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Heterothallism and sexual reproduction in the Iranian isolates of *Aspergillus flavus*

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Abstract: Ascomycota includes a large number of species that lack a known sexual stage but have a hidden potential for sexual reproduction, among which is *Aspergillus flavus*, an opportunistic aflatoxin-producing pathogen. The sexual stage of this heterothallic fungus results from crossing between strains with opposite mating types that belong to different vegetative compatibility groups. Here, twenty *A. flavus* Iranian isolates were investigated for heterothallism and sexual reproduction. The mating type genes *MAT1-1* and *MAT1-2* were explored using specific primers and multiplex PCR. The results indicated an equal frequency of mating types (1:1) in the investigated *A. flavus* isolates, indicating heterothallism, and possible sexual reproduction. The cross-cultures on Mixed Cereal Agar (MCA) and Aspergillus Complete Medium **(**ACM) induced the sexual phase. After 6 to 11 months, 33.16% of the crosses led to sexual reproduction and the sexual reproductive organ of the fungus appeared at the contact lines of the crosses. Scanning Electron Microscopy (SEM) analyses revealed multiple asci and ascospores, which were produced in the pseudoparenchymatous stromata. Ascospore production was more efficient in ACM than in the MCA. Our findings place the Iranian *A. flavus* in the genus *Petromyces*, as *Petromyces flavus*.

Keywords: Heterothallism, Mating type, *MAT1-1*, *MAT1-2*, Cross-culture, *Petromyces flavus.*

INTRODUCTION

Aspergillus is an ascomycetous genus, with over 330 species, which are dispersed globally and have pathogenic potentials with deep impacts on human, animal, and plant health (Bennet 2010, Geiser et al. 2007, Golparyan et al. 2018, Soltani 2016, Soltani 2017). *Aspergillus* species produce vast primary and secondary metabolites, which have applications in the bioindustry (Erfandoust et al. 2020, Soltani 2016, Soltani & Hosseyni Moghaddam 2014). Aflatoxins, produced by some *Aspergillus* species including *Aspergillus flavus* Link, are among the most carcinogenic polyketide-derived mycotoxins (Turner et al. 2003).

A. flavus, from the section *Flavi* (Peterson 2008), was identified in 1809 and was first reported as a plant pathogen in 1920 (Leslie et al. 1988). As a cosmopolitan and ubiquitous species, it has adapted different lifestyles from saprophyte in the soil and endophyte in the plants (Golparyan et al. 2018, Soltani 2017, Soltani & Hosseyni Moghaddam 2014), to a toxicogenic contaminant of important crops before and after harvest, and from a plant pathogen to an opportunistic human pathogen (Campbell 1994). It commonly infects cereal grains, legumes, and nuts. Postharvest infections in these crops is associated with mycotoxin production (Agrios 2005, Chang et al. 2009, Hedayati et al. 2007), among which aflatoxins including B_1, B_2, G_1, and G_2 are the most toxic ones (Horn et al. 1996, Kumar et al. 2017, Li et al. 2022).

There is a vast genetic diversity in the populations of *A. flavus*, as is reflected in their morphology, mycotoxins production (Horn et al. 1996), vegetative compatibility groups (Wicklow et al. 1998), and hosts (Amaike & Keller, 2011). Recombination and sexual reproduction could be involved in this genetic diversity. If so, sexual and vegetative compatibilities

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are essential to the fungus biology (Dyer et al. 1992). These systems are crucial for gene flows among the members of a species (Taylor et al. 1999a,b). Vegetative compatibility in *A. flavus* is under the control of *het* loci (Leslie 1993), while sexual compatibility is regulated by *MAT1-1* and *MAT1-2* genes, located in the same locus (Debuchy & Turgeon 2006).

Although the majority of *Aspergillus* species do not have a known sexual stage, many have hidden potential for sexual reproduction (Horn et al. 2009, Dyer & O'Gorman 2011). Indeed, evidence in *A. flavus* suggests a hidden heterothallism in its life cycle, i.e. a sexual stage (Horn et al. 2009). Indeed, *Petromyces* was identified as the teleomorph of *A. flavus*, in which the ascospores develop within sclerotia (Horn et al. 2009). This heterothallic fungus enters the sexual cycle when sexually compatible isolates of opposite mating types, belonging to different vegetative compatibility groups, are cross-cultured (Coppin et al. 1997 Ramirez-Prado et al. 2008). *Petromyces* is phylogenetically related to the section *Flavi*. Ascospores of this genus may take several months to mature (Eagle 2009). Considering the importance of this carcinogenic fungal species in agriculture and medicine and its wide presence in Iran's pistachio and nut industry, investigating the presence of sexual cycle and its biology provides invaluable information for genetic analyses of its pathogenicity and resistance against fungicides and leads to the improvement of plant disease management practices.

To the best of our knowledge, limited studies have been conducted on the genetic structure of the populations of *A. flavus* in Iran (Houshyarfard et al. 2014a,b, 2015) and there is no information on its possible sexual reproduction. Therefore, in the present study, the presence of the mating types and the possibility of inducing the sexual stage of the Iranian isolates of this fungus in laboratory conditions were investigated and documented.

