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Morphological and Molecular Characterization of Ochratoxin A Producing Black Aspergilli from Grape Pomace

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Abstract

Grape pomace (GP), a winery by-product is increasingly being explored as food ingredients. Ochratoxin A (OTA), a natural toxigenic metabolite frequently found in wine and its by-products. Black Aspergilli are mainly responsible for OTA build-up and contamination of grapes and winery by-products. The fungal population in GP of five grape cultivars were enumerated and characterized. Fungal population ranged from 4.27±0.05 to 5.35±0.04 Log CFU/mL with GP from Chardonnay being the most contaminated. *Aspergillus niger* (81.1%) was found to be the major source of contamination and most frequently isolated fungal species. Other fungal isolates were *A. carbonarius* (13.51%) and *A fumigatus* (5.39%). Fungal contamination of GP correlated with the type of grape cultivars used for the pomace. Fourteen identified mold isolates were confirmed by PCR using primer pairs ITS1/NIG, ITS1/CAR and ITS1/FUM. Random amplified polymorphic DNA (RAPD) analysis with eight primers OPC-04, OPC-06, OPC-08, OPC-10, OPC-11, OPC-12, OPC-13 and OPC-14 revealed similarity in band patterns between the isolates and the control. Clustering of banding patterns generated from amplification with primer OPC-12 using Pearson's coefficient detected similarity at 99.10%, 97.60%, 86.30% and 99.40%, 99.10%, 87.60%, 78.50% among *Aspergillus niger* and *Aspergillus carbonarious* strains, respectively, confirming the identification of potential ochratoxigenic black *Aspergillus* strains in the GP. The findings from this study suggest that GP obtained from some grape cultivars could be unsafe as food ingredients due to contamination by ochratoxigenic-producing molds, which is an indicative factor for the presence of ochratoxin A and other mycotoxins.

Keywords: grape pomace (GP), ochratoxin A (OTA), fungal contamination, *Aspergillus carbonarius*, *Aspergillus niger*, internal transcription spacer (ITS), RAPD-PCR

1. Introduction

GP is a by-product of grapes derives from wine processing. In recent times, grape pomace (GP) is gaining use in food products. Many studies have estimated pomace to be 20-30% of the original weight of grape processed into wine (Chand et al., 2009; Yu & Ahmedna, 2013). About 75% of harvested grape fruit is used for wine production. The remaining 25 %, which consist of skin, seed and pulp referred to as GP, is typically composted as fertilizer, or used in animal feed (Dwyer et al., 2014). Numerous studies have also shown that GP could serve as a functional food ingredient for the prevention of diet related diseases such as cardiovascular diseases, obesity, and certain cancers (Bousetta et al., 2009). Due to its high chemical and rich nutrients content, GP also has a potential to support the growth of microorganisms (Borrero et al., 2004, 2005). Over a decade, GP has increasingly been used as food ingredient in products such as sourdough, cookies, minced fish, extruded snacks, frankfurters, and muffins (Canett Romero et al., 2004; Altan et al., 2008; Sánchez-Alonso et al., 2008; Mildner-Szkudlarz et al., 2011a, 2015a).

Ochratoxin A (OTA), a naturally occurring toxin, with nephrotoxic, carcinogenic, immunotoxic, genotoxic, teratogenic effects (Pfohl-Leszkowicz & Manderville, 2007; Reddy & Bhoola 2010; Matsuda et al., 2013), and

Balkan Endemic Nephropathy tendencies (Abozied et al., 2002; Stefanović et al., 2009) found in a variety of different agricultural products and their by-products, including GP are creating food safety concerns. Members of *Aspergillus* section *Nigri* (black aspergilli) are mainly responsible for OTA accumulation in GP. Numerous studies showed that grapes could be contaminated from different fungal species during harvesting or processing by pressing for wine making, or sun drying for raisings (Somma et al., 2012). Fungal contamination of grape is mainly by *Aspergillus*, *Botrytis*, and *Penicillium* species (Guzev et al., 2008, Terra et al., 2012). However, *Aspergillus* section *Nigri* is considered to be principal source of ochratoxin A contamination in grapes and wine in various regions around the world. *A. carbonarius* and *A. niger* aggregate are the major OTA producer isolated and observed more frequently from grapes (Bau et al., 2006; Perrone et al., 2011). Although *A. niger* aggregate is more commonly found in grapes, the ability to produce OTA is lower than that of *A. carbonarius*. In addition, *A. fumigatus*, which belong to *A. niger* aggregate, isolated from grapes has also been found to be pathogenic. *A. fumigatus* has been report to be the cause of most invasive aspergillosis (IA) cases (Serrano et al., 2011). Worldwide IA cases was estimated to be only a few thousand per year, with an overall fatality of >50% (Latge, 1999). *Aspergillus* has the ability to penetrate the lung causing pulmonary conditions such allergic brochopulmonary aspergillosis, sinus infections, aspergilloma and invasive aspergillosis, which may spread to other organs such as brain, heart and kidney (Lasker, 2002; Vargas, 2006).

