BIOLOGICAL ACTIVITES AND FUNDAMENTAL VARIATIONS BETWEEN FUNGAL ISOLATES BELONG TO ASCOMYCETES

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ABSTRACT:

Sexuality in fungi has long been a matter of concern and debates that always necessitated extensive analysis of the relationship between organisms assumed to represent different developmental forms of the same organism. Random amplification of polymorphic DNA (RAPD), amino acids, fatty acids and secondary metabolites profiles were performed for four isolates belong to ascomycetes fungi; *Aspergillus nidulans* and *Aspergillus chevalieri* and their teleomorph *Emercilla nidulans* and *Eurotium chevalieri*. Comparison of their activities as antibacterial and antifungal potency against many of Gram positive, Gram negative bacterial and several fungal species including yeast were determinate. Changes in fatty acid, secondary metabolite and RAPD profiles of sexual and their corresponding asexual isolates were observed. Oleic acid was of lower concentrations in *A.nidulans* and *A. chevalieri* than in *E. nidulans* and *E. chevalieri* while the opposite was observed for linoleic and linolenic acids. RAPD bands of molecular weights of 559 and 790 bp were the only different ones between *A. chevalieri* and *E. chevalieri* using primer 5 while those of molecular weights of 1239 and 1757 bp using primer 3 as well as that of 1209 bp using primer 5 represented the only different bands between *A. nidulans* and *E. nidulans*. Some intra- and extracellular secondary metabolites were undetected in the imperfect isolates while were detected in the corresponding perfect ones as contract with amino acids percentage detection where exhibited in imperfect isolates much more than perfect one. Deep relationship among cleistothecia formation, amino acids, fatty acid and secondary metabolite biosynthesis has been shown. Study of biological potency of four ascomycetes fungi exhibited great activities of *Emercilla nidulans* and *Eurotium chevalieri* (cleistothecia producing isolates) against Gram negative bacterial growth and yeast like fungi on contrast *Aspergillus nidulans* and *Aspergillus chevalieri* (two non-cleistothecia producing ones) show high inhibition of growth of Gram positive bacterial and filamentous fungi isolates.

Key words: Sexuality in fungi, Teleomorph, Cleistothecium Formation, Aspergillus, Ascomycetes**,** Amino Acids, Fatty Acids, Secondary Metabolites, RAPD-PCR.

INTRODUCTION

Fungi are ubiquitous eukaryotes that are estimated to comprise a quarter of the entire biomass on earth and consist of nearly 1.5 million species. They are enrichment with intra and extra cellular chemical compounds including protein and enzymes where they are the primary degraders of cellulose and lignin and devastating pathogens of plants and animals (**Pitt and Hocking, 1997**). The genus *Aspergillus* was first described almost 300 years ago and is an important genus in foods, both from the point of view of spoilage, and because many species produce mycotoxins which can cause liver carcinogen and effect on the both central nervous system and kidney function. Although a few species have been used in production of fermented food and the early discovery of their ability to produce organic acids were made at the turn of the century. It is therefore

not surprising that the aspergilli used or encountered in biotechnology play a significant role. They are extremely common in stored commodities such as grains, nuts and spices, and occur more frequently in tropical and subtropical than in temperate climates **(Calvo *et al*., 2001).**

A unique property of many fungi is their ability to propagate by both sexual and asexual spores. The integration of the teleomorph (meiotic sexual morph) and anamorph (mitotic asexual morph) stages, *Aspergillus nidulans* (teleomorph: *Emericella nidulans*) and *Aspergillus chevalieri* (teleomorph: *Eurotium chevalieri*) are a homothallic ascomycete with a defined asexual and sexual cycle that has long served as model system for understanding the genetic regulation of asexual development and secondary metabolism in filamentous fungi. The asexual cycle is characterized by the production of haploid conidiophores that bear single-cell asexual spores called conidia. Sexual development commences with the formation of multinucleate globular cells, called Hülle cells that surround the cleistothecium, the ascocarp that contains sexual spores called ascospores **(Calvo *et al*., 2008).**

