A multi-aspect analysis of two analogous aspergillus spp. belonging to section Flavi: aspergillus flavus and aspergillus oryzae

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Abstract

Microfungal isolates were routinely identified depending on both macro and micro morphological characteristics, sometimes, some fungal isolates appeared to be similar and such cases caused severe confusion for mycologists during the preliminary identification. During our previous studies dealing with isolation of fungi for some biotechnological applications; two mystifying species *Aspergillus flavus* and *Aspergillus oryzae* showed similar cultural and macroscopic features. Therefore, the current study aimed to easily distinguish between these two species depending on simple approaches which are routinely followed by a large segment of researchers. Investigation of the macroscopic features was performed to check the fungal growth on four different media (PDA, MEA, YES, and CYA) followed by microscopic examination using an ordinary light microscope, and scanning electron microscope SEM. Also, screening of secondary metabolites for both strains was preliminarily identified to find out the difference between their metabolic profiles. Finally, ITS rDNA was involved to clarify the molecular differences along their partial sequence. Conclusively, the BLAST strategy confirmed the similarity of ITS rDNA segments of both fungal strains that supported our hypothesis. The color of the fungal growth is a very critical factor whereas it is extensively influenced by the type of cultivation media. Accordingly, the YES medium was an inspiring tool assisting in prompt differentiation during the culture investigation step whereas *A. oryzae* and *A. flavus* appeared significant mustard yellow and olive green respectively. During the microscopic examination, the CYA medium also had a robust effect on the formation of the conidial chain whereas the knit long chain was observed in *A. oryzae* while the conidia appeared scattered and not in a chain in the case of *A. flavus*. Likewise, both two strains possessed different metabolic profiles where *A. oryzae* is not an *Afla* toxin producer, unlike *A. flavus*.

Keywords Analogous strains, *Aspergillus flavus*, *Aspergillus oryzae*, SEM, Secondary metabolites, ITS rDNA

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Introduction

Aspergilli are great genera that include more than 180 identified anamorphs, plus the other nine genera with teleomorphs [1]. *Aspergillus* is classified into seven subgenera, which are subsequently subdivided into Sect. [2]. *Aspergillus* classification is complicated and ever-developing as well as the rest genera of other fungi. The identification of genus level is easily performed through recognition of its typical conidiophore, nonetheless, the identification of species level is intricate, for it is classically dependent on different morphological topographies. Cultural characteristics include color, the reverse color of mycelial growth, growth diameter, and secretion of dissolvable pigments and exudates. Microscopic characterization in *Aspergillus* is principally based on the shape and size of the vesicle, conidia, and seriation as well as characteristics of cleistothecia and ascospores. Moreover, all macroscopic and microscopic investigations must be conducted under standardized certain laboratory conditions [3].

Aspergillus flavus group was specifically referred to as *Aspergillus* Subgenus *Circumdati* Section *Flavi*, it has interesting aspects due to its industrial applications and toxigenicity. The section *Flavi* was classically subdivided into two groups; the aflatoxigenic species (*A. flavus*, *A. parasiticus*, and *A. nomius*) which give rise to severe agricultural and health problems overall in the universe. The second group comprises the non-aflatoxigenic species (*A. oryzae*, *A. sojae*, and *A. tamarii*) which were traditionally applied in fermented foods production in Asia [4]. Aspergilli could secrete several biologically active chemical compounds such as mycotoxins, immune-suppressants, antibiotics, and cholesterol-lowering agents [5]. Subgenus *circumdati* also could contribute to the industrial field, particularly in biotransformation processes.

Isolation and identification of *A. oryzae* have been reported to be tricky due to the intra-and interspecies variability of isolates and the morphological similarities with *A, flavus* [6]. The distinguishing of *A. flavus* from *A. oryzae* is a noteworthy challenge in all laboratories of mycology, particularly which use the classical techniques depending upon macroscopic and microscopic examination [7]. Some previous research articles [8] deeply studied the differentiation between these two species with highly sophisticated protocols such as (MALDI-TOF MS).

During our previous study dealing with isolation of fungi for the production of oil [9–12] from different agricultural wastes, in addition to screening and biodiversity of fungal communities along the coastline of Alexandria, Egypt [13–15], and screening of some fungi for their ability to produce biologically active enzymes [16], it was considerably noticed that some fungal species appear to be similar to each other in their culture characteristics especially two isolates; *A. flavus* and *A. oryzae.* Hence, this study aimed to find the simplest way to easily distinguish between those two tricky species *A. flavus* and *A. oryzae* through the investigation of their cultural and microscopical features on 4 different media, followed by screening of their secondary metabolic profiles, and finally ITS molecular identification to find out the significant differences. All these strategies were routinely used by a large segment of mycologists.