MATERIALS AND METHODS

Fungal isolates

Twenty isolates of *A. flavus* including isolates 22-8, 2-15, 63, 81, 83, 87, 121, 124, 1-124, 125, 126, 137, 139, 141, 142, 151, 152, 153, 165 and 200 which were formerly isolated and identified by molecular means at the Plant Protection Department, Vali-e-Asr University of Rafsanjan, Kerman, Iran (*E. Sedaghati*, personal communications, unpublished) were obtained and used. The fungi were subcultured, and grown at 28 ˚C.

Molecular *MAT* gene analyses

DNA Extraction

The fungal isolates were grown in Malt Extract broth medium (containing 30 g Malt Extract, 1 g Peptone, in 1 L water), for 5-7 days at 27 ˚C, and 120 rpm. The mycelial masses were separated from the medium, washed, and used for DNA extractions according to Murray and Thompson, 1980, as well as the SDS-CTAB protocol of Zhang et al., 1996.

Multiplex PCR

To track *MAT1-1* and *MAT1-2* genes, specific primers were used in multiplex PCR for the amplification of *MAT1-2* HMG and *MAT1-1* α-box conserved region genes. The primers included MAT1F (5'-ATTGCCCATTTGGCCTTGAA-3'), MAT1R (5'-TTGATGACCATGCCACCAGA-3'), MAT2F (5'-GCATTCATCCTTTATCGTCAGC-3'), and MAT2R (5'-GCTTCTTTTCGGATGGCTTGCG-3') (Ramirez-Prado et al. 2008). All four primers MAT1F, MAT1R, MAT2F, and MAT2R were included in each reaction. The primer for *MAT1-1* amplifies a 369 bp fragment that contains 52 bp of a conserved intron, and the primer for *MAT1-2* targets a 270 bp fragment that contains 51 bp of the second conserved intron. The concentration of the primers made with sterile distilled water was set at 25 pmol μl^{-1} and kept in a freezer at -20 ºC.

A Master Mix (Amplicon Red, Pishgam Co., Iran) was used to perform multiplex PCR. In a total volume of 25 µL, 12.5 µL of PCR Master Mix, 1 µL of each primer (25 pm μL^{-1}), 2 µL of DNA (50 ng), and 6.5 µL of sterile double distilled water were added. Thermocycler (Techne TC-572) was programmed for one cycle of 5 min at 95ºC, followed by 40 cycles of 30 sec at 95ºC, 1 min at 54ºC, 45 sec at 72ºC, and a final cycle of 10 min at 72ºC. Amplicons were visualized on a 1.5% agarose gel stained and viewed under UV light. The PCR products were sequenced (Biomagic Co., Iran) using the same primers, and the sequences were processed using MEGA-X software, blasted, and compared to sequences in NCBI GenBank. The most similar sequences with the lowest E-values were selected and used as references.

Sexual stage in *A. flavus*

Formation of sclerotia, stromata, and ascocarps

All *A. flavus* isolates were cross-cultured mutually on specific culture media, i.e. Mixed Cereal Agar (MCA) and Aspergillus Complete Medium (ACM), and kept for 6-11 months at 27-30˚C. The above-mentioned twenty isolates were crossed on culture media one to one according to all possible conditions (Burgess et al. 2009). In this scheme, positive intersections indicated the presence of sclerotia or stromata, and negative intersections indicated the absence of sexual structures.

To monitor the fungal structures, 10 ml of sterile distilled water was added to each culture and ascocarps, and sclerotia were separated with a sterile needle or loop and washed again in sterile distilled water with a vortex to remove the conidia from the surface of the culture. Then they were placed on Whatman No. 4 paper to dry. The samples were observed by a Carl Zeiss (Axiostar plus) microscope and photographed by a Canon Powershot sx220 Hs camera.

Scanning electron microscopy (SEM)

To explore ascospore production in fungal sclerotia and stromata, SEM was used. The samples were first

fixed in 2.5% neutral buffered glutaraldehyde in 0.1 M phosphate buffer, and then in 1.0% osmium tetroxide. Graded ethanol series were applied to dehydrate the samples, which were further dried at room temperature, and mounted onto stubs. The prepared samples were sputter-coated with gold and examined in a JEOL JSM-840 scanning electron microscope operating at an accelerating voltage of 6 kV (Soltani et al. 2023).

RESULTS

In the present study, several methods were used for DNA extraction from *A. flavus* (data not shown). However, two extraction protocols, i.e. Murray and Thompson (1980) and the SDS-CTAB protocol (Zhang et al. 1996), were more effective than other methods. Moreover, the quantity and quality of DNAs extracted by the SDS protocol were higher than those of the Murray and Thompson method (data not shown).

Identification of *MAT* genes using multiplex PCR

Multiplex PCR on twenty Iranian isolates of *A. flavus* indicated that each isolate either carried the *MAT1-1* gene or the *MAT1-2* gene (Fig. 1), which indicates the heterothallism of the isolates. This is in accordance with the former findings in the USA (Ramirez-Prado et al. 2008). The results indicated an equal frequency of mating types (1:1) in the investigated *A. flavus* isolates. As seen in Fig. 1, isolates 8-2, 15-2, 63, 124, 125, 137, 139, 141, 153, and 200 harbored *MAT1-1* gene and isolates 81, 83, 87, 121, 124-1, 126, 142, 151, 152, and 165 harbored *MAT1-2* gene.

