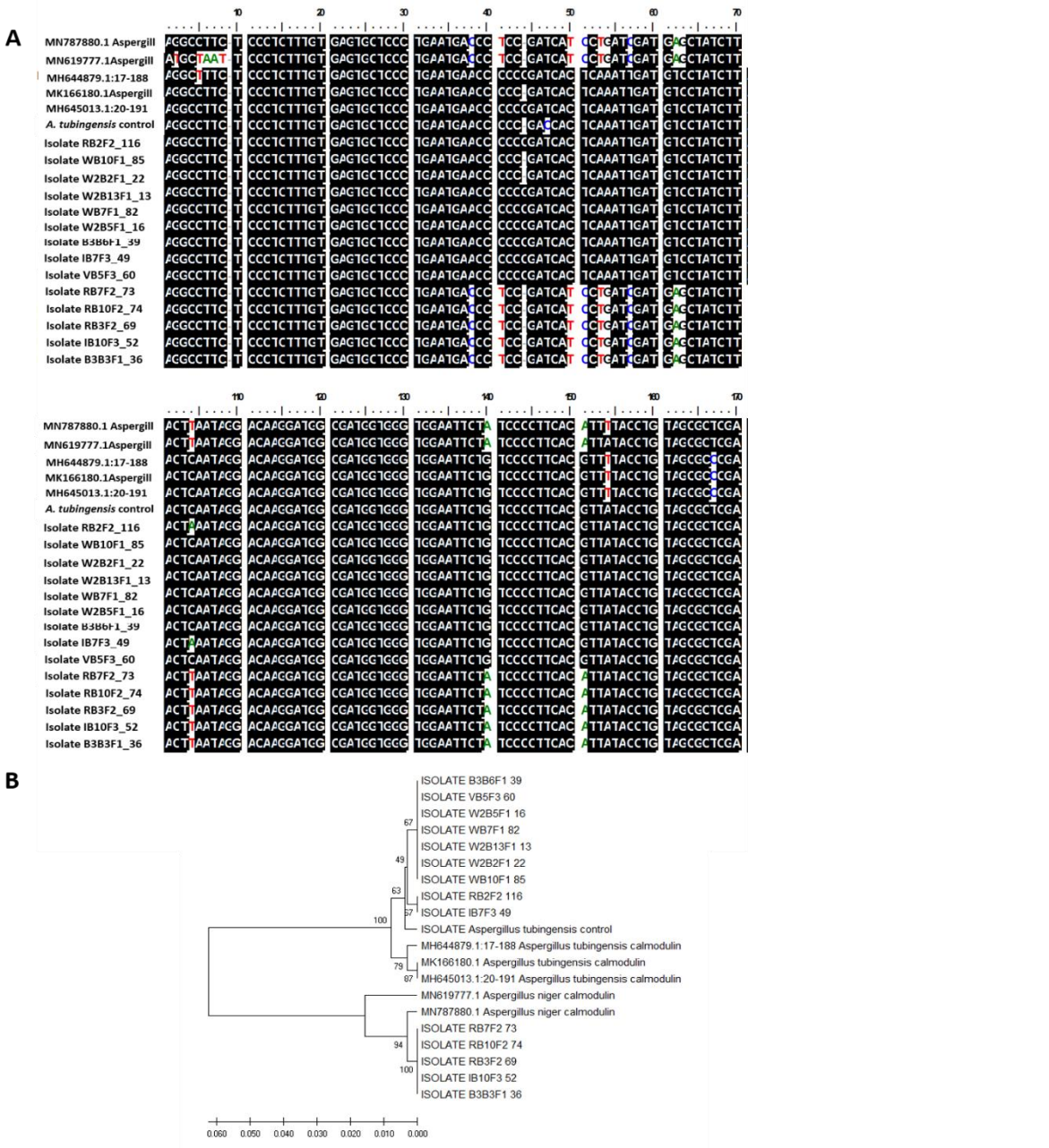

Figure 14 Sequence alignment of the qPCR products amplified with primers *NI-cal* from representative isolates of *Aspergillus* spp. collected from grapes, with the NCBI *Aspergillus* spp. showing highest homology **A**) BLAST alignment of part of calmodulin gene sequence related to *A. niger*, *A. tubingensis* with the qPCR amplicons sequence from isolates **B)** Phylogenetic tree based on calmodulin sequence of *Aspergillus* spp. Numbers next to nodes are bootstrap values. The *Aspergillus* spp. isolates collected from grapes, show the relationships with the NCBI calmodulin sequences selected as references.