Materials and methods
Fungal strains
The two species *A. flavus* and *A. oryzae* were provided by the unit of culture collection, the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo-Egypt. They were cultivated onto four different media that are commonly involved in fungal enumeration.

Cultivation media
Potato dextrose agar (PDA) medium
PDA medium (NutriSelect® Basic) composed of potato extract 4 g/L, dextrose 20 g/L, and agar 15 g/L. it was prepared by dissolving 39 g of the ready-made medium, and cooked in one liter of distilled water [17, 18].

Malt extract agar (MEA) medium
MEA medium was prepared by suspending 33.6 g of readymade medium (Thermo Fisher Scientific) in one liter of distilled water [19, 20].

Yeast extract sucrose agar (YES) medium
YES medium (HIMEDIA, M1797) was prepared by dissolving 40.5 g of the readymade medium into one liter of distilled water [21].

Czapek yeast autolysate Agar (CYA) medium
CYA medium (HIMEDIA, M2061) was prepared by dissolving 54.75 g of the readymade medium into one liter of distilled water [22].

All media were sterilized by autoclaving at 1.5 Pa, 121 °C, for 15 min. then poured into Petri dishes for inoculation. Each fungal inoculum [23, 24] was spread regularly onto the agar surface of each medium, incubated at 30 ± 2 °C, and checked daily for suitable fungal growth.

Investigation of morphological characteristics
Culture and microscopic examination
Fungal cultures were submitted for confirmative identification based on their morphological characteristics on malt extract agar medium. The macroscopic features including colony growth, color, texture, and reverse color were reported. Also, the hyphae were checked for their aerial growth on the medium, and the slides were

observed under a microscope (400X) [25]. Conidiophores, conidia, vesicles, and branching patterns were identified under a microscope using the identification characteristics key by Klich (2002) [26].

Scanning electron microscopy (SEM)

Four-day cultures of both two analogous fungi were submitted to be investigated by SEM, the medium chosen was MEA as the most common growth medium. SEM technique was performed stepwise including fixation by osmium tetraoxide (OsO$_4$), followed by tissue processor (Sciences tissue processor model Lynx) for dehydration through immersion in gradual concentrations of ethyl alcohol followed by gradual concentrations of acetone. The third step involved the critical point dryer (EMS 850 apparatus) in which acetone was replaced by carbon dioxide, mounted on stubs to be ready for the gold coating in order to good conductivity by diode gold sputter coater (SPI Module™ Sputter Coater), and finally, the fugal specimen getting ready to be examined by high-vacuum mode of a JEOL JSM-5500LV Scanning Electron Microscope [27, 28].

Molecular differences based on ITS rDNA identification

Fungal mycelia were inoculated in yeast extract sucrose broth for 10 days with agitation at 160 rpm, then the fungal pellets were harvested and squashed to a fine powder in a mortar with an appropriate amount of liquid nitrogen, and extraction of DNA was performed using DNeasy kit (Qiagene, Germany) [13], the PCR amplification was carried out in 25 µL as a total volume containing genomic DNA (20 ng), PCR buffer (1X), dNTPs (0.2 mM), Taq DNA polymerase (0.2 U) (Roche Holding AG, Basel, Switzerland), and the primers (10 pmol); ITS1 (5`TCC-GTAGGTGAACCTGCGG3`), ITS4 (5`TCCTCCGCT-TATTGATATGC3`) [29]. The temperature pattern of the PCR amplification process was conducted as follows: general denaturation step at 94°C for 2 min, amplification step for 40 cycles of 60 s at 94°C, 90 s at 52°C, and 2 min at 72°C, and a final extension step at 72°C for 10 min.

Screening of secondary metabolites profiles by thin-layer chromatography

Both two fungal cultures (21-day culture age) were homogenized and macerated in chloroform-methanol 2:1 (v/v) for intracellular and intracellular extraction of secondary metabolites. After filtration, both fungal extracts were subsequently kept at room temperature to allow all solvents to be evaporated, then their dried residues were resuspended in an appropriate volume of methanol and filtered. Thin-layer chromatography (TLC) plate (10×10 cm Merck aluminum sheet, silica gel 60, layer thickness 0.2 mm) was loaded with both fungal extracts against Griseofulvin as an authentic reference standard.

