Egypt. J. Bot. **Vol. 57,** No. 2, pp. 395 -404 (2017)

Studies on the Sclerotia of Some Species in The Genus *Aspergillus*

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THIS study is part of a large study concerning with the sclerotia of some species of genus *Aspergillus*. In the present study, the sclerotia of different aspergilli (*Aspergillus sclerotioniger*, *A. sclerotiorum*, *A. candidus*, *A. flavus, A. piperis*, *A. ochraceus, A. robustus*, *A. sepultus*, *A. petrakii*, *A. melleus*, *A. parasiticus* and *A. sclerotiicarbonarius*) were examined concerning their morphological and anatomical characters. The mature sclerotia in the cultures of the used aspergilli appeared with different shapes (globose, sub-globose and oval). Transverse sections of mature sclerotia revealed two regions; the outer region, considered as rind, while the inner region proposed as medulla. These aspergilli were tested also by polymerase chain reaction (PCR) technique for the presence of sclerotium regulator (SclR) gene. The results indicated that eight of them contain this gene; these were *A. petrakii*, *A. sepultus*, *A. robustus*, *A. sclerotiicarbonarius*, *A. melleus*, *A. sclerotioniger*, *A. parasiticus* and *A. sclerotiorum*. These species exhibited bands with different molecular weights when tested for presence of SclR gene. Each *Aspergillus* species showed one band with specific molecular weight, these bands ranged in size; the highest was 436 bp (*A. parasiticus*) while the lowest was 134 bp (*A. sclerotiicarbonarius*).

Keywords: *Aspergillus*, Sclerotia, PCR and (SclR) gene.

Introduction

Some fungi are known to form sclerotia as a part of their life cycles. Sclerotia are defined as resting bodies which enable the fungus to survive and withstand environmental adverse conditions. These resting bodies could be produced by certain pathogenic and saprophytic fungal species such as those of some of the genera *Claviceps*, *Sclerotinia*, *Sclerotium*, *Aspergillus*, *Penicillium*, etc.

Sclerotia are resting bodies produced by many fungi as a mechanism for their long-term survival and propagation of the species Gloer et al. (1988) and Wicklow et al. (1988).

On the other hand, Calvo (2008) mentioned that sclerotia resemble cleistothecia in both their morphology and the genetic control of their development. This suggests that the two structures may be homologous but sclerotia being vestigial cleistothecia that lost the capacity to produce spores.

According to Georgiou et al. (2006), three substates in sclerotia biogenesis were distinguished:

(1) *Sclerotial initial*
Distinct initials formed from highly proliferating interwoven hyphae.

(2) *Sclerotial development*
The initials increase in size.

(3) *Sclerotial maturation*
Characterized by surface delimitation, internal consolidation, melanin pigmentation, and often associated with droplet excretion (exudate).

Aspergillus species are widespread in different environments and climates as saprophytes and parasites. Several aspergilli such as groups of *A. niger* (black aspergilli), *A. flavus*, *A. ochraceus* contain many species which produce sclerotia during their life cycles.

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DOI: 10.21608/ejbo.2017.683.1036

Raper & Thom (1949), Raper & Fennell(1965) and Christensen (1981) reported that synnemata and sclerotia are important morphological characters for identifying some species of *Aspergillus, Penicillium* and related genera.

Bojovic-Cvetic & Vujicic (1988) stated that the sclerotia of *A. flavus* are comprised of a rind and a sub-rind and central medullary cells. The rind is made of flattened cells with darkly-pigmented walls and empty lumina; sub- rind cells have partially pigmented walls and cytoplasm containing glycogen particles. The thick walled medullary cells contain mostly polysaccharides.

Moreover Samson et al. (2007) illustrated that *A. sclerotioniger* is able to produce sclerotia and give positive result with Ehrlish reagent at sclerotia regions which means that the sclerotia contain alkaloids.

Bacha et al. (2009) studied *Aspergillus westerdijkiae* which is the main producer of several biologically active polyketide metabolites including isoasperlactone and asperlactone. A 5298 bp polyketide synthase gene (*aomsas*) has been cloned in *A. westerdijkiae* by using gene walking approach and RACE-PCR. Moreover, Passone et al. (2010) quantified *Aspergillus* section *Flavi* population in peanuts using a real-time PCR system directed against the *nor*-1 gene of the aflatoxin biosynthetic pathway.