The program of asexual and sexual sporulation is characterized by many developmental stages including temporal and spatial regulation of gene expression, cell specialization and intercellular communication. *A. nidulans* asexual reproductive cycle can be divided into at least three different stages: (i) a growth phase required for cells to acquire the ability to respond to induction signals (competence phenomenon), (ii) initiation of the developmental pathway and (iii) execution of the developmentally regulated events leading to sporulation. A development-specific array of transcription factors is activated that control the expression of multiple sets of genes required for conidiophore morphogenesis. Sexual fruiting body formation is influenced by several environmental and genetic determinants; however, the molecular pathways for this developmental stage are not well dissected (**Braus *et al*., 2002**).

Many studies are focusing on the characterization of mutants defective in sexual reproduction. Normal sexual and asexual development in *A. nidulans* and *A. chevalieri* requires the function of the velvet (*veA*) gene. *VeA* is known to have a role in activating sexual development and/or inhibiting asexual development, since asexual sporulation in the *veA1* mutant is promoted and increased, while sexual development is significantly delayed and reduced (**Sambrook *et al*. 1989**). Furthermore, *veA1* mutants do not exhibit light-dependent development of conidia and ascospores in contrast to the wild type where light induces asexual and delays and reduces sexual spore production. The *veA1* mutant gene differs from that of the wild type by one nucleotide in the initiation codon resulting in a putative truncated protein by 37 amino acids. *veA* null (*ΔveA*) mutants do not form cleistothecia; by contrast, overexpression of *veA* gene leads to formation of cleistothecia even in liquid culture and to formation of fewer conidial heads than in a wild type on solid media (**Yager, 1992)**.

Calvo *et al.* (2001) identified two mutants (*acoB202, acoC193*) in *A. nidulans* that fail to become competent and are blocked in both sexual and asexual sporulation. These mutants overproduce hormone-like fatty acid derived oxylipins collectively termed psi factor (precocious sexual inducer). Psi factor serves as signals that modulate sexual and asexual sporulation by affecting the timing and balance of asexual and sexual spore development.

Diseases caused by bacteria and fungi are common worldwide and major causes of death, disability, and social and economic hindrance for millions of people. According to World Health Organization, over 9.5 million people die each year due to

infectious diseases and up to 19% patients are infected from hospital visits throughout the world. Hospital-acquired infections cause a wide range of severe infections including pneumonia, infections of the bloodstream, urinary tract among other organs of the body. Most of the nosocomial pathogens are difficult to treat because they are resistant to many antibiotics (**Talbot *et al.*, 2006**).

The treatment of infections caused by pathogenic fungi also faces enormous challenges. In the past 30 years, the incidence of fungal infections has significantly increased around the world, and current antifungal drugs such as polyene macrolides (amphotericin B), azoles fluconazole, miconazole, itraconazole, and voriconazole), flucytosine, and the candins (caspofungin acetate and micafungin), are not ideal in terms of efficacy, antifungal spectrum, or safety. Furthermore, recent reports support that invasive candidiasis and aspergillosis has increased dramatically and spread rapidly (**Gullo, 2009**). Amphotericin B is efficacious against both candidiasis and aspergillosis. However, it exhibits severe side effects such as renal toxicity (**Maschmeyer and Ruhnke, 2004**). Hence, the urgent need of new agents to combat bacterial and fungal infections is immense. Fungi offer a treasure trove for the discovery of structurally unique natural products with potential biomedical applications. Recently, we isolated and identified eight secondary metabolites from the fungus aspergillus (**Gao *et al.*, 2012**). In this study, we report the antibacterial and antifungal activities of these compounds.