***MAT* gene sequences**

The PCR products from isolate 8-2 representing the MAT1-1 group and from 121 representing the MAT1-2 group were sequenced and analyzed. The resulting sequences were aligned in the NCBI database by BLAST software. The result showed that the MAT1-1 sequence from isolate no. 2-8 had 99% similarity to mating type protein *MAT1* α-box gene in *A. flavus* (Accession No: EU357934.1) and 100% similarity to mating type gene *MAT1-1* in *A. flavus* isolate NRRL 28987 (Accession No: HM803055.1). MAT1-2 sequence from isolate n.121 had 92% similarity to the *MAT1-2* protein HMG box gene in *A. flavus* (Accession No: EU357936.1) and 100% similarity to its homologous gene in *A. tamarii* (Accession No: HQ002124.1).

Formation of sclerotia, stromata, and ascocarps

Tables 1 and 2, and figures 2-7 show results of the cross between *A. flavus* isolates containing opposite mating types, i.e. MAT1-1 and MAT1-2, on specific culture media, kept for 6-11 months at 27-30 ˚C. Positive crosses result in sclerotia or stromata, and negative crosses indicate the absence of sexual structures. A number of crosses induced the formation of sexual organs (Table 1). Crosses between isolates with different mating types in *A. flavus* were different in terms of fertility (Table 2). Some crosses produced no ascocarps, others produced few sterile ascocarps, and some produced a large number of fertile ascocarps (Table 2). The number of sclerotia or stromata ranged between 1-170 (Table 2), and the number of ascocarp-containing sclerotia/stromata bodies ranged between 0-25 in our assay (Table 2). Indeed, in some cases the formation of sclerotia/stromata was naive, and so were the ascocarps. But, in crosses that a huge number of sclerotia/stromata were produced, e.g., isolate 125 × isolate 150, or isolate 125 × isolate 151, a large number of ascocarps could also be observed. However, it is thought that many pre-zygotic and post-zygotic impediments in fungi constrain the completion of the sexual cycle despite sexual compatibility (Anderson et al., 1992). After the specified period of incubation, velvety to fluffy and mostly brown colonies were seen (Figure 2). The cross lines of the mycelia of the compatible isolates were dense and wild (Figure 2), and also more density of sexual organs could be seen at the cross lines.

Sexual reproduction bodies of the fungus were observed in one-third (33.16%) of the cultures. The sclerotia were scattered, but the stromata were mostly formed between two colonies, which were similar to each other in terms of external appearance, and were round to oval in shape. After 6 to 11 months, the average dimensions of sclerotia were (450-1350) µm × (450-1250) µm, and they were light brown in the beginning and dark brown or black in the end (Fig. 3-7). The inner matrix was light to dark brown and contained pseudoparenchymatous tissue (Figure 6). These results were consistent with the findings of Horn et al. 2009, in which the sizes of stromata and sclerotia were reported as 1400×1250-300×250 µm. However, inn their research, the outer and inner colors of the textures were identical.

Ascocarps were round to oval or irregular, without pores, and were formed inside the stromata. The inner tissue was seen in white color (Figure 4). Each stroma consisted of several fertile ascocarps (i.e. containing ascospores) or several infertile ascocarps. In some cases, a combination of both types of ascocarps could be seen in the stromata. In most samples, between 1-3 ascocarps were seen in each stroma. The ascocarp peridium was thin and brown to crimson (Fig. 6).

Asci were round to oval and contained 4 ascospores, which were placed together in no particular order (Fig. 6, 7). Ascospores were round and broad, colorless to light brown, and often surrounded by an oil drop. They varied in size from 12 × 6.5 µm to 14 × 8.05 µm (Fig. 7).

Although 1-7 ascocarp per stroma was reported before (Horn et al., 2009), we observed a maximum of 3 ascocarps in each stroma. However, the appearance and size of ascospores in our observations were very similar to that of Horn et al., 2009. Moreover, the majority of asci in our observations contained 4 ascospores, while in the former report, it was observed to be 8 (Horn et al., 2009). Despite this, SEM indicated the high similarity of the structure of ascospores with the former report, as the ascospores were round and wide with an edge in the central area,

and small decorations were observed on the surface of each spore (Fig. 7). Moreover, the size of ascospores in our research was 12.0×6.5 µm to 14.0×8.05 µm, which was in accordance with former reports in which the size of ascospores was 12.5×5.0 µm to 14.0×8.75 µm (Horn et al., 2009).

Fig. 1. Multiplex PCR amplifications of *MAT1-1* (expected band: 369 bp) and *MAT1-2* (expected band: 270 bp) gene segments in 20 Iranian isolates of *A. flavus*. Each isolate carried either the *MAT1-1* gene or the *MAT1-2* gene.

Table 1. *A. flavus* isolates used in cross cultures. Sclerotia or stromata were seen in positive cultures after 6-11 months. Nothing was observed in negative cultures.