Recently, many mycologists have stepped up their studies on the sclerotia of different species of genus *Aspergillus* where the aspergilli are distributed in diverse environments. This work was designed to study the sclerotia of (*A. sclerotioniger*, *A. sclerotiorum*, *A. candidus*, *A. flavus*, *A. piperis*, *A. ochraceus*, *A. robustus*, *A. sepultus*, *A. petrakii*, *A. melleus*, *A. parasiticus* and *A. sclerotiicarbonarius*) concerning their morphological and anatomical characters. These aspergillii were tested by PCR technique for the presence of SclR gene which encodes basic helix- loop-helix transcription factor which regulates the hyphal morphology and promotes sclerotial formation in *Aspergillus oryzae*.

Materials and Methods

The tested fungi

In the present study, sclerotia forming aspergilli were selected. *Aspergillus sclerotioniger*, *A. sclerotiorum*, *A. candidus, A. flavus, A. piperis, A. ochraceus, A. robustus, A. sepultus, A. petrakii, A. melleus, A. parasiticus* and A. *sclerotiicarbonarius* were purchased from the mycological center, Assiut University, Egypt and maintained on Czapek's medium (CZA), which was used for the growth of the selected species whenever needed.

Czapek's agar medium (CZA)

Sucrose: 30 g
Sodium Nitrate ($NaNO_3$): 3 g
Potassium dihydrogen ortho phosphate (KH_2PO_4): 1g
Magnesium sulphate ($MgSO_4$): 0.5 g
Potassium chloride (KCl): 0.5 g
Ferrous sulphate ($FeSO_4$): 0.01 g
Agar: 20 g
Dist. H_2O: 1000 ml

Morphology and anatomy of mature sclerotia of the tested aspergilli

This experiment was carried out using modified method adopted by Ryan et al. (2003). Mature sclerotia were gently picked from six days old cultures of *A. flavus, A. melleus, A. ochraceus, A. robustus*, *A. sclerotiicarbonarius*, *A. sclerotioniger* and *A. sclerotiorum* separately; however, these were collected from one month old culture of *A. petrakii* after which sclerotia were established. For *A. candidus*, *A. parasiticus*, *A. piperis* and *A. sepultus* sclerotial detection was achieved from 2 months old cultures, then could be harvested. The tested sclerotia were subsequently placed in a sieve and washed several times with distilled water to remove any remained attached mycelia, thereafter the sclerotia could be described morphologically.

For anatomical studies the method of Thakur et al. (1984) was conducted; thin transverse hand sections were established for the mature sclerotia, mounted in water, examined by light microscope and photographed.

Detection for the presence of SclR gene in different aspergilli using PCR analysis

Jin et al. (2011) examined the SclR gene which encodes basic helix-loop-helix transcription factor which regulates the hyphal morphology and promotes sclerotial formation in *Aspergillus oryzae*. In the present experiment, the presence of this gene in the different tested aspergilli was tested using PCR technique as follow:

DNA extraction

DNA was extracted and purified from the hyphae of bulked samples of the tested aspergilli separately using Gene JET Plant Genomic DNA purification

minikit. This was adopted following the protocol of the manufacturer [Maxi Hot start PCR Master Mix (2X)].

Optimization of PCR conditions

According to Jin et al. (2011) the primer sequence of SclR gene is: QRT215-F (187): GAGAACTTCGCTCTCGATGTGC which is the forward strand and QRT215-R (311): CCGAAGTGATAGAACCGGCAT which is the Reverse strand.

Each isolated genomic DNA was used as template in the amplification reactions. A total of 12 µl reaction mix was prepared. Amplification conditions were optimized using a gradient thermal cycler (Biometera Uno thermal cycler, Germany). After several experiments for optimizing the best conditions, a program for PCR was standardized with the following settings: Initial denaturation at 95°C for 4 min, followed by 35 cycles of 1 min at 94°C for denaturation, annealing temperature according to each primer for 45 s, and 2 min at 72°C for extension and a final extension of 5 min at 72°C and then temperature was set at 4°C till removal of PCR tubes within 12 h.

Separation of PCR amplification products

The amplification products were separated by mixing 10 µl of the PCR- products of each primer and 2 µl of selected buffer then loading the mix into the agarose wells. Electrophoresis was made in 1.7 % agarose gel prepared in 0.5 X TAE buffer at 70 V for 3 h. The PCR fingerprinting was visualized using a Gel Works 1D advanced gel documentation system (UVP, UK) and photographed under UV light. The size of each band was estimated using 100 bp DNA ladder (Fermentas) as a standard marker.