Determination of the mycoflora and OTA-producing mold in GP is vital in determining the potential OTA contamination of the GP and subsequently, leading to the development of appropriate storage and treatments of GP to ensure safety of consumers of such products. Although many work were reported on the world- wide occurrence of ochratoxin-producing *Aspergillus* species in grape and many other agricultural products no work has been published on contamination of GP with OTA producing fungi. Therefore, the aims of this study were to determine fungal population of the selected GP cultivars, to isolate and characterize ochratoxigenic black *Aspergilli* from GP.

2. Materials and Methods

2.1 Samples Collection

GP from five grape cultivars namely, Cabernet Franc, Cabernet Sauvignon, Merlot, Chardonnay and Sangiovesse were collected from two grape farms/wineries in North Carolina (USA). Samples weighing between 2-4 kg each were collected in sterile plastic bags, transported in cool boxes and frozen immediately at -20 ^{o}C for analyses.

2.2 Mycological Analyses

Thirty (30 g) of GP were mixed with 0.1% peptone water and blended with a stomach blender for 2 minutes, serially diluted and 100 µl of appropriate dilutions extract were plated in triplicates on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Oxoid, USA), Dichloran Glycerol 18% (DG18, Oxoid, USA) and Potato Dextrose Agar (PDA, Oxoid, USA) plates. Three grams (3 g) of each GP samples was also directly plated on DRBC to compare the fungal population. Both sets of samples were then incubated at 25 ^{o}C for 7 days in the dark. The total colonies of yeast and mold were enumerated and results were reported in colony forming units CFU/ml from average fungal counts (Pitt & Hocking, 1997; Dachoupakan et al., 2009; US Food and Drug Bacteriological Analytical Methods, FDA-BAM).

2.3 Isolation and Maintenance of Culture

Colonies of mold that appeared to look different were selected and coded. Selected molds were isolated by three point inoculation onto PDA (potato dextrose agar), MEA (malt extract agar) and CYA (Czapek yeast extract), 5 g $NaNO_3$; 1.0 g K_2HPO_4; 0.5 g KCl; 0.5 g $MgSO_4 \cdot 7H_2O$; 5.0 g yeast extract; 30.0 g sucrose; and 20.0 g agar in 1 liter water at pH 6.5 incubated for 7 days at 25 ^{o}C in the dark. Mold isolates were then maintained on Dichloran Glycerol 18% (DG18, Oxoid, USA) plates for further analysis (Pitt & Hocking, 1999; Dachoupakan et al., 2009; US Food and Drug Bacteriological Analytical Methods, FDA-BAM).

2.4. Identification of Mold

Molds isolates were identified morphologically according to Pitt and Hocking (1997) based on colony appearance, color, exudates and colony diameter on CYA, PDA, and MEA.

2.5 DNA Extraction

Samples taken from three point inoculated CYA plates were aseptically transferred into the labeled 1.5-ml microcentrifuge tubes. Approximately 100 mg of lyophilized mycelium extract was mixed in 1 ml of ChargeSwitch lysis buffer (Invitrogen, Carlsbad, CA, USA) lysis buffer containing reagent A. Four microliters

(4 µl) RNase A was added to each sample homogenized and centrifuged at 13,000 rpm for 5 min. One hundred microliters (100 µl) of 10% SDS was added to lysate and incubated at room temperature for 5 mins. Four hundred (400 ul) microliters of precipitation buffer (N5) (ChargeSwitch Plant DNA Kit) was added and centrifuged at 13,000 rpm for 3 min. Batches of 1200 ml of cleared supernatant were carefully transferred to a 1.5-ml centrifuge tubes for washing and purification followed by eluting according to the manufacturer's instruction to complete DNA extraction (Dachoupakan et al., 2009; Peronne et al., 2010).

2.6 Molecular Detection of OTA Producing Aspergillus Strains

2.6.1 PCR Amplification

PCR analysis was performed according to the method by Dachoupakan et al. (2009) with a slight modification using GoTaq PCR Core Systems I (Madison, WI, USA). ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') was used in combination with each of the following primers: CAR (5'-AGACAGGGGGACGGC-3'), NIG (5'-AGACAGGGGGACGGC-3'), FUM (5'-CGG CCC TTA AAT AGC CCG GTC-3') ELL (5'-CCCGGGATGGGGGACGG-3'), HET (5'-GCAAAATGGTTGGAGAGGTGC-3'), and JAP (5'-GAGAAGATTGGGGGTCGAGG-3'). A total PCR mixture of 25 µL containing: 14.75 µL deionized water, 2.5 µl of 5x buffer green, 2.5 µL of $MgCl_2$ (25mM), 1 µL dNTP (10 pmol/µl), 1 µl of primer ITS1 (10pmol/ µL), 1 µl of primer NIG for *A. niger* or 1 µl of primer CAR (10 pmol/ µl) for *A. carbonarius* or 1 µl of primer FUM (10 pmol/ µl) for *A. fumigatus*, 0.25 µL of Taq DNA polymerase and 2 µL of DNA template. PCR amplification was performed in a Eppendorf Master Pro (Applied Biosystem, Califonia, USA) Cycler set at 1 cycle of 4 min 30 s at 95 ^{o}C, 25 cycles of 30 s at 95 ^{o}C (denaturation) 25 s at 66 (annealing), 40 s at 72 ^{o}C (extension) and finally 1 cycle of 5 min at 72 ^{o}C (Dachoupakan et al., 2009; Gonzalez-Salgado et al., 2005), and 4 ^{o}C 10 mins (hold). The PCR products were separated on 2.5 % (w/v) agarose (Bio-Rad, California, USA) stained with 0.5µg/ml ethidium bromide and visualize with a UV trans-illuminator. A 1 Kb plus DNA ladder Generuler (Thermo Scientific, California, USA) was used as a standard marker to compare molecular masses of amplified DNA.