×	8-2	15-2	63	81	83	87	121	124	124-1	125	126	137	139	141	142	151	152	153	165	200
8-2		-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
15-2			-	-	-	-	-	+	-	+	-	-	+	+	+	-	-	-	-	-
63				-	-	-	-	+	-	+	-	+	+	+	-	-	-	+	+	-
81					-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
83						-	-	+	+	-	+	+	+	+	-	-	-	-	-	-
87							-	+	-	-	-	-	+	-	-	-	-	-	-	-
121								+	+	+	-	+	+	+	-	-	-	-	-	-
124									-	+	+	+	+	+	+	+	+	-	+	-
124-1										-	-	+	+	+	-	-	-	-	-	-
125											-	-	+	+	+	+	+	-	+	+
126												+	+	-	-	-	-	-	-	-
137													+	+	+	+	-	+	-	-
139														+	+	+	-	-	+	-
141															-	+	-	-	+	+
142																-	-	-	-	-
151																	-	+	-	-
152																		-	-	+
153																			-	-
165																				-
200																				

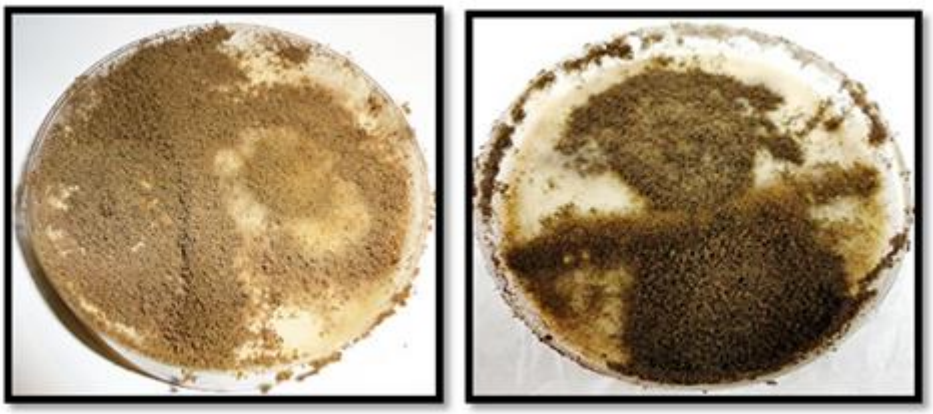

Fig. 2. The surface of the crossed colonies after a few months and the cross points of the mycelia in the middle.

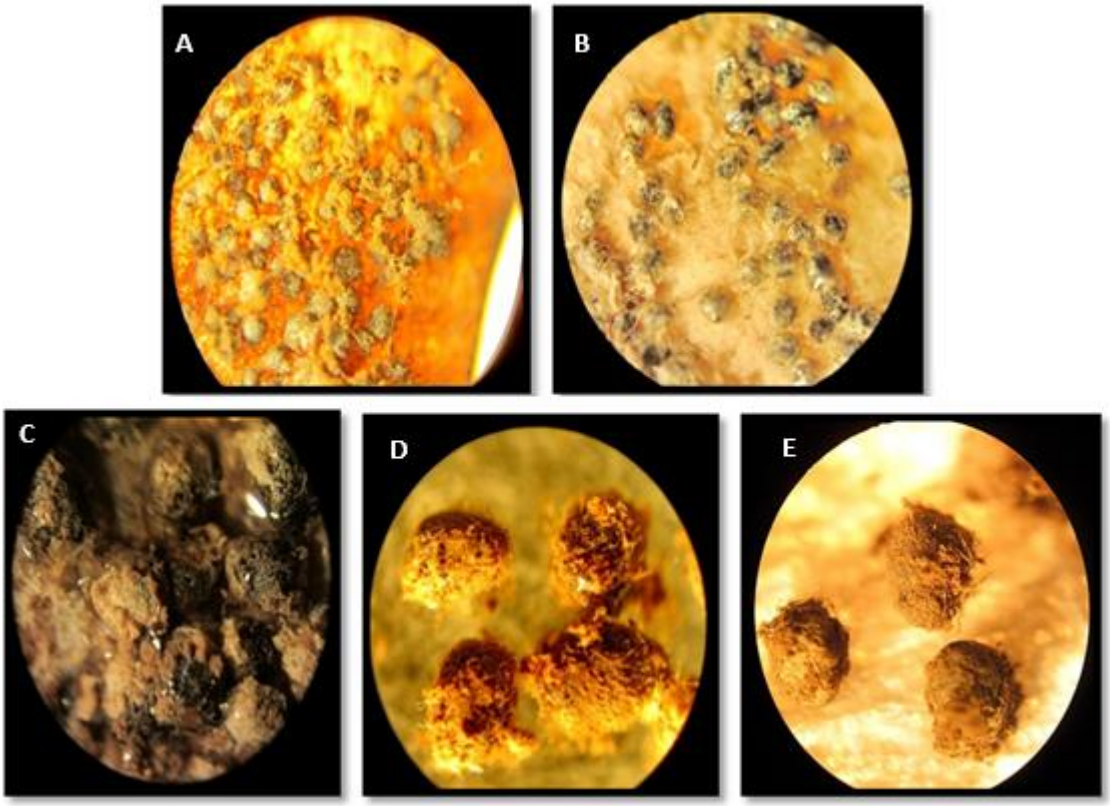

Fig. 3. A-B) Sclerotia formed on the surface of some cultures; C-D) Stromata formed in compatible crosses, 10× magnification