To prepare the gel, 100 ml 0.5 X TE buffer were added to 1.7 g agarose and the mixture was heated in a microwave tell complete melting and left to cool to 60ºC then ethidium bromide was added to the solution in a concentration of 0.2 µg/ml.

Results and Discussion

Morphological studies of mature sclerotia of the tested aspergilli

Table 1 and Fig.1 illustrate the morphological characters (shape, color, size and compactness) of mature sclerotia of the tested aspergilli. The mature sclerotia in the cultures of the used aspergilli appeared with different shapes (globose, sub-globose and oval). The sclerotia of *A. sepultus* and *A. melleus* were too small to be recognized in the culture by naked eye so they were examined and photographed using microscope (Fig. 1: J& K). The sclerotia of *A. melleus*, *A. candidus* and *A. petrakii* were globose (Fig. 1: K, F& L respectively).

The color of sclerotia of *A. melleus*, *A. candidus* and *A. petrakii* were reddish brown, brown and brownish cinnamon respectively. The size of sclerotia of *A. candidus* and *A. petrakii* were medium in size; they measured 0.43×0.43 mm and 0.62×0.62 mm respectively. On the other hand, *A. melleus* sclerotia were too small to be only detected microscopically; measuring 0.3×0.3 mm.

These results mostly in accordance with Christensen, (1982) who reported that, *A. melleus* colonies on malt agar were characterized by production of yellow conidial heads and sclerotia were mostly of 0.350.4- mm diameter and creamy to golden color. However, Samson & Mouchacca (1975) described the sclerotia of *A. melleus* on Czapek agar as hard, more or less globose, 0.1-0.2 mm in diameter, white at first, later yellow to brownish. Concerning sclerotia of *A. petrakii* our results discrepant that of Christensen (1982) who did not observe any sclerotia in *A. petrakii*. While Varga et al. (2007) described the sclerotia of *A. candidus* when produced at first take white color then quickly becoming reddish purple to black consisting of thick walled parenchyma like cells.

The present study revealed that sclerotia of *A. piperis, A. sclerotiicarbonarius* and *A. sclerotioniger* were globose to sub-globose (Fig. 1: B, C& A, respectively). The colors of sclerotia of these species were found to be brownish red, black and light to dark brown with surface exudate droplets respectively. Sclerotia of *A. sclerotioniger, A. sclerotiicarbonarius* were medium in size; they measured 0.55×0.59 mm to 0.73×0.73 mm, 0.43 × 0.57 mm respectively; but *A. piperis* sclerotia were larger (1.14×1.147 mm to 1.53×1.55 mm). Many researchers studied sclerotia of black aspergilli; Samson et al. (2007) reported that the sclerotia of *A. sclerotioniger* and *A. sclerotiicarbonarius* were yellow to orange to red- brown while sclerotia of *A. piperis* were yellow to pink brown (0.5 - 0.8 mm). In the meantime, Noonim et al. (2008) stated that the sclerotia of *A. sclerotiicarbonarius* were produced abundantly in most isolates of this species, and were globose, sub globose to ellipsoidal with yellow to orange to red–brown color and diameter measuring 0.6 to 1.6 mm.

TABLE 1. Morphological characters of mature sclerotia of the tested aspergilli.

Species / Characters	*A.sclero-tioniger*	*A.piperis*	*A.sclero-tiicarb-onarius*	*A.flavus*	*A.parasiticus*	*A.candidus*	*A.ochraceus*	*A.sclerotiorum*	*A.robustus*	*A.sepultus*	*A.melleus*	*A.petrakii*
shape	Globose to sub-globose	Globose to sub-globose	Globose to sub-globose	Oval	Globose to sub-globose and oval	Globose	Sub-globose and oval	Globose and oval	Globose to sub-globose and oval	Irregular shape	Globose	Globose
Color	Light to dark brown with surface exudate droplets.	Browni-sh red	Black	Black with surface exudate droplets.	Black	Brown	Black with surface exudate droplets.	Black	Black	Reddish brown	Reddish brown	Brownish cinnamon
Size(mm)	0.55×0.59 To 0.73×0.73	1.14×1.147 To 1.53×1.55	0.43 × 0.57	0.25 × 0.35 To 0.63×1	0.36×0.38 To 0.55×0.65	0.43×0.43	0.22×0.25 To 0.85×1.4& 0.9×1.4	0.31×0.31 To 0.89×1.13& 0.85×1.5	0.22×0.25 To 0.61 × 0.8	Not measured	0.3×0.3	0.62
Compact-ness	Hard	Hard	Hard	Hard	Hard	Hard but brettle	Hard	Hard	Hard	Not detected	Hard	Hard