2.6.2 RAPD-PCR Analysis

Amplification of fungal DNA was performed according to the method described by Dachoupakan et al., (2009) with slight modifications using each of the following random primers (Applied Biosystems, California, USA): OPC-04 (5'-CCGCATCTAC-3'), OPC-06 (5'-GAACGGACTC-3'), OPC-08 (5'-TGGACCGGTG-3'), OPC-10 (5'-TGTCTGGGTG-3'), OPC-11 (5'-TGTCTGGGTG-3'), OPC-12 (5'-TGTCATCCCC-3'), OPC-13 (5'-AAGCCTCGTC 3'), and OPC-14 (5'-TGCGTGCTTG-3'). PCR was performed using GoTaq PCR Core Systems I (Madison, WI, USA) in a total volume of 25 µL containing: 12.5 µL deionized water, 2.5uL 5x buffer green, 2.5 µL25mM MgCl, 1 µL nucleotide mixture, 2 µL primer (10 pmol/ µL), 4 µL template DNA. Parameters for PCR were as follow: 1 cycle of 2 min at 92 ^{o}C for *A. carbonarius* and at 94 ^{o}C for *A. niger* isolates, 45 cycles for *A. carbonarius* and 35 cycles for *A. niger* aggregate of 1 min at 92 ^{o}C (denaturation), 1 min at 35 ^{o}C annealing, 2 min at 72 ^{o}C (extension) and finally 1 cycle of 5 min at 72 ^{o}C (Dachoupakan et al., 2009). The amplified DNA were separated by electrophoresis in 1 % (w/v) agarose (Bio-Rad, California, USA) in 10X TBE buffer and stained with ethidium bromide solution (10mg/mL) (Promega, Madison, WI, USA) and visualized under UV light. The molecular masses of amplified DNA were estimated by comparing with a 1 Kb plus DNA ladder (Thermo Scientific, California, USA).

2.7 Data Analysis

Statistical analysis of the random amplified polymorphic DNA (RAPD) analysis was carried out using GelCompare software (Applied Maths BVBA, Belgium). Similarity in band patterns was computed by cluster analysis using tree clustering with unweighted pair-group averaging (UPGMA) algorithm and corresponding tools in Biometrics.

3. Results and Discussion

3.1 Determination of Fungal Population

The yeast and mold counts ranged from 4.27±0.05 to 5.35±0.04 log CFU/mL (Table 1) with *Aspergillus* spp., being the predominant fungi detected. The highest population of mold (5.35±0.04 log CFUml-1) was detected in pomace derived from Chardonnay grape cultivar, which also showed lowest water activity (0.979±0.01) and highest pH (3.93±0.01). According to Diaz et al. (2009), ochratoxigenic species grow between water activity of 0.900 and 0.996. This indicates the role of water activity and pH in the growth of ochratoxigenic mold. It was observed that pomace from Chardonnay cultivar was the most contaminated with all the three strains of *Aspergillus* detected namely, *A niger, A. carbonarius and A. fumigatus.* The Chardonnay grape cultivar from

which this pomace was produced is used mainly for white wine production. The average total soluble sugar content (TSSC), which is a measure of the soluble sugar content for white and red grape cultivar are 78.15% and 26.03% respectively (Jiang et al., 2010). In white wine processing, grape peels and seeds are not fermented together with juice leading to relatively higher sugar content of GP derived from Chardonnay grape cultivar used in white wine processing. High sugar content could increase carbon source that promote fungal growth. No mold was detected in pomace from Cabernet Franc grape cultivars. The absence of mold in pomace from Cabernet Franc grapes cultivars could be due to its relative high acidity and low water activity (Figures not shown). Slightly higher fungal population was found on PDA compared to DRBC and DG18 agar (Table 1).

Table 1. Fungal population and percentage contamination of grape pomace cultivars on DRBC, PDA and DG18 for 7 days at 25 ^{o}C

GP	Yeast and mold counts (Log CFU mL^{-1})			Contamination by mold (%)		
	DRBC	PDA	DG18	*A. niger*	*A. carbonarius*	*A. fumigatus*
Cabernet Franc	NG	NG	NG	ND	ND	ND
Cabernet Sauvignon	<10	<10	<10	100	-	-
Merlot	<10	<10	<10	66.67	16.67	16.67
Chardonnay	4.47±0.56	5.35±0.04	4.27±0.05	81.10	13.51	5.39
Sangiovesse	NG	NG	NG	ND	ND	ND

NG-no growth; ND-not determined; <10 = less than 10 colonies/mL.