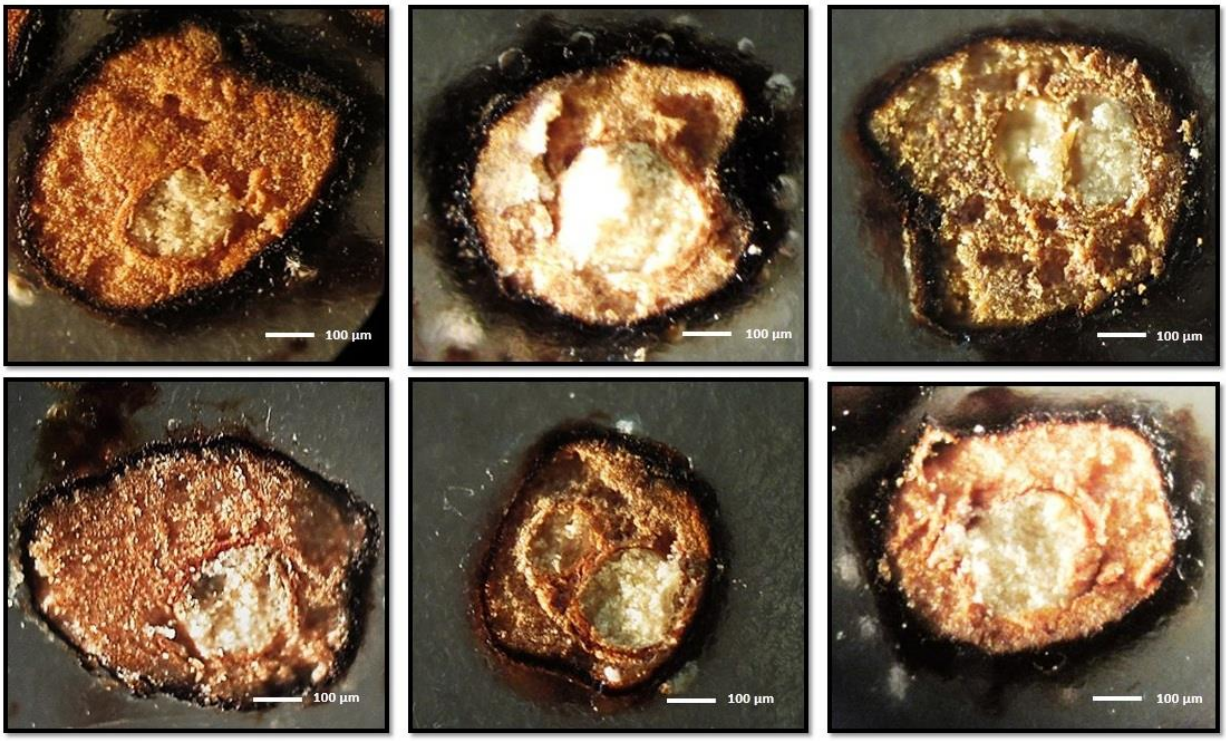

Fig. 4. Transverse section of the stromata formed on the surface of cross cultures of *A. flavus* (40× magnification).

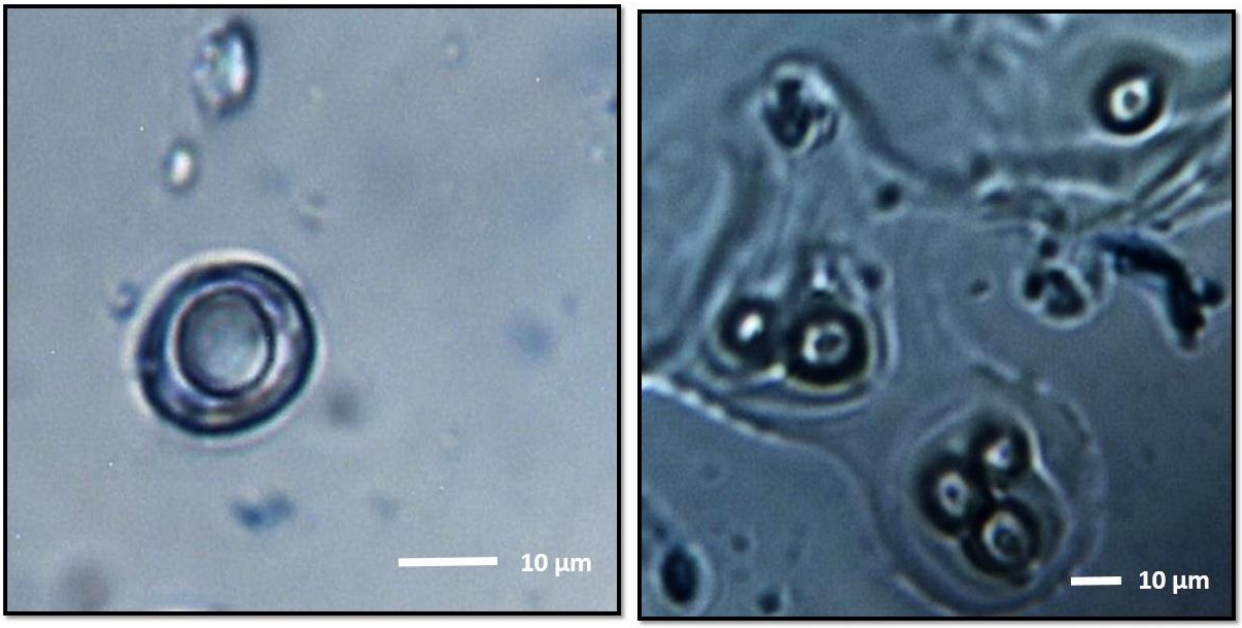

Fig. 5. View of *A. flavus* ascospores containing oil droplets (Left) and asci containing ascospores (Right) (100× magnification).