This result is highlighted by the HRM analysis which splits the isolates homologous to *A. niger* into a separate cluster from those identified as *A. tubingensis* (**Figure 15**).

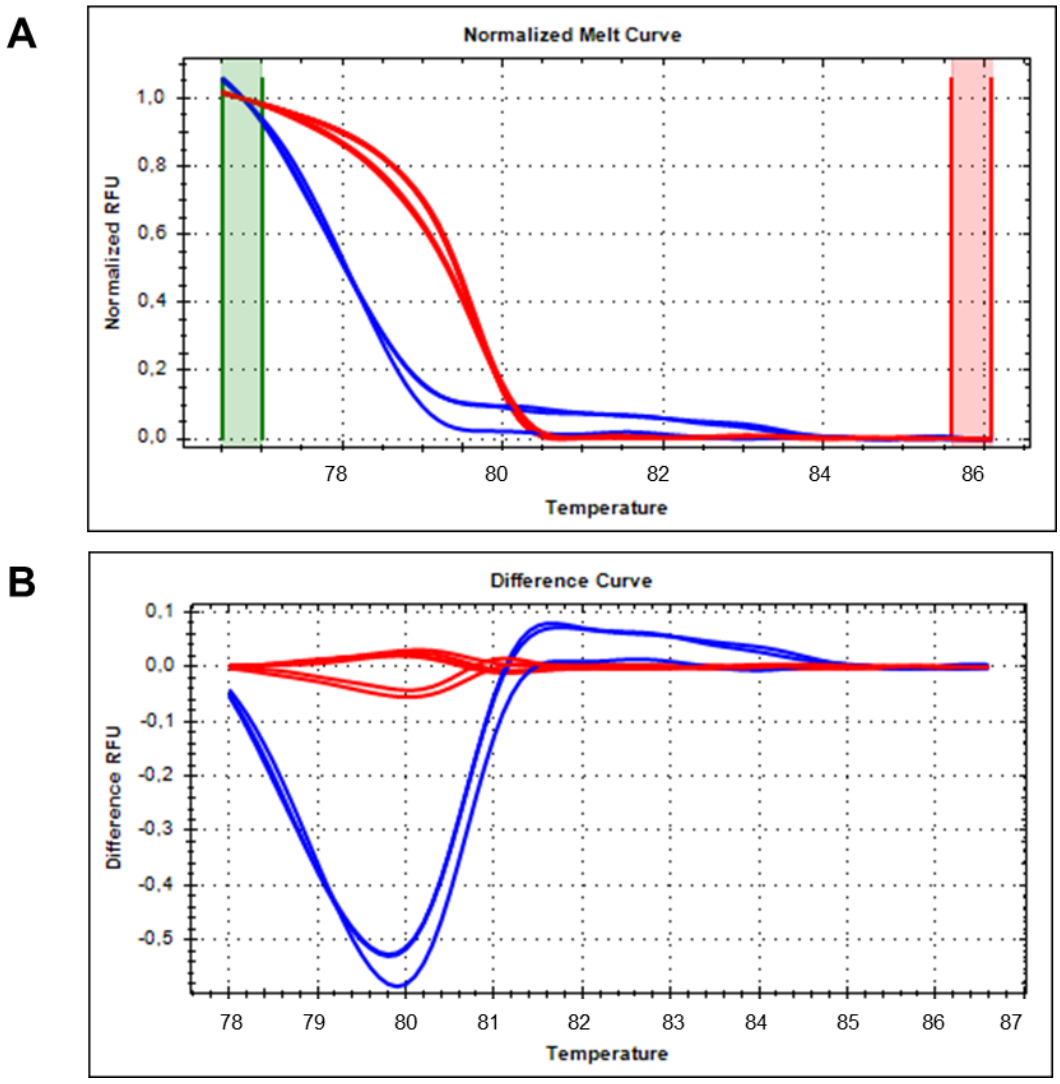

Figure 15. qPCR-high-resolution melting (HRM) analysis to discriminate between *A. tubingensis* (blue lines) and *A. niger* (red lines) and using *NT-cal* primers. Typical genotyping patterns as normalized melting curves (**A**) and normalized difference plots (**B**) are shown. RFU: relative fluorescence units.