3.2 Determination of Contamination and Comparing the Morphological Characteristics of Different Molds

3.2.1 Percentage Contamination

Percentage contamination of the GP was determined. Pomace derived from Chardonnay was found to have the most contamination from mold. Merlot and Cabernet Sauvignon were also contaminated but to a lesser extent. Predominant fungal population belonging to black *Aspergillus* species made up 94.61 % of the total fungal population. *Aspergillus niger* (81.10%) was found to be the major source of contamination and most frequently isolated fungal species. Other fungal isolates were *A. carbonarius* (13.51%) and *A fumigatus* (5.39%). Several studies reported black *Aspergillus* as primary sources of contamination and major OTA producers in grape and related products (Bau et al., 2005; Medina et al., 2005; Lucchetta et al., 2010; Somma et al., 2012) from a span of studies on grapes and related products. Fungal contamination of the pomace correlated with the type of grape cultivar from which the pomace was derived.

3.2.2 Identification of Mold Isolates

A total of 7 out of the 14 previously selected isolates (Figure 1) including *A. carbonarius, A. niger and A. fumigatus* obtained from the GP samples collected from two winery in North Carolina (Figure 1) were further studied morphologically (Table 2, 3, 4) using three different culture media and molecularly by RAPD analysis.

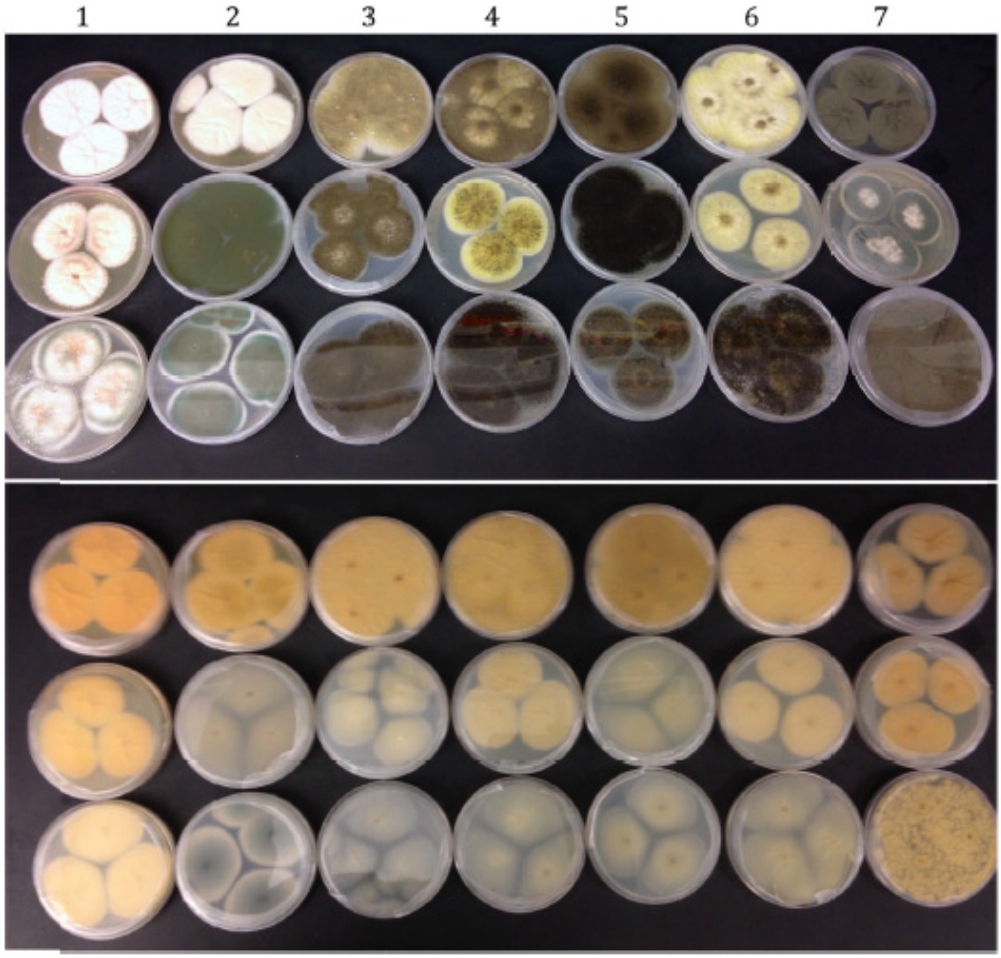

Figure 1. Front and reverse views of different mold (from left to right) isolated on CYA (lane 1), PDA (lane 2) and MEA (lane 3). *Aspergillus niger* (4,6); *Aspergillus carbonarius* (3, 5); *Aspegillus fumigatus* (1, 2, 7)

Table 2. Morphological identification of *A. carbonarius* isolates on CYA for 7 days at 25 ^{o}C

Morphology characteristics		Average colony diameter (mm) on		
Colony Appearance	Light brown and dark brown	PDA	CYA	MEA
Wrinkle colony	Not wrinkled	65	78	71
Reverse coloration	Cream to yellow			
Exudate production	Nil			

Table 3. Morphological identification of *A. niger* isolates on CYA for 7 days at 25 ^{o}C