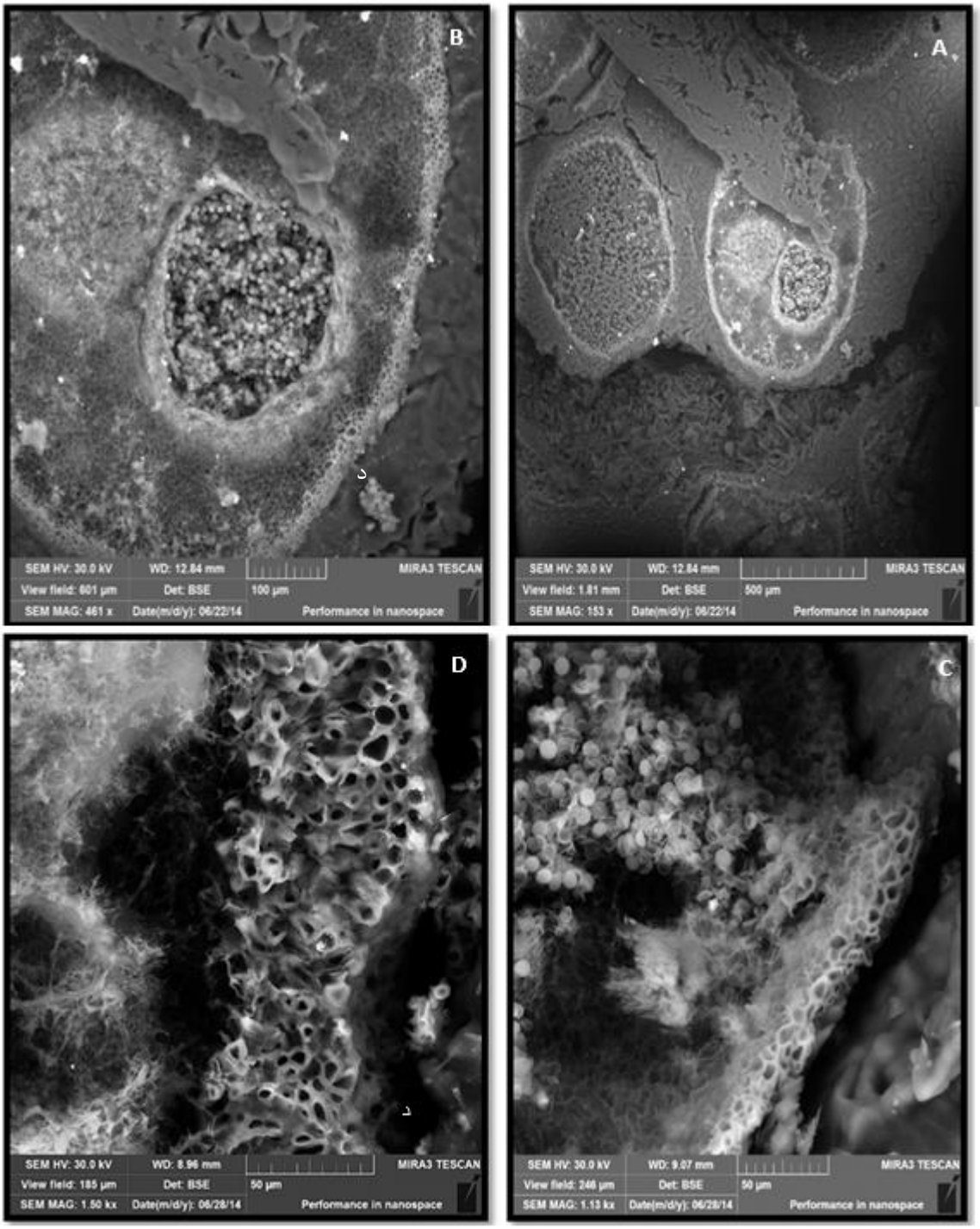

Fig. 6. Scanning Electron Microscopy (SEM). A and B: cross-sections of *A. flavus* stroma containing a sterile ascocarp and a fertile ascocarp, C: cross-section of external peridium and pseudoparenchyma matrix tissue and D: peridium of ascocarp separating ascocarp matrix and tissue