Chromatographic bands were allowed to be developed by (TEF) toluene - ethyl acetate- formic acid 5:4:1 (v/v/v) as an elution buffer for a while, pursued by multiple ultraviolet scanning at different wavelengths; visible light, long UV$_{365}$, short UV$_{254}$, long UV$_{365}$. 0.5%p-anisaldehyde dissolved in (Conc. H$_2$SO$_4$ – acetic acid – acetone 5:10:85) was used for spraying the loaded TLC plate which was subsequently heated at (105 °C) in the oven for 10 min. Data of the resulted bands (color, Rf, and shape) was analyzed according to Paterson & Bridge [30, 31] to primarily predict the chemical constituents of the fungal extracts.

Results and discussion

Macroscopic and microscopic features of both fungal strains on different media

Both *A. flavus* and *A. oryzae* were grown on four different cultivation media (PDA, MEA, YES, and CYA) to show the difference in their behavior. Figure (1) exhibited a substantial difference in the culture color which is olive green with white margins in the case of *A. flavus* with condensed growth on YES medium followed by MEA, CYA, and PDA respectively. In the case of *A. oryzae*, the culture color was mustard yellow and the white edges were observed only on CYA, and PDA media while lacking in both YES, and MEA media. The growth of fungal biomass is interestingly affected by the type of medium included for cultivation. Those four media types are commonly used as aspergilli growth media, several studies approved the effect of media types on the growth, sporulation [32], also the growth rate, colony characteristics, and sporulation patterns of 10 fungal isolates that were cultivated on three different media were greatly influenced where lignocellulose agar medium promotes the growth rate for some isolated and the other isolates were promoted by PDA, and CYA media [33].

Regarding the sporulation, it was clear to observe the tight conidial chain formed in *A. oryzae* in which the conidiospores appeared connected to form a clear tight chain. Based on the Fig. (2), the sporulation and formation of the conidial chain are weakly affected by the media type, but the most effective medium to show the clear tight chain was MEA, and the least medium was PDA, the vesicle appeared to be well-developed and grow normally when the fungus was sub-cultured on CYA compared to the size of vesicle on the other media types. Referring to *A. flavus*, Fig. (3) exhibited that the vesicle of *A. flavus* was well developed with distinctive phialides when it was cultivated on CYA and MEA. Based on our results, the vesicle size of *A. flavus* appeared larger than its counterpart in *A. oryzae.* Cultivation media highly affected the asexual sporulation in *A. flavus* unlike what happened in *A. oryzae.* Sporulation was distinctively established in presence of MEA but it was rarely established in the presence of PDA. Conclusively, based on the

Fig. 1 Differences in fungal features were cultivated on four different media for 7 days. The upper row represents the growth of *A. flavus* on YES, MEA, PDA, and CYA (from left to right), and the lower row represents the growth of *A. oryzae* on YES, MEA, PDA, and CYA (from left to right)

culture features, CYA could differentiate easily between two analogous fungi through the color, and also, the other media involved could participate in this comparison to differentiate between these two fungi through the color of colonies and the formation of white edges. As for light microscopy, it succeeded in explaining the influence of media types on the development of the vesicle and phialides as well as the sporulation process, and the results of the light microscope did not recommend PDA to investigate *Aspergillus* spp.

Morphological identification is one of the traditional means of distinguishing the species in the *Aspergillus* section *Flavi*, nonetheless, it lacks meticulousness due to the close similitude of these species [34]. Nevertheless, the grouping of *Aspergillus* isolates into sections

is necessary to facilitate examination using innovative methods for characterization such as molecular and biochemical tools [35]. The nutritional media could attain adequate requirements for fungal growth, colony development, and other macroscopic and microscopic features to have readiness for phenotypic assessment [36].

Microscopic examination by SEM
Regarding the examination of microscopic examination of 4 days-cultures of both *A. oryzae*, and *A. flavus*, SEM approved the same results of light microscopy by which *A. oryzae*, and its vesicle appeared bigger with intensive growing conidiospores that attached forming a tight conidial chain as shown in Fig. (4-A1 & 4-A2). On the other side, the vesicle of *A. flavus* appeared smaller in size than its counterpart in *A. oryzae*. Although the small size of the vesicle but the phialides appeared to be well developed and those phialides could be easily observed under light microscopy may be due to the scarcity of spore formation if compared with sporulation in *A. oryzae* (Fig. 4-B1 & 4-B2).