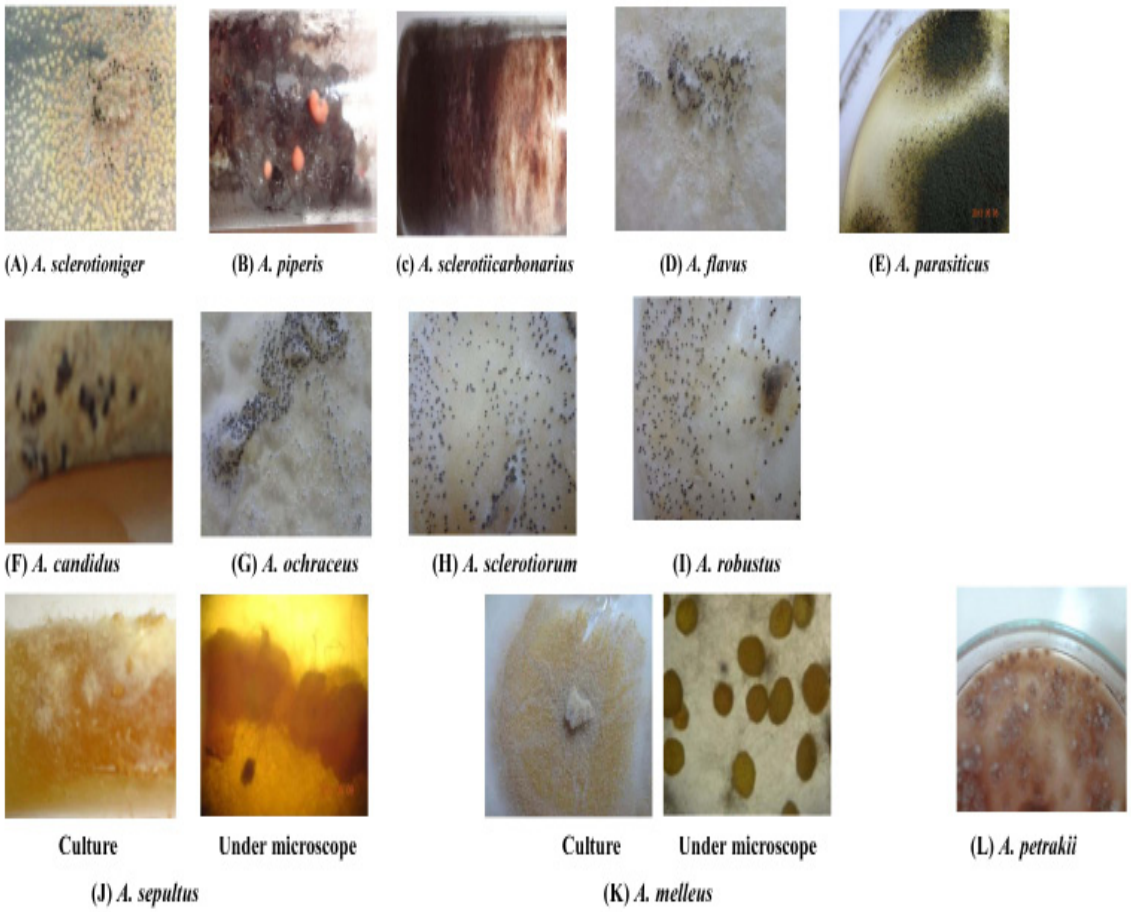


Fig.1. Appearence of mature sclerotia of tested aspergilli.