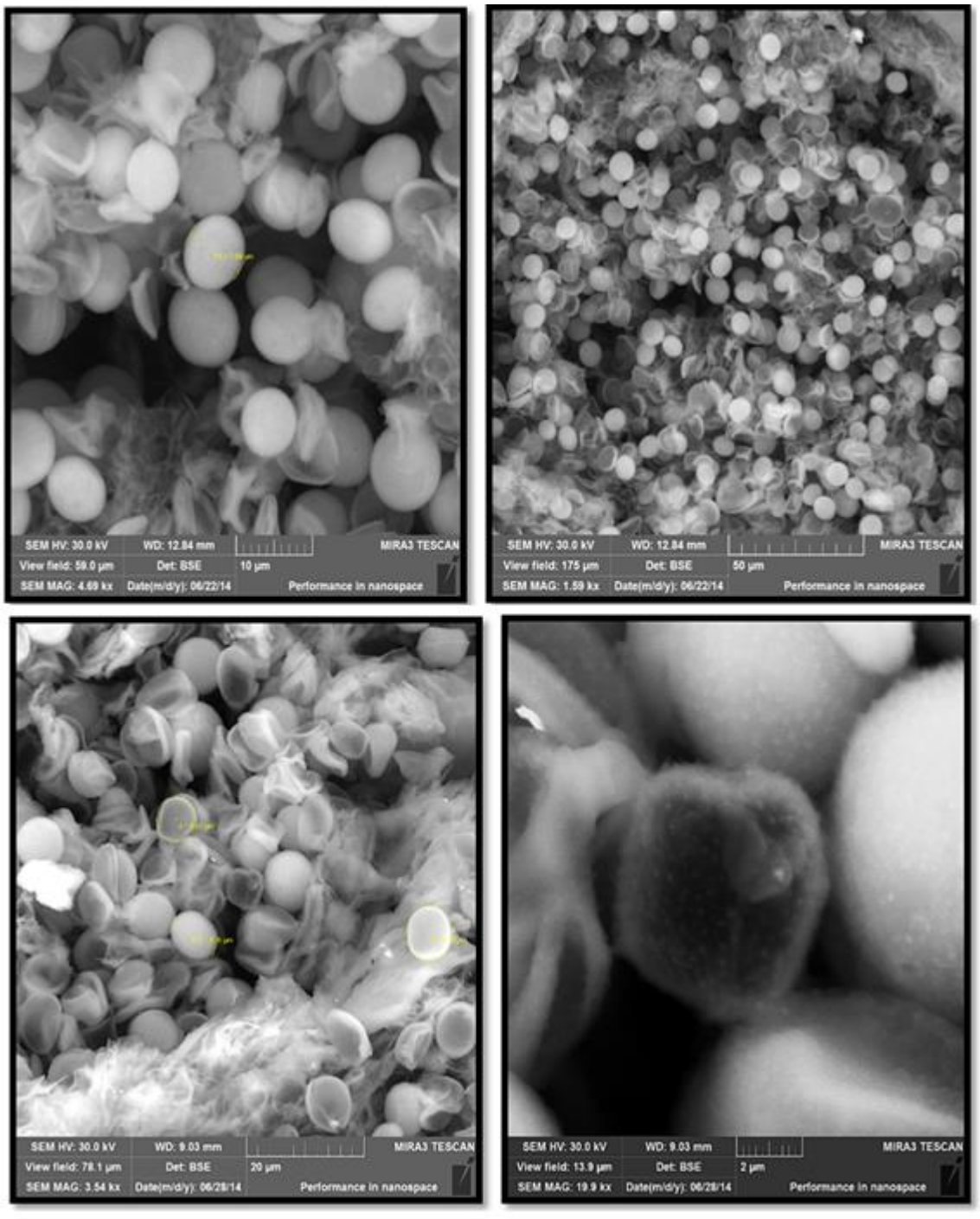

Fig. 7. Scanning Electron Microscopy (SEM). Close-up of *A. flavus* ascospores and their surface decorations, 4000-10000×

Table 2. Examples of positive crosses between sexually compatible isolates of *A. flavus* with different number of sclerotia or stromata, produced after 6-11 months incubations at 28˚C.

MAT1-1 isolate number	MAT1-2 isolate number	No. of sclerotia or stromata produced in each cross culture	No. of sclerotia or stromata examined	Ascocarp-bearing (fertile) stromata
124	87	2	2	0
141	81	50	50	11
139	124-1	120	100	23
137	124-1	1	1	0
139	126	2	2	0
125	151	117	100	25
125	165	170	100	22

DISCUSSION

Despite the immense importance of *A. flavus* in agriculture, medicine, and bioindustry, many biological aspects of it are still unknown. The observation of high biodiversity in this apparently asexual species suggests the possibility of pre-meiosis recombination or a hidden sexual stage (Geiser 2009). In fact, evidence has shown that many asexual organisms including fungi have the potential for sexual reproduction, and investigations in fungi have revealed teleomorphs for some asexual plant pathogens (Lucas et al. 2000). In addition, population genetic analyses have shown evidence of recombination among several asexual species (Taylor et al. 1999a,b, Geiser et al. 1998). For example, genomic analyzes and molecular experiments revealed the presence of sex in *Candida albicans*, which was thought to be asexual for a long time (Magee & Magee 2004). In similar works in *A. fumigatus*, the existence of genes involved in sexual reproduction, including those involved in the process of mating and copulation, signaling pathways, reproductive organ formation, and meiosis is documented (Varga 2003).