The analysis qPCR performed with the previously validated primers *AN-tub, AnPKS* specific for *A. niger* and *WE-cal* specific for *A.* welwitschiae species, confirmed the isolates *Aspergillus* spp. identification.

3.3.3. *Aspergillus* spp. identification

The initial identification of the *Aspergillus* isolates based on colony and conidial morphologies was compared with the outcomes of qPCR analyses and a majority of the isolates were also confirmed by qPCR amplicon sequence analyses.

Based on molecular identification techniques, the 118 fungal isolates were confirmed to belong to *A. carbonarius* (66.1%), *A. niger* (12.7%) and *A. tubingensis* (20.33%). The isolates collected from different cultivars of Takelsa vineyard (**Table 4**) were identified as *A. carbonarius* (71.87%), *A. niger* (15.62%) and *A. tubingensis* (9.37%). The occurrence of isolates from Michele Palieri cultivar was 91.6% and 8.4% *A. tubingensis,* for Victoria was 85.71% and 14.3% of *A. carbonarius* and *A. tubingensis* respectively, while for the cultivar Red Globe the occurrence was 41.6%, 50% and 8.3% of *A. niger, A. carbonarius* and *A. tubingensis,* respectively.

Table 4. Molecular identification and morphological characteristics of *Aspergillus* spp. isolates from ripened table grapes from Takelsa vineyard in Tunisia.

Isolates	Grape host cultivar	Molecular identification	Fungal structures (µm)[1]		
			Conidial head	Conidiophore	Conidia
MB1F2	Michele Palieri	*Aspergillus carbonarius*	-	-	-
MB2F2	Michele Palieri	*A. carbonarius*	-	-	-
MB3F2	Michele Palieri	*A. carbonarius*	-	-	
MB4F2	Michele Palieri	*A. carbonarius*	144.9±12.8	827.9±12.8	4.8±0.1
MB5F2	Michele Palieri	*A. carbonarius*	-	-	-
MB7F2	Michele Palieri	*A. carbonarius*	-	-	-
MB8F2	Michele Palieri	*A. carbonarius*	-	-	-
MB9F2	Michele Palieri	*A. carbonarius*	-	-	-
MB10F2	Michele Palieri	*A. carbonarius*	-	-	-
MB11F2	Michele Palieri	*A. carbonarius*	-	-	-
MB12F2	Michele Palieri	*A. carbonarius*	-	-	-
MB13F2	Michele Palieri	*A. tubingensis*	60.2±5.2	289.2±20.5	2.4±0.2
VB1F2	Victoria	*A. carbonarius*	139.7±13.5	960.±98	5.1±0.6

VB2F2	Victoria	*A. carbonarius*	-	-	-
VB3F2	Victoria	*A. carbonarius*	-	-	-
VB5F2	Victoria	*A. carbonarius*	-	-	-
VB6F2	Victoria	*A. tubingensis*	-	-	-
VB7F2	Victoria	*A. carbonarius*	67.9±7	466 ±19.1	2.2±0.2
VB8F2	Victoria	*A. carbonarius*	-	-	-
VB10F2	Victoria	-	-	-	-
RB11F2	Red Globe	*A. niger*	-	-	-
RB12F2	Red Globe	*A. carbonarius*	-	-	-
RB3F2	Red Globe	*A. niger*	-	-	-
RB4F2	Red Globe	*A. niger*	-	-	-
RB8F2	Red Globe	*A. carbonarius*	-	-	-
RB6F2	Red Globe	*A. carbonarius*	75.1±4.7	889.6±49.2	5.7±0.8
RB7F2	Red Globe	*A. niger*	-	-	-
RB10F2	Red Globe	*A. niger*	-	-	-
RB5F2	Red Globe	*A. carbonarius*	117.6±11.7	799.5±52.8	5.6±0.1
RB1F2	Red Globe	*A. carbonarius*	-	-	-
RB9F2	Red Globe	*A. carbonarius*	-	-	-
RB2F2	Red Globe	*A. tubingensis*	-	-	-

[1].Data are means ±SD. (-) not measured.