Resemblances in morphological features among both interspecific and intraspecific levels within the *Aspergilli* crucible lead to identification errors [37]. Many studies addressed scrutiny of *Aspergillus* spp. by SEM [38] to examine mycelia development, conidiophores, conidia, vesicles, and phialides [39]. Polyphasic tactics were endorsed as the ideal standards for Aspergilli classification because of the close similarity of their morphology, thus, sometimes morphology went wrong to identify strictly related species. However, noteworthy efforts recommended the use of various approaches, including phenotypic, molecular characterization, and advanced MALDI-TOF to distinguish *A. flavus* from *A. oryzae* successfully [7].

Difference(s) in the level of ITS in both strains
According to Molecular differences based on ITS rDNA identification, the ITS segment alone does not appear to be a particularly useful gene for species differentiation of Aspergillus spp., especially between closely related species. An analysis of Beta-tubulin (*ben*A), RNA polymerase II second largest subunit (*rpb*2) and Calmodulin (*Ca*M), well-known DNA barcoding genes, on a multilocus method will yield more accurate results [40, 41]. But in this present study working on a small scale, ITS was selected as a simple, common, and most available method for a large segment of junior researchers.

Mostly, the first step in studies related to *Aspergillus* species identification relied on the sequencing of ITS amplicons as a typical marker in filamentous fungi [42]. Amplicons of the two fungal strains were found to be 1145, and 1052 bp for *A. flavus* and *A. oryzae*, respectively. Both ITS sequences were submitted to GenBank
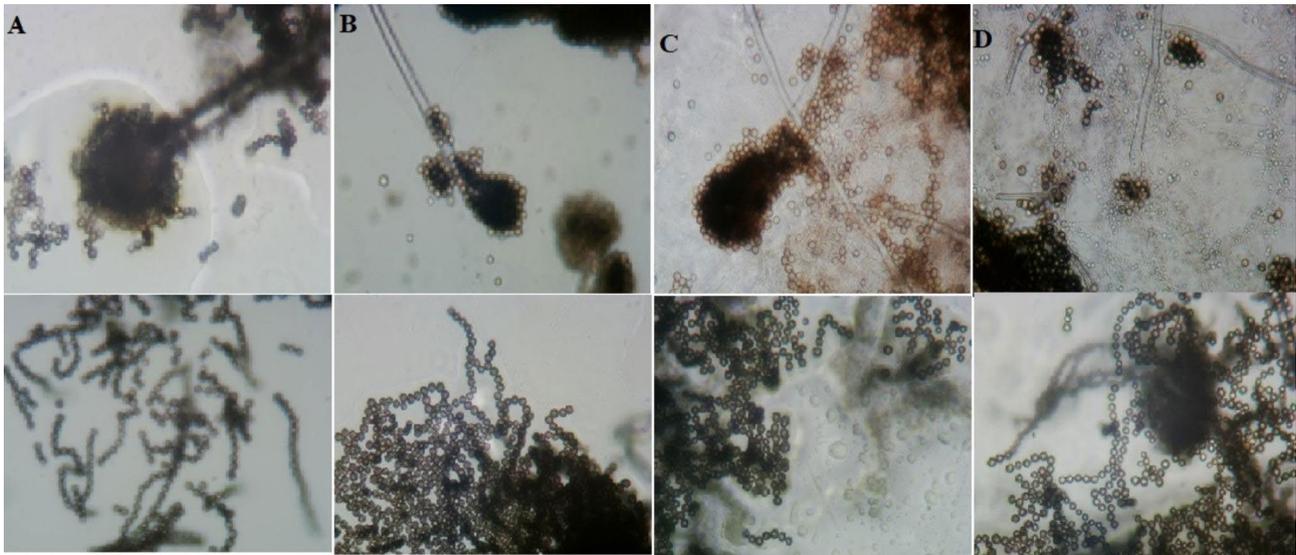
Fig. 2 Light microscopy shows the influence of different growth media on the microscopic characteristics of *A. oryzae*. **A**; the vesicle on CYA, and the shape of the conidial chain, **B**; the vesicle on MEA, and the shape of the conidial chain, **C**; the vesicle on PDA, and the shape of conidia chain, **D**; the vesicle on YES, and the shape of the conidial chain
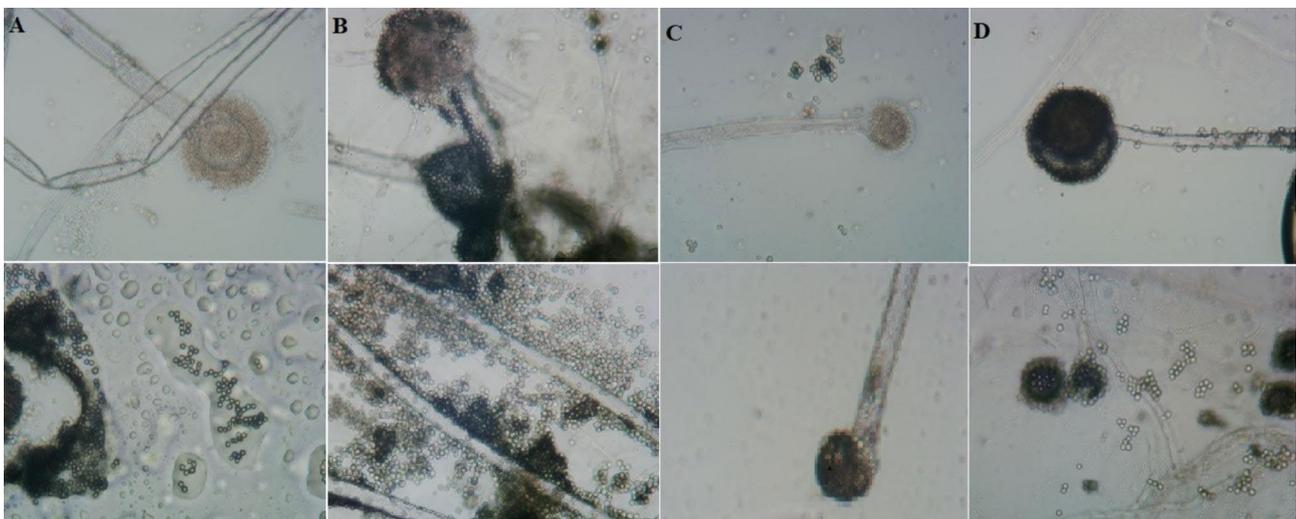