To induce sexual reproduction in heterothallic fungi, the presence of opposite mating types is necessary (Dyer et al., 1992). Mating types in filamentous ascomycetes are identified by genes in the *MAT* locus, which are either *MAT1-1* or *MAT1-2* idiomorphs (Metzenberg & Glass 1990, Turgeon & Yoder 2000). The *MAT1-1* idiomorph is an α-box specific gene, while *MAT1-2* encodes a high-mobility group HMG (Coppin et al. 1997). Former findings indicate that *MAT1-1* genes in *A. flavus* and *A. parasiticus* were homologous to *MAT1-1* in *A. fumigatus*, and *A. nidulans* and to *MATA-1* in *Neurospora crassa* (Ramírez-Prado et al. 2008). Furthermore, *MAT1-2* genes in *A. flavus* and *A. parasiticus* were homologous to *MAT1-2* in *A. fumigatus*, *A. nidulans*, and *Sclerotinia sclerotiorum*, and to MATa-1 in *N. crassa*. The high similarity of *MAT1-1* and *MAT1-2* sequences among these species along with conservation of transcription direction, chromosomal position, and synteny supports the hypothesis of the functionality of mating type genes in *A. flavus*. Indeed, observations indicated that when opposite mating types of *A. flavus* were cultured together sexual reproduction occurred (Ramirez-Prado et al. 2008), and *Petromyces* was identified as the sexual stage of *A. flavus* (Horn et al. 2009).

In Iran aflatoxins produced by *A. flavus* in pistachio (*Pistacia vera* L.), peanuts (*Arachis hypogaea* L.) and corn (*Zea mays* L.) are a major cause of economic losses for the growers (Cheraghali et al. 2007, Hedayati et al. 2010, Ghiasian et al. 2011). However, there is no information on possible sexual reproduction of the fungus. In this research, twenty isolates of *A. flavus* from Iran were investigated for possible heterothallism and sexual stage. Multiplex PCR indicated that half of the isolates belonged to the MAT1-1 group and the rest belonged to the MAT1-2 group. The *MAT1-1* sequence in our investigation showed 99-100% similarity to the *MAT1-1* gene in *A. flavus* sequences deposited in the GenBank. *MAT1-2* sequence showed 92% similarity to the *MAT1-2* gene in *A. flavus* and 100% similarity to its homologous gene in *A. tamarii*.

To enhance sexual reproduction, crosses were made between compatible isolates on MCA and ACM culture mediums, which led to the production of ascocarps, asci, and ascospores. The sexual stage of *A. flavus* with the formation of several unopened ascocarps inside the stromata, in our investigation, locates the investigated fungal isolates in the genus *Petromyces* as *Petromyces flavus*. This is the first report of the sexual stage of *A. flavus* isolates recovered in Iran. Here, in addition to the Mixed

Cereal Agar (MCA) medium used by Horn et al., 2009, Aspergillus Complete Medium **(**ACM) was also used, and the sexual organ of the fungus was formed in this medium. Indeed, ACM contains all the necessary materials and elements for the growth and development of *Aspergillus* species. Results indicated that the number of fertile ascocarps containing ascospores was higher in the ACM medium than in MCA (data not shown). This may be related to the presence of several microelements in this medium. So, we introduce ACM as a more efficient medium for enhancing ascospore production in *A. flavus*. In conclusion, due to the vast prevalence of the fungus in the nuts industry of Iran, our findings might be of high value in future disease management practices in the country.

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هتروتالیسم و تولیدمثل جنسی در جدایه‌های ایرانی قارچ *Aspergillus flavus*

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چکیده: Ascomycota گونه‌های زیادی دارد که مرحله جنسی شناخته شده نداشته، اما پتانسیل پنهان برای تولید مثل جنسی دارند. از جمله‌ی آن‌ها *Aspergillus flavus* می‌باشد که بیمارگری فرصت‌طلب و آفلاتوکسین‌زا است. مرحله جنسی این قارچ هتروتالیک از تلاقی بین سویه‌هایی با تیپ‌آمیزشی مخالف که به گروه‌های مختلف سازگاری رویشی تعلق دارند، حاصل می‌شود. در پژوهش حاضر، ۲۰ جدایه ایرانی *A. flavus* از نظر هتروتالیسم و تولید مثل جنسی مورد بررسی قرار گرفتند. ژن‌های تیپ‌آمیزشی MAT1-1و MAT1-2 با استفاده از پرایمرهای اختصاصی و Multiplex PCR مورد بررسی قرار گرفتند. نتایج نشان‌دهنده‌ی فراوانی برابر تیپ‌های‌آمیزشی (۱:۱) در جدایه‌های *A. flavus* مورد بررسی بود که نشان از هتروتالیسم و تولیدمثل جنسی احتمالی داشت. کشت متقابل روی محیط آگار غلات مخلوط (MCA) و محیط کامل آسپرژیلوس (ACM) فاز جنسی را القا کرد. پس از ۶ تا ۱۱ ماه، حدود ۳۳٪ از تلاقی‌ها منجر به تولیدمثل جنسی شد و اندام‌های جنسی قارچ در خطوط تلاقی کلنی‌ها ظاهر شدند. بررسی‌های میکروسکوپ الکترونی روبشی (SEM) آسک و آسکوسپورهای متعددی را نشان داد که در استروماهای شبه‌پارانشیمی تولید شده بودند. تولید آسکوسپور در محیط کشت ACM کارآمدتر از MCA بود. یافته‌های ما *A. flavus* ایرانی را در جنس *Petromyces* با عنوان *Petromyces flavus* قرار می‌دهد.

کلمات کلیدی: هتروتالیسم، تیپ‌آمیزشی، *MAT1-1*، *MAT1-2*، کشت متقابل، *Petromyces flavus*

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