The occurrence of the isolates collected from different cultivars of Medjez El Bab vineyard (**Table 5**) were 53.48%, 37.2% and 9.3% of *A. carbonarius, A. tubingensis* and *A. niger,* respectively. In particular, 69.2% of *A. tubingensis* and 30.8% of *A. carbonarius* for the isolates from ARR34 cultivar collected at veraison stage. The ARRA32 isolates collected in the same stage was distributed as 40%, 40% and 20% of *A. carbonarius, A. tubingensis* and *A. niger,* respectively. For the isolates from the ripened cultivar ARRA4 the distribution was 62.5%, 25% and 12.5% of *A. carbonarius, A. tubingensis* and *A. niger,* respectively. For the isolates from the ripened cultivar ARRA27 the distribution was 83.3% and 16.66% of *A. carbonarius* and *A. tubingensis,* respectively. While, for ripened ARRA32 isolates the distribution was 83.3% of *A. carbonarius* and 16.66% of *A. niger.*

Table 5. Molecular identification and morphological characteristics of *Aspergillus* spp. isolates from table grapes from Medjez El Bab vineyard in Tunisia.

Isolates	Grape host cultivar	Molecular identification	Fungal structures (µm)[1]		
			Conidial head	Conidiophore	Conidia
W2B13F1	ARRA34*	*A. tubingensis*	97.4±7.8	781±45	2.5±0.2
W2B10F1	ARRA34*	*A. tubingensis*	-	-	-
W2B11F1	ARRA34*	*A. carbonarius*	-	-	-
W2B5F1	ARRA34*	*A. tubingensis*	-	-	-
W2B6F1	ARRA34*	*A. tubingensis*	-	-	-
W2B7F1	ARRA34*	*A. tubingensis*	-	-	-
W2B8F1	ARRA34*	*A. tubingensis*	-	-	-
W2B12F1	ARRA34*	*A. tubingensis*	-	-	-
W2B1F1	ARRA34*	*A. carbonarius*	-	-	-
W2B2F1	ARRA34*	*A. tubingensis*	-	-	-
W2B3F1	ARRA34*	*A. carbonarius*	-	-	-
W2B14F1	ARRA34*	*A. carbonarius*	216.9±29.2	877.8±67.2	5.5±0.1
W2B4F1	ARRA34*	*A. tubingensis*	-	-	-
B3B1F1	ARRA32*	*A. tubingensis*	-	-	-
B3B2F1	ARRA32*	*A. carbonarius*	-	-	-
B3B3F1	ARRA32*	*Aspergillus niger*	-	-	-
B3B4F1	ARRA32*	*A. tubingensis*	-	-	-
B3B5F1	ARRA32*	*A. carbonarius*	-	-	-
B3B6F1	ARRA32*	*A. tubingensis*	74.9±5	458.7±11.2	2.3±0.3
B3B7F1	ARRA32*	*A. tubingensis*	-	-	-
B3B8F1	ARRA32*	*A. niger*	79.6±3.6	345.6±15.1	2.3±0.3
B3B9F1	ARRA32*	*A. carbonarius*	-	-	-
B3B10F1	ARRA32*	*A. carbonarius*	-	-	-
WB1F1	ARRA4**	*A. carbonarius*	-	-	-