It has been noted since the earliest days of fungal manipulation that many species of filamentous fungi readily synthesize complex compounds named secondary metabolites that are putatively helpful but not necessary for survival and whose production is presumably costly to maintain. Natural products are often produced late in fungal development, and their biosynthesis is complex. This complexity is due to a number of factors that affect secondary metabolite production. These include (i) the influence of a number of external and internal factors on natural product biosynthesis, (ii) the involvement of many sequential enzymatic reactions required for converting primary building blocks into natural products, (iii) tight regulation of natural product enzymatic gene expression by one or more transcriptional activators, (iv) close association of natural product biosynthesis with primary metabolism, and (v) close association of natural products with later stages of fungal development, particularly sporulation. Furthermore, the genes required for biosynthesis of some natural products are clustered, perhaps as a consequence of these factors **(Calvo *et al.*, 2008).**

Lipids have been shown to regulate virulence and development, including both spore and secondary metabolite production in fungi. Shared intracellular signaling pathways for sporulation and secondary metabolite production suggest a common trigger(s) for both these processes. Lipid signals also affect secondary metabolites and sporulation. Among lipid signals, of particular note are the previously mentioned oxylipins. Additionally, the processes of sporulation has been demonstrated to share common regulatory elements, for instance, in *Colletotrichum lagenarium*, deletion of a mitogen-activated kinase (MAPK) gene lowers both production of conidia and expression of melanin genes (**Brodhagen and Keller, 2006).**

The genus Aspergillus is a relatively large taxon among the Hyphomycetes, and still many aspects of its biology have not been fully understood. With the increasing awareness of the beneficial and deleterious impacts of aspergilli, not only screening for new members in the genus is required, but also multi-disciplinary investigation of the currently identifiable members ranks high in the field of mycology. Techniques from

molecular biology have provided a series of new tools for the analysis of sexuality in fungi, thus in the current study, fatty acid and secondary metabolite profiles as well as RAPD patterns and biological activities as antifungal, antibacterial activities of two Aspergillus cultures uncapable of producing sexual spores (*A. nidulans* and A. chevalieri) and two other perfect ones (*E. nidulans* and *E. chevalieri*) were investigated in an attempt to figure out reasons for cleistothecia unproductivity by the imperfect cultures taking into account these three interconnected parameters.

MATERIALS AND METHODS

A: Fungal isolates used: Aspergillus isolates (*Aspergillus chevalieri, Eurotium chevalieri, Aspergillus nidulans* and *Emercilla nidulans*) and all bacterial and fungal isolates used in the present assay for biological activity were obtained from the culture collection of The Regional Center for Mycology and Biotechnology.

B: DNA- Based Techniques:

1- Fungal DNA Extraction Using Qiagen kit: The mycelial growth from 5–7 day old cultures on Malt Extract Agar (MEA) slopes were scraped by using 2 ml of sterile distilled water. The two ml of spore suspension were used to inoculate a 100 ml of Yeast Extract Sucrose (YES) medium in a universal 250ml flask and incubated with gentle shaking (180 rpm at 22<C for 48hrs). The mycelia from the flasks were harvested by filtration under aseptic conditions using a microcloth, washed with sterile distilled water and stored at -20 overnight in a sterile Petri dishes. The mycelia were lyophilized in a Heto lyophilizer system model Maxi Dry Plus. The freeze-dried mycelia were ground in a mortar using a sterile pestle, and the powdery samples were placed in eppendorf tubes (1.5 ml). DNA extraction was conducted using DNeasy kit (Qiagen-Germany) (**Sambrook *et al*., 1989 and Scazzocchio, 2006).**

2- RAPD-PCR: Ready to go PCR beads kit (purchased from Amershambioscience) was used to amplify DNA genomic fragments using a thermal cycler machine (gradient Robocycler 96 Stratagene, USA) by combining the lyophilized bead, 25 pmole of each primers, 100 ng DNA as a template in 25ml of total reaction volume. The mixture was then placed to the thermal cycler machine directly to start the appropriate PCR program including a universal denaturation cycle (5 min at 94°C), 45 cycles of annealing/extension reactions (1 min at 94°C, 1 min at an optimum annealing temperature 36°C for each used universal primer and 2 min at 72°C) and cycle of final extension step (5 min at 72°C) was followed by soaking at 4°C. The primers used in this study were supplied with the Ready to go kit and are of the following sequences: Primer3: 5'-d {GTAGACCCGT}; Primer5: 5'- {AACGCGCAAC}; Primer 6: 5 '-d{CCCGTCAGCA} (**Sambrook *et al*., 1989 and Scazzocchio, 2006).**