Morphology characteristics		Average colony diameter (mm) on:		
Colony Appearance	Yellow and black	PDA	CYA	MEA
Wrinkle colony	Wrinkled	58	78	72
Reverse coloration	Florescent Yellow			
Exudate production	Nil			

Table 4. Morphological identification of *A. fumigatus* isolates on CYA for 7 days at 25 ^{o}C

Morphology		Average colony diameter (mm) on		
Colony Appearance	Light brown, dark brown, and dark green	PDA	CYA	MEA
Wrinkle colony	Wrinkled	97	64	57
Reverse coloration	Uncolored to dull yellow			
Exudate production	Nil			

3.3 Molecular Characterization of Fungal Isolates

Specific primers were used to amplify specific regions to confirm morphological identification of the isolates. In all, a total of four *A. niger*, seven *A. carbonarius* and three *A. fumigatus* isolates were tested for amplification using each primer pairs ITS1/NIG and ITS1/CAR and ITS1/FUM respectively. Single fragments estimated to be 400-500 bp amplified was detected only for suspected *A. carbonarius* when ITS1/CAR was tested (Figure 2).

Isolates that did not amplify previously were suspected to be either *A. niger* isolates or *A. fumigatus*, which were further amplified using ITS1/NIG also showed single bands of between 400-500 bp (Figure 3) and were identified as *A. niger*. Isolates that failed to amplify using ITS1/CAR and ITS1/NIG were confirmed as *A. fumigatus* using ITS1/FUM primer set. The remaining sets: ITS1/JAP, ITS1/HET, and ITS1/ELL showed no amplification of the genomic DNA for *A. carbonarius, A. niger*, and *A. fumigatus* (Table 3).

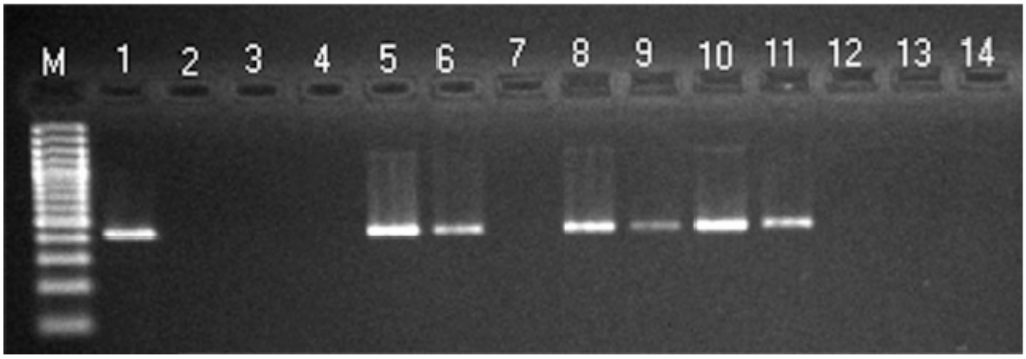

Figure 2. PCR amplification using ITS1/CAR and DNA from *A. carbonarius* isolates; Lane 1: *A. carbonarius* control (ATCC MYA-4641); Lanes 2, 3, 4 & 7: *A. niger* isolates; Lanes 5, 6, 8, 9, 10 & 11: *A carbonarius isolates*; Lanes 12, 13, 14: *A. fumigatus* isolates Lanes; M: DNA marker

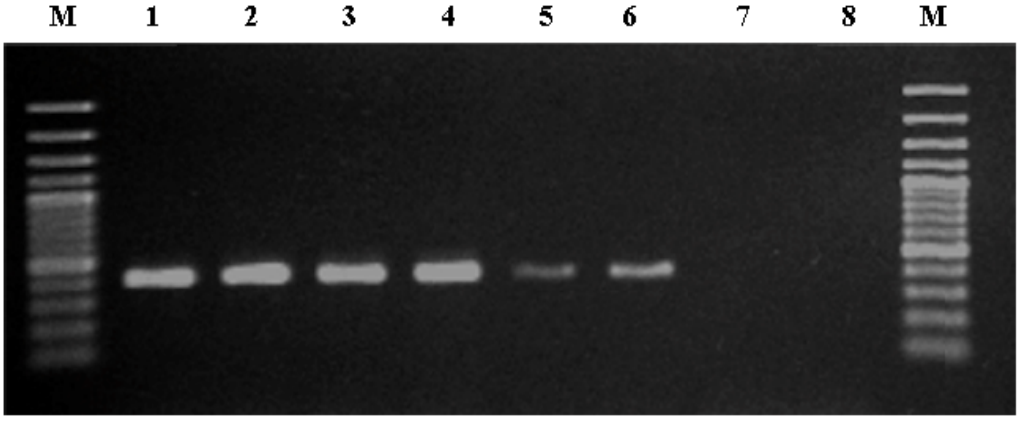

Figure 3. PCR amplification using ITS1/NIG and DNA from *Aspergillus niger* isolates; Lanes 1: *A. niger* control (ATCC 1015D-2); Lanes 2-6: *A. niger* isolates; Lanes 7, 8: *A. fumigatus*; Lanes M: DNA marker