Fig. 3 Light microscopy shows the influence of different growth media on the microscopic characteristics of *A. flavus*. **A**; the vesicle on CYA, and the shape of conidia, **B**; the vesicle on MEA, and the shape of conidia, **C**; the vesicle on PDA, and the shape of conidia, **D**; the vesicle on YES, and the shape of conidia

to get accession numbers OL685258.1 for *A. flavus* (Fig. 5), and OL685252.1 for *A. oryzae* (Fig. 6). When both sequences aligned against each other by BLAST strategy, it was observed that there is a compatible fragment covered an anchor to the inquiry in a percentage of 33.8–36.7% and this fragment is identical in a percentage of 92.9–98.2%, that support our hypothesis of the high similarity of both two fungi even on the genome level.

Complete genome sequencing of both two fungi is available, providing the prospect to study somewhat genomic variances that might elucidate both fungal niches and possibly recognize virulence factors in *A. flavus*. Both *A. flavus* and *A. oryzae* are very analogous in their genome size and the number of projected genes. The assessed genome size (36·7 Mb) and projected gene number (12097) for *A. oryzae* are similar to that of *A. flavus* (36·8 Mb and 12179, respectively) [43]. Based on our knowledge regarding the molecular tool, the genome analyses succeeded to clarify any variation even intraspecies level variation rather than interspecies level differences. Chacón-Vargas et al. (2021) provide evidence of independent evolution of the replication of the α-amylase gene, and the genomic analyses disclose significant phenotypic and genome variation within *A. oryzae* [44].
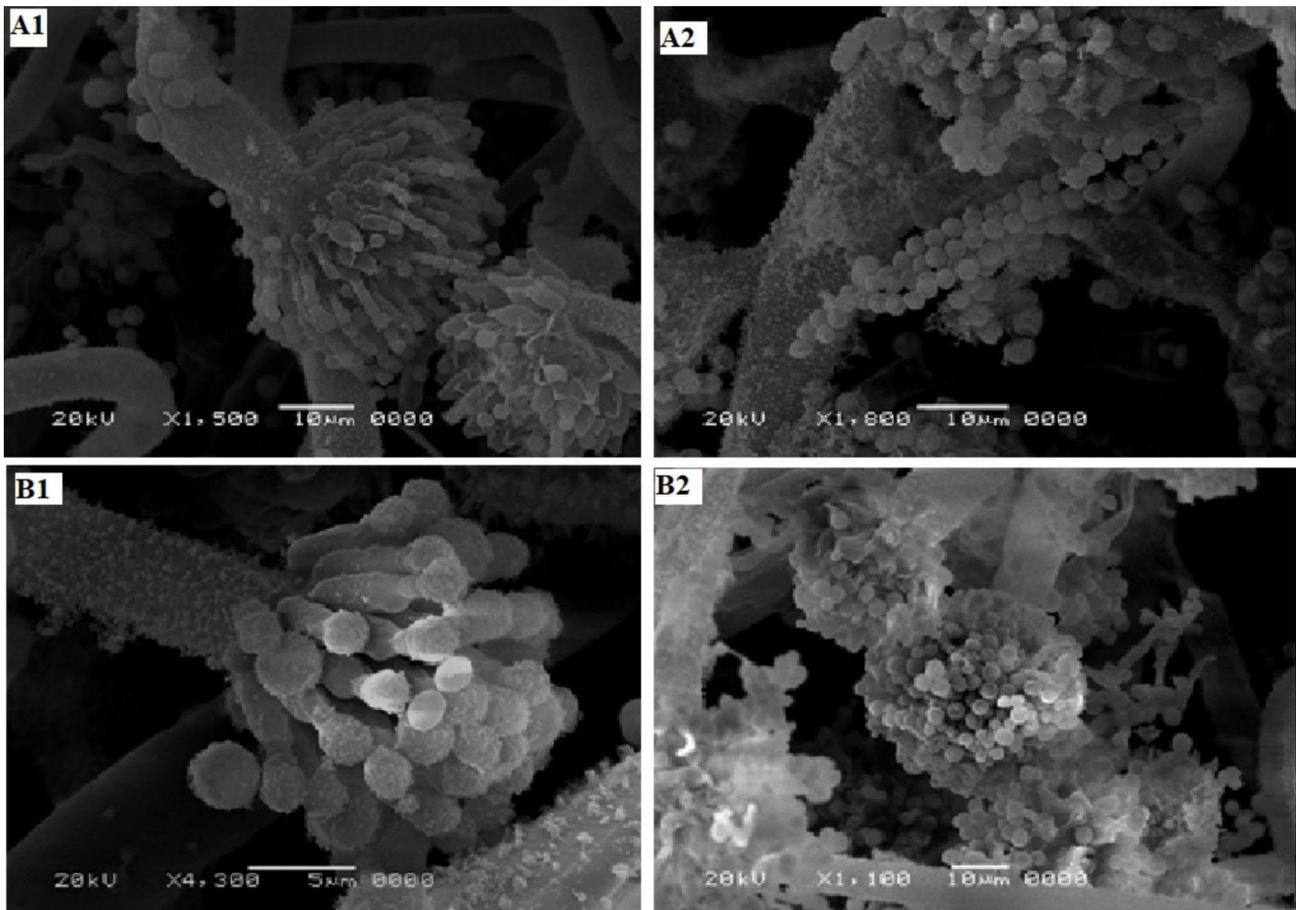
Fig. 4 Scanning electron microscopy illustrating the pattern of both *A. oryzae*, and *A. flavus* growing on MEA. A1; the shape of vesicle and sterigmata of *A. oryzae*, A2; the obvious conidial tight chain of *A. oryzae*, B1; the small vesicle and well-organized sterigmata of *A. flavus*, B2; the conidiospores attached to the sterigmata
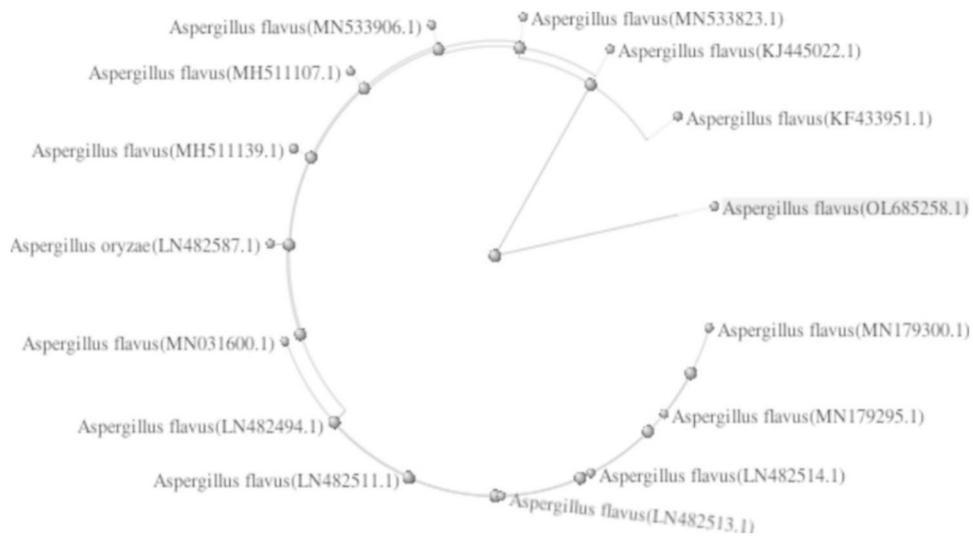