3- Agarose Gel Electrophoresis: The desired amount of agarose 2% was added to 100 ml (1X) of electrophoresis buffer (10X TBE, tris-base 108g/l; boric acid, 55g/l; 40 ml of 0.5M EDTA (pH8)). The gel was boiled and ethidium bromide solution (10 mg/ml) was added at 55°C, then poured into sealed gel tray and the appropriate comb was inserted (**Scazzocchio, 2006).**

D- Secondary Metabolite Analyses:

Seven day old fungal isolates were grown on 100 ml of YES medium for the determination of extracellular secondary metabolites while CYA medium was used for the determination of intracellular secondary metabolites using agar plug technique, incubate for 21 days at 28^{o}C. Extraction was carried whereas the flasks were aseptically filtered and the filtrate was concentrated using speed vacuum device (Maxi Dry Plus). Methanol/chloroform (1:2, v/v) was used for extraction of fungal secondary metabolites. Concentrated YES broth obtained from fungal cultures was mixed individually with chloroform/methanol. The mixture was shaken vigorously in a separating funnel and left to settle down forming a dense lower aqueous layer containing the secondary metabolites. Each extract was concentrated by evaporation of solvent under reduced pressure using an evaporator at a temperature not exceeding than 50 (±2) °C. Analysis and identification of intracellular and extracellular metabolites were carried out according **Paterson and Bridge (1994)**, using the automatic HPTLC system (CAMAG, model scanner 3- Switzerland).

C- Amino acids Analysis:

Preparation of cell-free extract for amino acids was analyzed according to **Barrett and Elmore (1998)**. Fifty ml sterile MEA medium were employed in each flask and inoculated with fungal spore suspension using 7 days old cultures for each fungus. The flasks were incubated for 7 days at 28ºC. Mycelia were harvested by filtration using a Buchner funnel. The mycelia were then washed thoroughly with distilled water. The harvested mycelia were then ground with clean glass using 70% ethyl alcohol. The obtained slurry was then centrifuged at 6000 rpm for 20 minutes. After centrifugation, the supernatant was decanted to be used for the analysis. Amino acid composition was carried out by using an amino acid analyzer (LC 3000 Eppendorf Biotronik).

E- Fatty Acid Analysis:

Intracellular fatty acids were extracted according **Peter and Michael (1996)**. Gas chromatography analysis was achieved using Dani GL\C-FID 1000 at the Central Laboratory of Ain Shams University. The fatty acid standard was manufactured by Supelco tm containing mixture of 37 fatty acid methyl esters (C4 – C24).

F- Biological activities assay:

Antimicrobial Screening: Disc diffusion method was used for the antimicrobial susceptibility testing. Antifungal potentialities were expressed as the diameter of inhibition zones. Intra and extra of fungal metabolites were examined as antimicrobial agent against twelve bacterial isolates (six Gram positive and other Gram negative) and sixteen fungal isolates including six isolates of yeast. Inoculum suspensions of all bacteria and fungi isolates were spread on the surface media [Nutrient agar "NA" medium for bacterial growth and Malt Glucose Agar "MGA" medium for fungal growth]. Six equidistant (1 cm diameter) holes were made in the agar using sterile cork borer in media plates (10x 10 cm), which had previously been seeded with bacteria and/or fungi tested, were filled by 100 µL with each fungal extracts (after evaporation of solvent). Control holes were failed with organic solvents, which were used in the extraction methods. Plates were left in a cooled incubator at 4 (±2)°C for one hour and then incubated at 37 (±2) °C for 24 hour for bacterial growth and 28ºC for 48 hours for fungal growth. Inhibition zones developed due to active fungal extract ingredients were measured after 24-48 hours of incubation **(Fagbemi *et al*., 2009)**.

RESULTS:

The current work studies biochemical and genomic differences between an asexual culture of *A. nidulans* and another capable of reproducing sexually (*E. nidulans*) as well as between *A. chevalieri* (asexual culture) and *E. chevalieri* (sexual culture) in an attempt to investigate genomic and metabolic differences between the sexual and the interestingly asexual culture through studying the three interconnected chosen parameters; fatty acids, intracellular and extracellular secondary metabolites representing the biochemical investigations, as well as RAPD analysis using three universal primers (primer 3, 5 and 6) representing the molecular investigations.