Sclerotia of *A. parasiticus* and *A. robustus* were globose to sub-globose to oval (Fig. 1: E&I, respectively); sclerotia of *A. flavus*, *A. ochraceus* and *A. sclerotiorum* were oval, sub-globose to oval and globose to oval as illustrated in Fig.1: D, G& H, respectively while sclerotia of *A. sepultus* were irregular in shape (Fig. 1: J). When the colors of sclerotia of *A. flavus*, *A. parasiticus*, *A. ochraceus*, *A. robustus* and *A. sclerotiorum* were examined they appeared to be black

A. flavus, *A. ochraceus*, *A. parasiticus*, *A. robustus* and *A. sclerotiorum* each exhibited different sizes of sclerotia. Of these smaller sizes measured 0.25×0.35 mm, 0.22×0.25 mm, 0.36×0.38 mm, 0.22×0.25 mm and 0.31× 0.31 mm, while larger sizes were oval and found to be 0.63×1 mm, 0.9×1.4 mm, 0.55×0.65 mm, 0.61×0.8 mm and 0.85×1.5 mm, respectively.

Christensen (1982) stated that *A. ochraceus* sclerotia were produced in some strains, irregular in form, up to 1 - 2 mm diameter, lavender to vinaceous at maturity. The same auther documented that sclerotia of *A. robustus* are yellow-brown to black, irregular in form, 0.2500.900- mm in longest dimension; in the meantime, sclerotia of *A. sclerotiorum* were produced in some strains, pale, 1 - 1.5 mm diameter. Earlier Neill (1940) reported that some strains of *A. ochraceus* produce abundant sclerotia which while sclerotia of *A. sclerotiorum* are abundant, first appearing in cultures 3 days old, globose or sub-globose, white at first, soon becoming light cream and then flesh pink. Also, the same author reported that sclerotia produced by some strains of *A. flavus* at first appeared as white weft of fine aerial hyphae, later becoming globose, black, to 0.7 mm diameter, frequently aggregated into masses. Moreover, Cotty (1989) recorded that different isolates of *A. flavus* can be categorized either as the typical L strain with sclerotia > 0.4 mm in diameter or as the S strain which is dominated by abundant small sclerotia < 0.4 mm in diameter.

Horn et al. (2009) stated that sclerotia of Petromyces parasiticus (which is the sexual state of *A. parasiticus*) produced sclerotia and stromata similar in external appearance, globose to ellipsoidal, with size

ranged from 0.25- 0.3 to 1.2 - 1.3 mm, white in color becoming pink brown and finally dark brown to black; inner matrix was light to dark brown, consisting of pseudoparenchymatous tissue.

In the present study, sclerotia of *A. sepultus* were difficultly detected in the culture for their minute size and were difficult to be measured (Fig. 1: J). Tuthill & Christensen, (1986) reported that sclerotia in this species were absent.

Anatomical studies of mature sclerotia of the tested aspergilli

Transverse sections of sclerotia of *A. sclerotioniger, A. piperis*, *A. sclerotiicarbonarius*, *A. candidus*, *A. melleus* and *A. petrakii* revealed two regions; the outer region, considered as rind, was brownish and hard while the inner region proposed as medulla appeared as white pseudoparenchymatous tissue, (Fig.2: A, B, C, F, K & L respectively). The mature sclerotia of *A. melleus* could not be recognized in the culture by naked eye but by reddish brown regions in the plate culture (Fig. 1: K). Microscopically these regions revealed globose to sub-globose sclerotia with reddish brown color.Anatomically two regions were clear; the outer region (rind) was reddish brown and the inner region (medulla) composed of colorless pseudoparenchymatous tissue (Fig. 2: K).