Fig. 5 A circular cladogram shows the distance of *A. flavus* from other relative species on GenBank
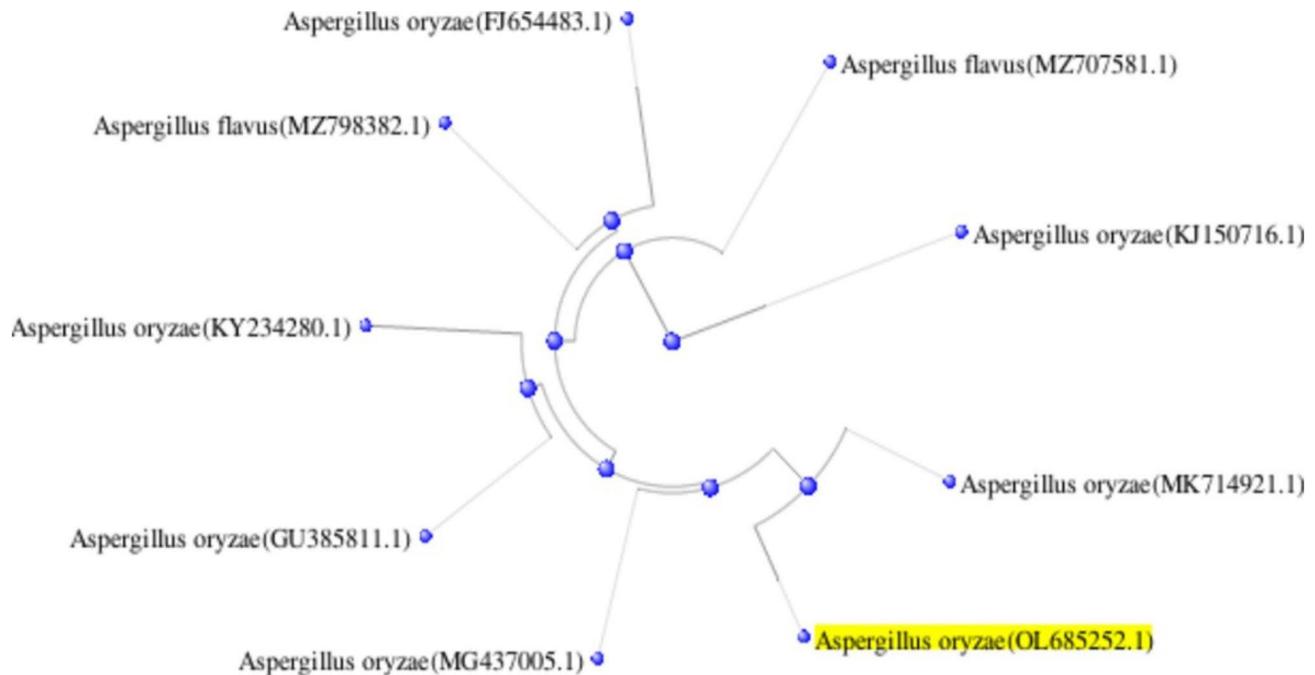
Fig. 6 A circular cladogram shows the distance of *A. oryzae* from other relative species on GenBank

Table 1 Secondary metabolic profile of both A. flavus and A. oryzae. By THC technique

No.	Rf	Color (L. UV)	Forecast	A. flavus	A. oryzae
1	5	Blue	2-Pyruvoylamino benzamide	+	-
2	10	Brown	Kojic acid	-	+
3	13	Green	Brevianamide A	+	+
4	20	Yellow/Brown	Gliotoxin	+	-
5	27	Yellow/Brown	Tenuazonic acid	+	-
6	31	Violet	ß-nitropropionic acid	+	+
7	39	Violet/Brown	Cyclopiazonic acid	+	+
8	43	Yellow	Aflatrem	+	-
9	45	Green/Blue	Afla toxin B1	+	-
10	53	Violet/Brown	Afla toxin G1	+	-
11	70	Brown	3,6-Dimethyl-6-hydroxyphthalide	+	-
12	77	Violet/Brown	Itaconic acid	+	-
Total				**12**	**11**	**4**