WB2F1	ARRA4**	*A. carbonarius*	-	-	-
WB3F1	ARRA4**	*A. niger*	209.1±17.4	857.2±51.8	3.1±0.0
WB4F1	ARRA4**	*A. carbonarius*	-	-	-
WB7F1	ARRA4**	*A. tubingensis*	-	-	-
WB8F1	ARRA4**	*A. carbonarius*	-	-	-
WB9F1	ARRA4**	*A. carbonarius*	72,86±3,71	905,13±55,31	5,41±0.0
WB10F1	ARRA4**	*A. tubingensis*	-	-	-
B1B2F1	ARRA27**	*A. carbonarius*	-	-	-
B1B6F1	ARRA27**	*A. carbonarius*	140.4±16.8	614.5±16.7	5,1±0.0
B1B3F1	ARRA27**	*A. carbonarius*	100.6±8.5	514.1±29.4	2.1±0.0
B1B5F1	ARRA27**	*A. carbonarius*	-	-	-
B1B4F1	ARRA27**	*A. carbonarius*	-	-	-
B1B1F1	ARRA27**	*A. tubingensis*	-	-	-
B2B4F1	ARRA32**	*A. carbonarius*	118.1±12.9	1126±24.6	6 ±0.0
B2B1F1	ARRA32**	*A. niger*	-	-	-
B2B2F1	ARRA32**	*A. carbonarius*	-	-	-
B2B5F1	ARRA32**	*A. carbonarius*	-	-	-
B2B6F1	ARRA32**	*A. carbonarius*	254.6±16.9	1164.5±45.7	5.5±0.0
B2B7F1	ARRA32**	*A. carbonarius*	-	-	-

* Table grapes berries collected from cultivar non ripened. ** Table grapes collected at ripening. [1].Data are means±SD. (-) not measured.

The isolates collected from different cultivars of Mornag vineyard (**Table 6**) were identified as *A. carbonarius* (66.66%), *A. niger* (18.18%) *and A. tubingensis* (15.15%). The occurrence of isolates from Victoria cultivar was 79%, 10.5% and 10.5% of *A. carbonarius, A. tubingensis* and *A. niger*, respectively. For the isolates from Italia cultivar the distribution was 44.5% 33.3% and 22.2% of *A. niger, A. tubingensis* and *A. carbonarius,* respectively. While all isolates from Michele Palieri were *A. carbonarius.* In addition, all isolates from the locality of Douala (**Table 7**) were identified as *A. carbonarius* (100%).

Table 6. Molecular identification and morphological characteristics of *Aspergillus* spp. isolates from ripened table grapes from Mornag vineyard in Tunisia.

Isolates	Grape host cultivar	Molecular identification	Fungal structures (µm)[1]		
			Conidial head	Conidiophore	Conidia
V2B2F4	Victoria	*A. carbonarius*	-	-	-
V2B7F4	Victoria	*A. carbonarius*	-	-	-
V2B3F4	Victoria	*A. carbonarius*	-	-	-
V2B4F4	Victoria	*A. carbonarius*	-	-	-
V2B5F4	Victoria	*A. carbonarius*	-	-	-
V2B6F4	Victoria	*A. carbonarius*	-	-	-
V2B9F4	Victoria	*A. carbonarius*	-	-	-
V2B10F4	Victoria	*A. carbonarius*	100,22±6,4	378,88±12,6	2,27±0,2
V2B8F4	Victoria	*A. carbonarius*	-	-	-
VB1F3	Victoria	*A. tubingensis*	-	-	-
VB3F3	Victoria	*A. carbonarius*	-	-	-
VB4F3	Victoria	*A. carbonarius*	-	-	-
VB5F3	Victoria	*A. tubingensis*	-	-	-
VB6F3	Victoria	*A. carbonarius*	-	-	-
VB7F3	Victoria	*A. carbonarius*	124,69±6,9	712,2±69,9	6,12±0,6
VB8F3	Victoria	*A. carbonarius*	-	-	-
VB9F3	Victoria	*A. carbonarius*	-	-	-
VB10F3	Victoria	*A. niger*	-	-	-
VB11F3	Victoria	*A. niger*	-	-	-
IB1F3	Italia	*A. niger*	-	-	-
IB2F3	Italia	*A. tubingensis*	-	-	-
IB4F3	Italia	*A. carbonarius*	-	-	-
IB5F3	Italia	*A. carbonarius*	185,77±17,8	979,07±55,5	5,05±0,7
IB6F3	Italia	*A. niger*	-	-	-
IB7F3	Italia	*A. tubingensis*	-	-	-

IB8F3	Italia	*A. tubingensis*	78,78±6,2	395,05±43,2	2,49±0,4
IB9F3	Italia	*A. niger*	-	-	-
IB10F3	Italia	*A. niger*	-	-	-
MB1F3	Michele Palieri	*A. carbonarius*	145,47±17,2	1125,73±77,3	5,26±0,7
MB3F3	Michele Palieri	*A. carbonarius*	-	-	-
MB4F3	Michele Palieri	*A. carbonarius*	-	-	-
MB5F3	Michele Palieri	*A. carbonarius*	-	-	-
MB6F3	Michele Palieri	*A. carbonarius*	-	-	-

[1].Data are means±SD. (-) not measured.