Teleomorphs are sexual, or perfect, states of fungi. Aspergillus anamorphs (imperfect states) are found in at least eight teleomorphic Ascomycete genera; however only three of these, Eurotium, Neosartorya and Emericella, occur in foods. All form cleistothecia. The cleistothecia are surrounded by Hülle cells, which are thick, walled, highly refractile, roughly spherical cells resembling chlamydoconidia. Eurotium species are the most common and significant of the foodborne genera with Aspergillus anamorphs. They produce bright yellow cleistothecia and pale yellow ascospores. Heads producing conidia are formed from phialides only **(figure 1)**.

RAPD-PCR: Three primers (primers 3, 5 and 6) were used to evaluate the genomic profile of each of *A. chevalieri; E. chevalieri; A. nidulans* as well as *E. nidulans*. Exactly the same RAPD pattern was obtained for both *A. chevalieri* and *E. chevalieri* using primer 3 (**Figure 2 and Table 1**). For primer 5, only two bands (559 bp and 790 bp) were different; the band of molecular weight of 559 bp was present in *A. chevalieri* while absent in *E. chevalieri*, on contrast the band of molecular weight of 790 bp present in *E. chevalieri* while absent in *A. chevalieri*, (**Figure 3 and Table 2**). Primer 6, like primer 3, resulted in exactly the same RAPD pattern (**Figure 4 and Table 3**).

In case of *A. nidulans* and *E. nidulans*, primer 3 resulted in differences in two bands of molecular weights of 1239 bp (present in *A. nidulans* while absent in *E. nidulans*) and 1757 bp (present in *E. nidulans* while absent in *A. nidulans*) (**Figure 5 and Table 4**). Amplification of their DNA using primer 5 resulted in differences in only one band of molecular weight of 1209 bp which was present in *E. nidulans* while absent in *A. nidulans* (**Figure 6 and Table 5**). Exactly the same RAPD pattern was developed using primer six (**Figure 7 and Table 6**).

Intracellular Secondary Metabolite Profiles: Eight intracellular secondary metabolites were detected. *E. chevalieri* being the richest in secondary metabolites for detecting seven of the eight secondary metabolites in its cell free extract whereas all secondary metabolites were found except Viridicatum toxin. While, *A. nidulans* represented the poorest isolate for possessing only three metabolites (ochratoxin A, carlosic acid and 2-carboxy -3, 5-dihydroxy phenyl acetyl carbinol). *E. nidulans* exceeded its anamorph by Viridicatum toxin. As well as, *E. chevalieri* exceeded its anamorph by schizopaltic, genestic and psoromic acids (**Table 7**).

Extracellular Secondary Metabolite Profiles: Six extracellular secondary metabolites were detected in the culture filtrate of the investigated aspergilla (**Table 8**). *E. chevalieri* was the richest for possessing five of the six detected metabolites followed by *E. nidulans*. Both Asexual cultures produced poor extracellular secondary metabolites whereas 2-pyruvoylaminobenzamide and rosepurpurine for *A. chevalieri* while alectronic and α-collatolic acids for *A. nidulans*.