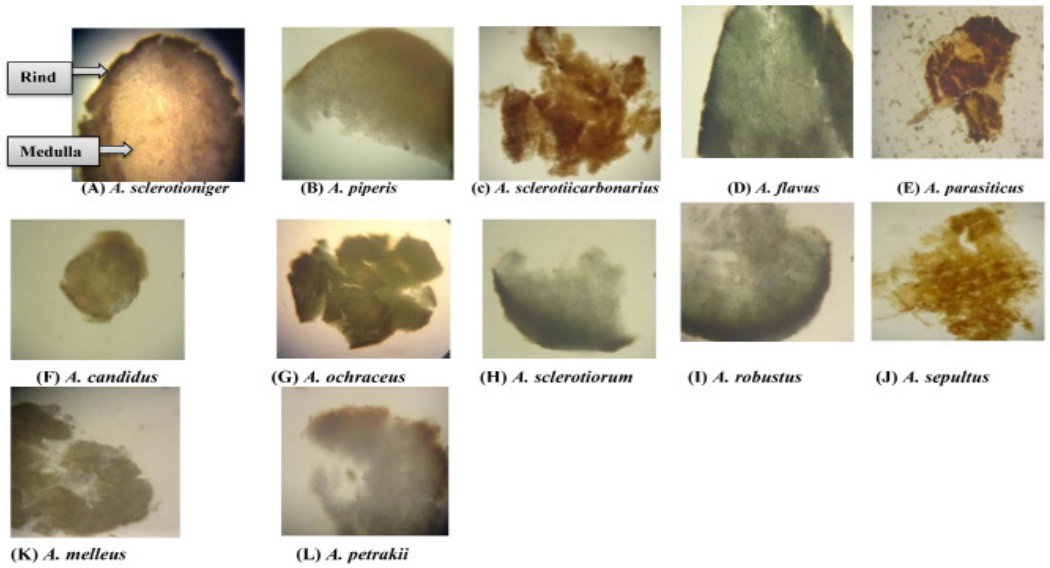


Fig.2. Anatomical features of mature sclerotia of the tested aspergilli.

On the other hand, the rinds of sclerotia in *A. flavus*, *A. ochraceus*, *A. robustus* and *A. sclerotiorum* were black while medullas of these species were composed of colourless pseudoparenchymatous tissues (Fig. 2: D, G, I & H, respectively). Under microscope the transverse section of sclerotia of *A. parasiticus* revealed dark brown rind and brownish yellow medulla, while the macerated sclerotium of *A. sepultus* appeared as woven mass of swollen tissues traversed by swollen hyphae (Fig. 2: J).

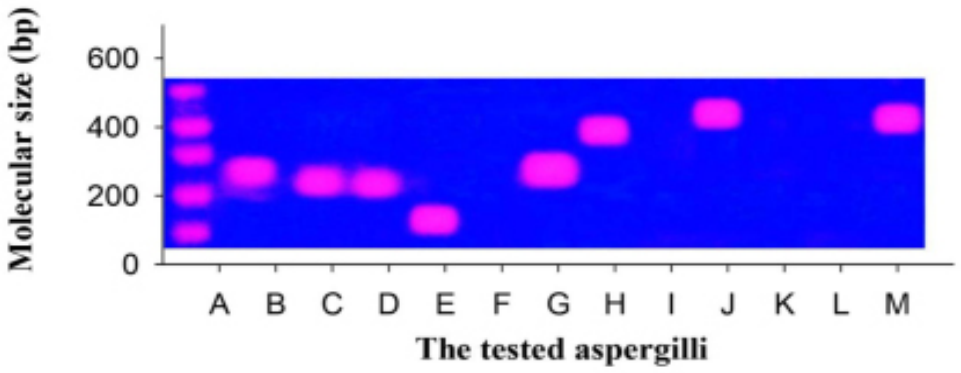
Detection of the presence of SclR gene in different used aspergilli by PCR analysis:

Formation of sclerotia in the genus *Aspergillus* was regulated by different genes. The present study dealing with the presence of only one gene (SclR gene) in the tested Aspergilli. Table 2 and Fig. 3 illustrate that eight of the tested aspergilli (*A. petrakii*, *A. sepultus*, *A. robustus*, *A. sclerotiicarbonarius*, *A. melleus*, *A. sclerotioniger*, *A. parasiticus* and *A. sclerotiorum*) exhibited bands with different molecular weights when tested for presence of SclR gene by the tested primer. Each *Aspergillus* species showed one band with specific molecular weight, these bands ranged in size; the highest was 436 bp (*A. parasiticus*) while the lowest was 134 bp (*A. sclerotiicarbonarius*). Jin et al. (2009) identified a novel bHLH protein encoding gene (SclR; AO090011000215) in *A. oryzae* by systematically deleting large chromosomal segments and further deletion analysis to screen a phenotype of dense conidia. Also, they mentioned that *A. oryzae* SclR shares high similarity with those

of putative proteins from genome sequencing projects of *A. niger*, *A. fumigatus* and *A. nidulans*. The gene-disrupted strain was found to produce dense conidia, but sparse sclerotia, relative to the parent strain, suggesting that it possibly plays an important role in morphology and growth.