Table 5. Fungal strains analyzed indicating, origin, species, host and the occurrence of PCR amplification product with primers: ITS1-NIG, ITS1-CAR, ITS1-FUM, ITS1-HET, ITS1-JAP and ITS1-ELL

Mold isolate	GP (Cultivar/source)	ITS1-NIG	ITS1-CAR	ITS1-FUM	ITS1-HET	ITS1-ELL	ITS1-JAP
101	Cabernet Franc	-	-	-	-	-	-
102	Cabernet Sauvignon	+	+	+	-	-	-
103	Chardonnay	+	+	+	-	-	-
104	Merlot	+	+	+	-	-	-
105	Sangiovesse	-	-	-	-	-	-

Aspergillus spp. have been isolated from many agricultural products include grape and its associated products such as wine (Somma et al., 2012; Guzev et al., 2008, Terra et al., 2012). Our findings corroborate with results obtained by other studies (Dachoupakan et al., 2009; Diaz et al., 2009; Gonzalez-Salgado et al., 2005).

3.4 Genotypic Studies

Series of RAPD analyses were conducted using 8 random primers (OPC-04, OPC-06, OPC-08, OPC-10, OPC-11, OPC-12, OPC-13, and OPC-14) for detecting similarity through amplified polymorphism between the genomic DNA of isolated *A. niger* and *A. carbonarius* strains and their respective control.

For *A. niger* isolates, similar banding patterns between bands 3 to 9 denoting random amplification of polymorphic DNA were detectable with primers OPC-06, OPC-08, OPC-10, OPC-11, OPC-12, and OPC-14 (Figure 4.) being the most interpretable. Primers OPC-04 and OPC-13 produced amplification that resulted into faint polymorphic bands. All isolates identified as *A. niger* showed identical sequence pattern that are also identical to banding in *A. niger* control samples. Primer OPC-11 displayed the faintest yet the highest discriminatory power (6) and OPC- 04 the least (3). Similar polymorphic banding was observed between isolates and *A. niger* control when primer OPC-12 was amplified with genomic DNA from *A. niger* isolates (Figure 7).

Similarly, *A. carbonarius* isolates also demonstrated similar banding with OPC primers with the highest (8) being with primer OPC 04 and the least (3) with OPC 06. Relatively more distinct bands were observed when primers OPC were used in amplification with genomic DNA of *A. carbonarius* isolates compare to *A. niger*. Except for primer OPC-11, all primers used produced distinct and interpretable polymorphic bands (Figure 5).

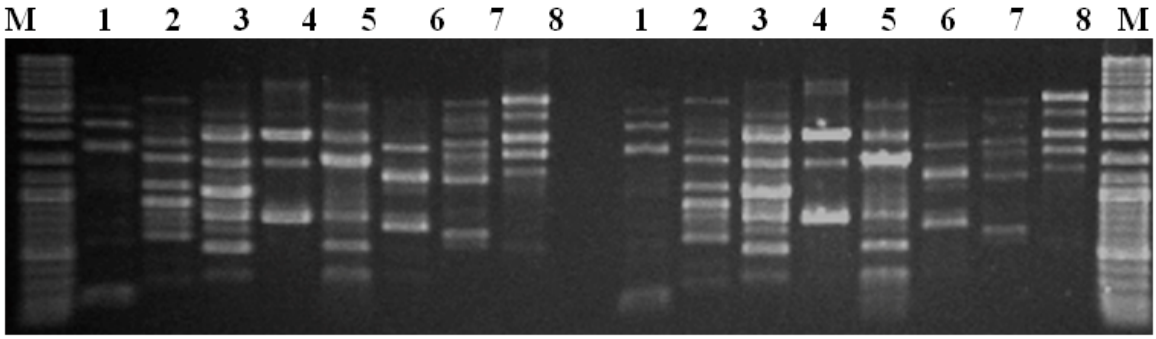

Figure 4. RAPD patterns of *A. niger* isolate, generated with OPC primers: Lane 1: OPC-04, Lane 2: OPC-06, Lane 3: OPC-08; Lane 4: OPC-10; Lane 5: OPC-11; Lane 6: OPC-12; Lane 7: OPC-13; Lane 8: OPC-14; M: DNA marker