Table 7. Molecular identification of *Aspergillus* spp. isolates from ripened table grapes from Douala vineyard in Tunisia.

Isolates	**Grape host cultivar**	**Molecular identification**
IB1F5	Italia	*A. carbonarius*
IB6F5	Italia	*A. carbonarius*
IB4F5	Italia	*A. carbonarius*
IB5F5	Italia	*A. carbonarius*
MB2F5	Michele Palieri	*A. carbonarius*
MB6F5	Michele Palieri	*A. carbonarius*
MB1F5	Michele Palieri	*A. carbonarius*
MB6F5	Michele Palieri	*A. carbonarius*
MB3F5	Michele Palieri	*A. carbonarius*
MB4F5	Michele Palieri	*A. carbonarius*

4. DISCUSSION AND CONCLUSIONS

Grapes (*Vitis vinifera* L.) are considered to be an important fruit crops. However, grapes are susceptible to several fungal diseases including *Aspergillus* black rot which can be associated with the contamination of grapes and grape products with mycotoxins (Lasram et al., 2012). '*Aspergillus* section Nigri' (black aspergilli) are considered to be a potential OTA producing fungi in grapes, a polyketide mycotoxin with carcinogenic, nephrotoxic, teratogenic, immunosuppressive and cytotoxic properties (Follmann and Lucas, 2003; Kamp et al., 2005).

In the present study, symptomatic black rot table grapes berries were collected from Tunisia vineyards in different areas. Based on the morphological characteristics, the isolates were identified belong to '*Aspergillus* section Nigri' (black *Aspergilli*), related to *A. carbonarius* and *A. niger* "aggregate". As reported in different studies, *A. carbonarius* can be easily distinguished from the other species based on size and conidial criteria, whose diameter varied from 7 to 9 µm (Silva et al., 2011). Our investigation confirms the morphological structure of *A. carbonarius* in several isolates investigated. The presence of several isolates belonging to '*A. niger* aggregate complex' was also confirmed by morphological criteria. '*A. niger* aggregate' was subgroup included on 'black *Aspergilli* group'. To date, this subgroup encompasses 15 morphologically indistinguishable species including *A. tubingensis*, and *A. niger* (Perrone et al., 2011; Hong et al., 2013; Gil-Serna et al., 2019)

It is known that singular species related to the '*A. niger* aggregate complex' were difficult to distinguish using only morphologic criteria (Samson et al., 2004). Indeed, *A. niger*, and *A. tubingensis* are morphological indistinguishable species (Silva et al., 2011). Identify the specific *Aspergillus* spp. is very important because each species can produce different toxins with a different impact on human health. At this regard, the most important species of 'black *Aspergilli*' in terms of food safety and human health are *A. carbonarius*, and *A. niger* and *A. welwitschiae*. Strains of these species produce OTA. In addition, strains of *A. niger* and *A. welwitschiae* produce fumonisin B_2, a polyketide mycotoxin that is carcinogenic and cytotoxic (Frisvad et al., 2011; Perrone et al., 2011). For these reasons, the molecular approaches are essential to reach a correct identification. For the '*Aspergillus niger* aggregate' their ability to produce this toxin seems to be limited to a small number of strains (Gil-Serna et al., 2018). Indeed, an accurate identification of ochratoxigenic fungi is crucial to estimate the risk of OTA contamination (Samson et al., 2004).