The contrast of their metabolic profiles by the TLC technique

Table (1) showed the presence of 12 different predicted compounds with different Rf values ranging from 5 to 77, it is clear to observe the abundant band detected in *A. flavus* in which 11 out of 12 bands were detected, while on the other hand, only four bands were detected in *A. oryzae*. Three out of 12 are common in both fungi; brevianamide A ($Rf=13$), ß-nitro propionic acid ($Rf=31$), and cyclopiazonic acid ($Rf=39$), but there is one forecast is unique for *A. oryzae* which was kojic acid with Rf of 10 with brown color at long UV ate 365 nm. As for *A. flavus*,

there are eight unique bands as follows; 2-pyruvoyl-amino benzamide ($Rf=5$), gliotoxin ($Rf=20$), tenuazonic acid ($Rf=27$), aflatrem ($Rf=43$), Aflatoxin B1 ($Rf=45$), Aflatoxin G1 ($Rf=53$), 3,6-dimethyl-6-hydroxyphthalide ($Rf=70$), and itaconic acid ($Rf=77$). Accordingly, the secondary metabolic profiles of both fungi could be easily distinguished at the first sight, toxicogenic profile of *A. flavus* is very clear in particular due to the presence of aflatoxins B1 and G1, while kojic acid is a unique component in the metabolic profile of *A. oryzae* might be the reason behind the recruitment of this fungal species in the cosmetics biotechnology. Although, kojic acid is unique for the current *A. oryzae* but sometimes *A. flavus* can do so [45].

Two marine *Aspergillus* strains were submitted to investigate their secondary metabolites profiles using HPTLC, it was observed the variation between axenic cultures and co-cultures in different ways, including some dramatical changes [46]. TLC was directed to emulate metabolic descriptions of the growing cultures in 1% and 2% glycerol. Remarkably, it showed some fluorescent compounds formed in the extracts of 2% glycerol-grown cultures relative to those produced in 1% glycerol [47]. Kojic acid is synthesized in bulky quantities by *Aspergillus oryzae* as a secondary metabolite, and it is commonly conducted in the cosmetic industry [48]. Cyclopiazonic acid, an indole-tetramic acid mycotoxin, is produced by many species of *Aspergillus* and *Penicillium* [49]. *Aspergillus* species are the dominant abundant fungi that spoil numerous foodstuffs and produce biochemicals known as mycotoxins. Aflatoxins (AFTs), aflatrem (AT), citrinin

(CIT), cyclopiazonic acid (CPA), gliotoxin (GT), ochratoxin A (OTA), patulin (PAT), secalonic acids (SA), sterigmatocystin (ST) and terrein (TR), and other toxins produced by *Aspergillus* spp. that represent a major part in food and human health [50].

Conclusion

Conclusively, the current study succeeded to provide a piece of interesting information that helped to distinguish *A. flavus* from *A. oryzae* in an easy and simple method. The nutritional media tremendously contributed to this comparison by presenting significant differences in both culture color, and culture margin color. As well, light microscopy exhibited a substantial distinction in the shape of the conidial chain in which the conidiospores bonded tightly to each other to form a clear streptococcal chain in *A. oryzae*, while, conidiospores were found scattered in the case of *A. flavus*, this difference was proved by SEM too. Additionally, the molecular tool was used to identify and align both ITSs, it displayed an identity of both sequences in a percentage of 33.8–36.7% which established our hypothesis that both strains are analogous. Finally, TLC primitively showed a noticeable difference in both secondary metabolites' profiles where the profile of *A. flavus* exhibited the presence of aflatoxins B1 and G1 as a major substantial difference. Furthermore, as a future perspective, a large group of section Flavi will be involved in order to represent a large sector of fungal isolates from different sources, countries, and habitats to maximize the benefit of this finding for the possibility to generalize this theory.

Data Availability
All datasets generated throughout this research are included in the manuscript. Regarding ITS rDNA, the sequences were submitted to GenBank on NCBI as a member of INSDC repository. Accession numbers are available on:
https://www.ncbi.nlm.nih.gov/nuccore/OL685258.1.
https://www.ncbi.nlm.nih.gov/nuccore/OL685252.1.

Declarations

Ethical approval and consent to participate
The ethics approval and consent to participate are not applicable.

Consent for publication
Not applicable.

Competing interests
The author declares that there is no conflict of interest concerning this article.

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