TABLE 2. Molecular weights of SclR gene in the tested aspergilli

Aspergillus	**Molecular size of SclR gene (bp)**
A. petrakii	259
A. sepultus	232
A. robustus	226
A. sclerotiicarbonarius	134
A.piperis	-
A. melleus	273
A. sclerotioniger	387
A.candidus	-
A. parasiticus	436
A.flavus	-
A.ochraceus	-
A. sclerotiorum	414



A: Marker
B: *A. petrakii*
C: *A. sepultus*
D: *A. robustus*
E: *A. sclerotiicarbonarius*
F: *A. piperis*
M: *A. sclerotiorum*
G: *A. melleus*
H: *A. sclerotioniger*
I: *A. candidus*
J: *A. parasiticus*
K: *A. flavus*
L: *A. ochraceus*

Fig. 3. Bands on agarose gel indicating the presence of SclR gene in 8 species of tested aspergilli with different molecular weights.

Furthermore Jin et al. (2011) reported the characterization of the SclR gene, along with analysis of its function in *A. oryzae*. Overexpression of the SclR gene led to abnormal hyphal morphology and sclerotial formation.

In related work Chang (2008) found that the deletion of a zinc finger calcineurin response gene, *crzA*, in *A. parasiticus* resulted in the production of mainly immature sclerotia.

Moreover Kale et al. (2008) examined the role of nuclear regulator (LaeA) in two mutants and wild type of *A. flavus* and found that LaeA had a major effect on *A. flavus* secondary metabolism where *ΔlaeA* and over-expression *laeA* strains yielded opposite phenotypes resulting in changes in secondary metabolite production. The two mutant strains also exhibited striking morphological phenotypes in the loss (increase) of sclerotial production in comparison to wild type. Transcriptional examination of the mutants showed LaeA negatively regulates expression of its recently identified nuclear partner VeA (another global regulator of secondary metabolites and sclerotia of *A. flavus*).

Conclusion

From the present study, it could be concluded that:

1. The mature sclerotia of the tested Aspergilli were markedly varied in shape, color, size, compactness and anatomical features.

2. The *SclR* gene was detected using PCR and was found with different molecular weights in eight species from the tested Aspergilli.

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Received: 23/ 3 / 2017
Accepted: 4 / 5/ 2017

دراسات على الأجسام الحجرية لبعض أنواع الجنس اسبرجيلس

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يهدف هذا البحث إلى دراسة الأجسام الحجرية لبعض أنواع جنس الاسبرجيلس وذلك من ناحية الشكل الظاهرى والتركيب التشريحى وكذلك دراستها جينيا. وعلى ذلك فقد تم اختيار وسائل ومواد مختلفة وذلك لزراعة الأنواع المختبرة من جنس الأسبرجيلس ووصف الأجسام الحجرية الخاصة بها ظاهريا وتشريحيا. أوضحت النتائج أن الأجسام الحجرية لأنواع **الأسبرجيلس** المختبرة تتميز بأشكال مختلفة منها الكروى وشبه الكروى والبيضاوى فيماعدا الأجسام الحجرية الخاصة بفطر (أسبرجيلس سيبألتس) فهى تتميز بشكل غير منتظم. تتميز الأجسام الحجرية للأنواع المختبرة بدرجات ألوان تتراوح من البنى إلى الأسود وتتميز أيضا بأحجام متباينة تتراوح بين 0.25×0.35 ميلليمتر الى 1.35×1.55 ميلليمتر. أما بالنسبة لدراسة التركيب التشريحى للأجسام الحجرية للأنواع المختبرة فقد بينت النتائج أن الجسم الحجرى لكل منهم يتركب من منطقتين: المنطقة الخارجية تسمى (Rind) والداخلية تسمى (medulla). كان هذا مميزا فى كل الأنواع المختبرة ماعدا الأجسام الحجرية لفطر أسبرجيللس سيبألتس فأنه يتكون من كتلة متداخلة من الأنسجة يعترضها بعض الخيوط المنتفخة.

كما استهدفت الدراسة الحالية أيضا تحديد وجود جين (*SclR*) فى خلايا خيوط أنواع **الأسبرجيلس** المختبرة باستخدام تقنية (PCR) وبينت النتائج أن هذا الجين يتواجد فى ثمانية أنواع هم (أسبرجيلس بيتركياى, أسبرجيلس سيبألتس, أسبرجيلس روبأستس, أسبرجيلس اسكليروتيكربوناريس, أسبرجيلس ميلليس, أسبرجيلس اسكليروشيونيجر, أسبرجيلس بارازيتيكس و أسبرجيلس اسكليروشيورم) وذلك بأوزان جزيئية مختلفة تتراوح من 134 bp فى النوع أسبرجيلس اسكيروتايكربوناريس إلى 436 bp فى أسبرجيلس بارازيتيكس.