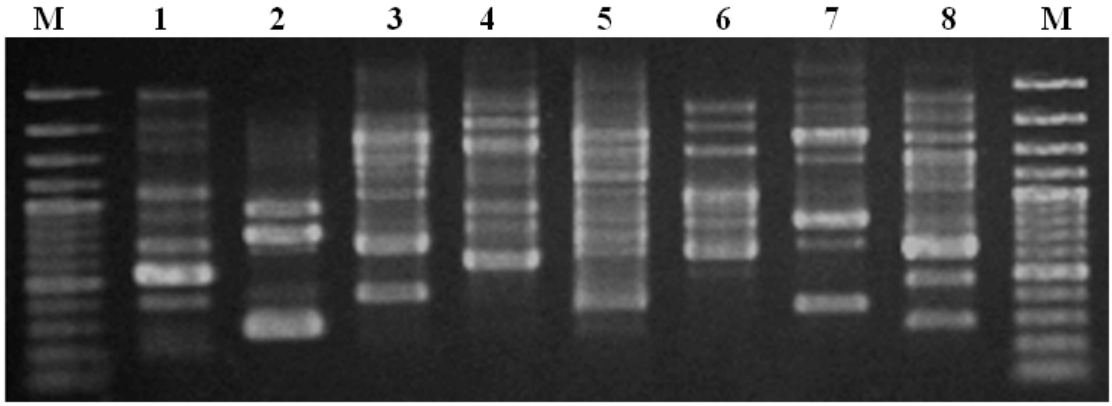

Figure 5. RAPD patterns of *A. carbonarius* isolate, generated with OPC primers: Lane 1: OPC-04; Lane 2: OPC-06; Lane 3: OPC-08; Lane 4: OPC-10; Lane 5: OPC-11; Lane6: OPC-12; Lane 7: OPC-13; Lane 8: OPC-14; M: DNA marker

Except for primers OPC 06 and OPC13 that showed distinct bands when used in amplification of genomic DNA of *A. fumigatus* isolates, all other primers used displayed faint bands (Figure 6). However, banding patterns observed in all isolates were similar to those found in respective controls (Figure 7).

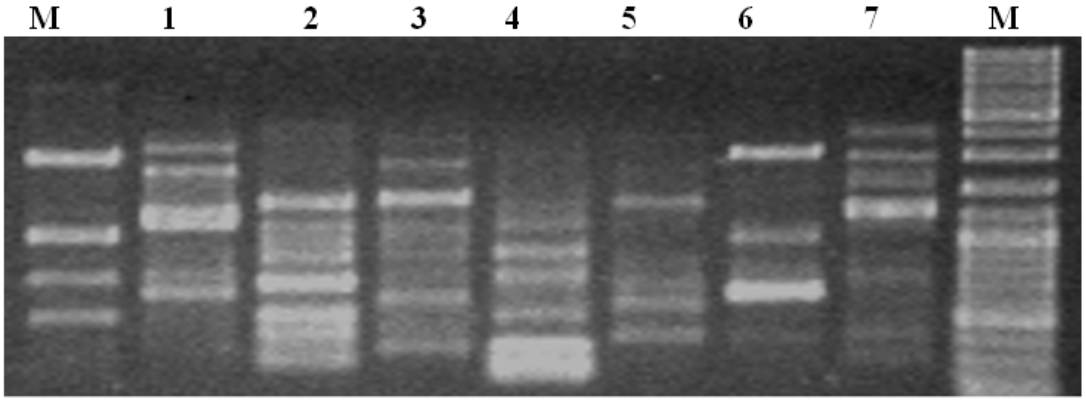

Figure 6. RAPD patterns of *A. fumigatus* isolate, generated with OPC primers: Lane 1: OPC-04; Lane 2: OPC-06; Lane 3: OPC-08; Lane 4: OPC-10; Lane 5: OPC-11; Lane 6: OPC-12; Lane 7: OPC-13; Lane 8: OPC-14; M: DNA marker

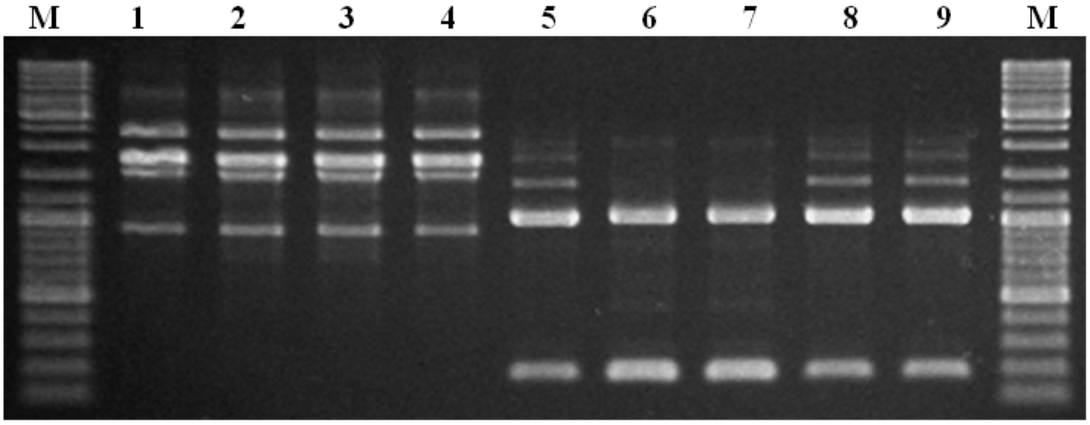

Figure 7. RAPD patterns of *A. niger* and *A. carbonarius* isolates and their respective controls, generated with primer OPC-12: Lane 1: *A. niger* control (ATCC 1015D-2); Lanes 2-4: *A. niger* isolates; Lane 5: *A. carbonarius* control (ATCC MYA-4641); Lanes 6-9: *A. carbonarius* isolates; M: DNA marker