In our study, the *A. niger* aggregate identification was validated according to molecular tools using qPCR. A set of primers, suitable for calmodulin, β-tubulin and PKS genes related to the main *Aspergillus* section *nigri* species involved in OTA production, were tested. Our experimental experience has highlighted the importance of analyzing different genes for a suitable identification of *Aspergillus* species. Several primers selected by BLAST tool specific for *Aspergillus* section *nigri* species, (i.e. *A. tubingensis* and *A. niger* species) not confirmed their specificity in qPCR test (data not shown). This confirm that the species belonging to *A. niger* "aggregate" species are very close each other.

Our study confirms the easy discrimination of *A. carbonarius* according to both, β-tubulin and PKSs genes using qPCR. This result was confirmed by sequence analyses of qPCR amplicons obtained for PKSs genes. The amplification of PKS genes included in the OTA production for all *A. carbonarius* isolates, confirm their ability to produce this toxin. In addition, to analyze several genes sequences, the post-qPCR high resolution melting (HRM) tool has been used, which would allow the discrimination of these species based on the highly accurate determination of the difference in melting temperature of the obtained amplicon. This approach was helpful to discriminate *A. niger* species from those of *A. tubingensis*. This method is specifically able to genotype and discriminate species, based on even single-nucleotide polymorphisms (SNPs) and small insertion/deletions, through the analysis of the melting behaviors of double-stranded DNA. Recently a similar approach was suggested for *A. carbonarius*, *A. tubingensis*, *A. niger*, *A. ibericus* and *A. japonicus* discrimination in grape samples artificially inoculated with conidia of these fungi according to ITS2 barcode gene (Xanthopoulou et al., 2019). This method was also used for *Penicillium* sensu strict and *Aspergillus* sect. *nigri* population discrimination from pomegranate fruit using β- tubulin gene (Mincuzzi et al., 2020).

Our results confirm that the molecular tools were crucial to identify *A. niger* aggregate species as *A. niger* and *A. tubingensis*, which were undistinguished by morphological criteria. However, the molecular analysis confirms the morphological results regarding the differences between *A. carbonarius* and '*A. niger* aggregate complex'.

Our study involve numerous *Aspergillus* spp. isolates collected from several grape cultivars in different regions of northern Tunisia known for its semi-arid climate. From all these isolates three species were identified that cause black rot of table grapes, *A. carbonarius, A.*

niger and *A. tubingensis. A. carbonarius* was the most abundant species (66.1%), followed by *A. tubingensis* (20.3%) and *A. niger* (12.7%). Previous investigation on Tunisia vineyard reported by Lasram et al. (2012) suggest that *Aspergillus niger* aggregate was the most dominant species. At the same way, recently *Aspergillus niger* aggregate were the most frequently found isolates reaching from 70% to 85% in dried grapes samples from regions of Kelibia, Sfax, and Rafraf, respectively (Chebil et al., 2020). Our investigation could suggest a new scenario about the occurrence of *Aspergillus* spp. in the main Tunisian grapes production areas. The high percentage of *A. carbonarius* identified in this study suggest a rapid spread of this specie in several localities in Tunisia.

This evidence suggests greater attention in the food supply chain involving grapes because the *A. carbonarius* are the main responsible fungi for the accumulation of OTA in grapes (Battilani and Camardo Leggieri, 2015). However, this study suggests some difference related to grape host cultivars that could be deepen. In the vineyard from Takelsa locality, the 'Michele Palieri' and 'Victoria' cultivars were mainly affected by *A. carbonarius* species (more than 90%), while the isolates collected from 'Red Globe' cultivar were 41.6% *A. niger*.

The occurrence of *Aspergillus* spp. observed in the vineyards located in Medjez El Bab area was interesting. Overall here, although the presence of *A. carbonarius* prevails (54.8%), especially for the isolates from ripened 'ARRA32' and 'ARRA27' cultivars (83%), a prevalence of *A. tubingensis* specie was observed in isolates from unripen berries of 'ARRA34' and 'ARRA32' cultivars (69.2% and 40% respectively). This result suggests that several aspects linked to host and geographic area could be affect the *Aspergillus* spp. occurrence in the vineyards. Underlining the epidemiological dynamism of *Aspergillus* section Nigri in the vineyards, this thesis was able to identify the different *Aspergillus* species present in different areas of Tunisia, however in the next step it will be significant to verify the quantitative and qualitative production of the toxins involved.

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