To further confirm the fungal species isolated from the pomace primer OPC-12 was used for RAPD analysis (Figure 7.) for both *A. niger* (N1, N2, N3, *A. niger* control: **N4**) and *A. carbonarius* (isolates: C1, C2, C3, C4; *A. carbonarius* control: **C5)** strains. Figure 8 shows the dendogram, which was generated from the RAPD banding patterns using unweighted pair group average linkage clustering revealed three main clusters within *A. carbonarius* and *A. niger* strains (Figure 8). *A. carbonarius* and *A. niger* isolates and their respective controls obtained by cluster analysis showed close degree of similarity among the strains. The similarity percentages ranged from 78.5-97.6% among *A. carbonarius* isolates, N1 and N2 clustered together showing similarity at 99.1% and with N3 at 97.6%. Similarly, all *A. niger* isolates showed similarity at 86.3% to the *A. niger* control **(N4**). Similarly, *A. carbonarius* isolates C1 and C2 clustered together at 99.1% similarity. Isolate C4 is similar to the *A. carbonarius* control (**C5**) at 99.4% similarity. All strains belonging to *A. carbonarius* and *A. niger* showed clustering at 2.2% similarity, indicating a wide genotypic variation between *A. carbonarius* and *A. niger*.

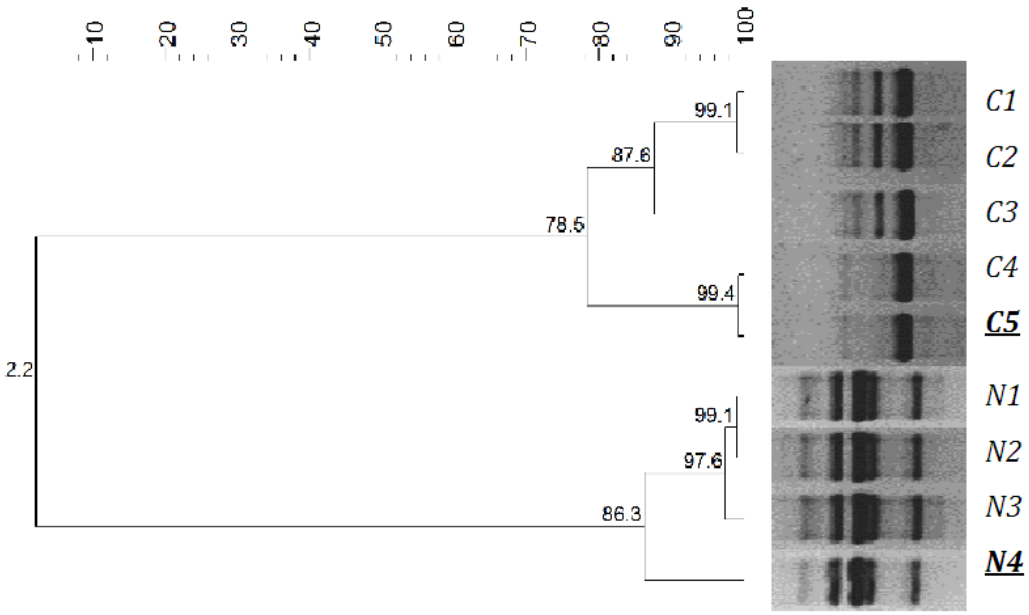

Figure 8. Dendogram based on UPGMA cluster analysis of *A. carbonarius* and *A. niger* isolates assessed from the comparison of RAPD fingerprints generated with primer OPC-12 using cluster analysis with percent similarity

Morphological group is indicated in letters: C1-C4 (*A. carbonarius* isolates); **C5** (*A. carbonarius* control); and N1-N3 (*A. niger* isolates); **N4** (*A. niger* control).

Black Aspergilli *are* considered primary producers and contaminant of OTA, a mycotoxin with lethal effects including immunosuppressive, teratogenic and carcinogenic consequences, in grapes and it's associated products (Varga & Kozakiewicz). *A. carbonarius* and *A. niger* have been the course of OTA production in grapes and its by-products (Cabanes et al., 2002; Varga et al., 2007; Peronne et al., 2007).

Our results agree with the study of Martinez et al. (2009) using ap-PCR and phylogenic analysis based on ITS and IGS sequences. The data obtained are also consistent with several other studies (Lasker, 2002; Varga et al., 2007; Peronne et al., 2008; Moslem et al., 2010). Therefore, a close relationship appeared to exist between the isolates and the control, confirming the identification of potential ochratoxin A-producing *A. niger,* and *A. carbonarius* in tested GP.

4. Conclusion

Our study found high levels of fungal population and contamination (94.61%) by potential ochratoxigenic *A. niger* and *A. carbonarious* in certain GP cultivars. Additionally, clear evidence of the presence of potential OTA producing *A. niger* and *A. carbonarius* isolates was established through PCR amplification of ITS region and close RAPD-PCR similarity matrix found between the isolates and their respective references. Our findings suggest that GP could be contaminated with high levels pathogenic black Aspergillus, which is an indicative factor for the presence of ochratoxin A and other mycotoxins rendering certain GP unsafe as food ingredients.

The contamination of grapes by mold is closely related to agricultural practices such as the use of fungicide and removal of grapes contaminated with mold before harvest, and handling after harvest. There is a pressing need to enhance awareness among wine processors to develop more stringent and appropriate protocols for proper handling of GP from vineyard to winery sites and to table to ensure they are safe as food products for both humans and animals.

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