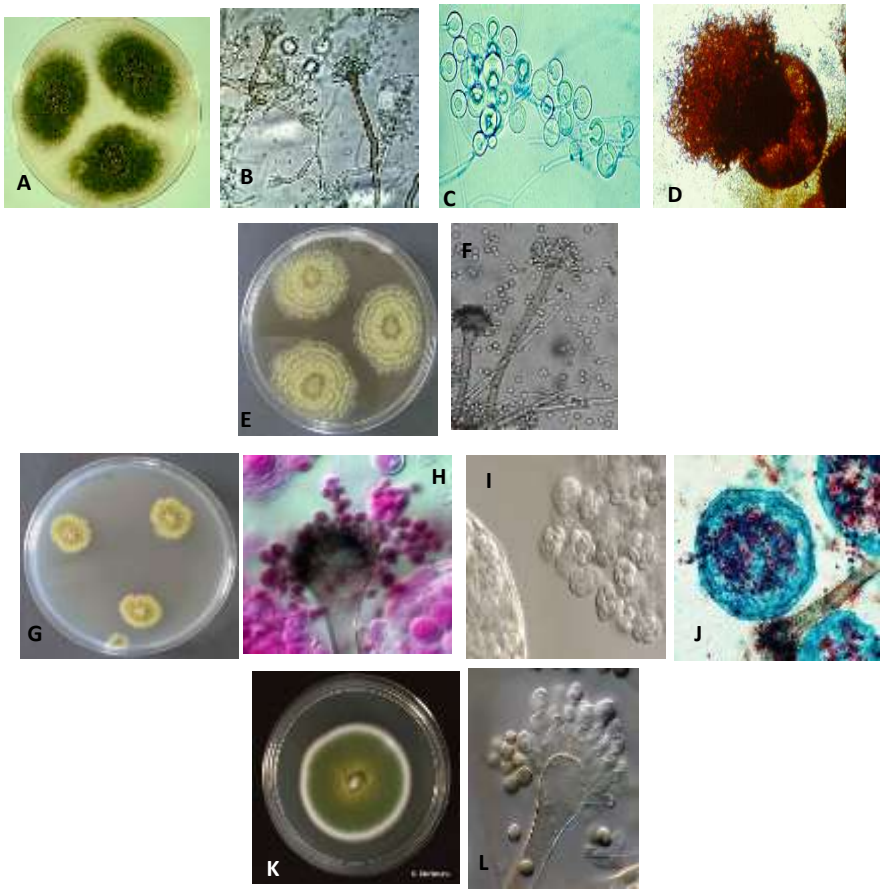

Figure 1: Culture and morphological features of *A. nidulans*, *E. nidulans as well as A. chevalieri; E. chevalieri* using image analyzer (X 40). (A) culture of *E. nidulans, (B):* heads of *E. nidulans*, (C): Hülle cells of *E. nidulans, (D):* cleistothecia of *E. nidulans*, (E) culture of *A. nidulans, (F):* heads of *A. nidulans*, (G) culture of *E. chevalieri*,*(H):* heads of *E. chevalieri*, (I): ascocspores of *E. chevalieri*,(J): cleistothecia of *E. chevalieri*, (K) culture of *A. chevalieri*,*(L):* heads of *A. chevalieri*,

Amino acids analysis: thirty one amino acids were detected. *A. nidulans* being the richest in amino acids for detecting twenty eight of amino acids in its cell free extract except Glycine; isoleucine and phenyl alanine followed *by A. chevalieri* were twenty four amino acids were exhibits except taurine; theronine; cysteine; arginine; γ- amino butric acid; tyrosine and 3- methyl histidine. In addition, *E. chevalieri* and *E. nidulans* represented the lowest isolate for possessing only twenty and nineteen amino acids respectively. Serine; alanine; 1- methyl- histidine were absent in *E. chevalieri* and *E. nidulans* while present in theirs anamorph that may explant might be used in clestithecia formation (sexual reproduction) (**Table 9**).

Fatty Acid Profiles: Sixteen fatty acids (eleven saturated and five unsaturated) were detected in the cell free extract of *A. chevalieri, A. nidulans* and their corresponding isolates capable of cleistothecia production; *E. chevalieri* and *E. nidulans*, respectively (**Table 10**).

Table 1: Molecular weights of fragments generated using primer 3 for *A. chevalieri* and *E. chevalieri*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. chevalieri*	*E. chevalieri*
300	+	+
713	+	+
1100	+	+
1500	+	+
1598	+	+
2019	+	+
2378	+	+

Table 2: Molecular weights of fragments generated using primer 5 for *A. chevalieri* and *E. chevalieri*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. chevalieri*	*E. chevalieri*
294	+	+
380	+	+
402	+	+
550	+	+
559	+	-
721	+	+
750	+	+
790	-	+
883	+	+
970	+	+
1014	+	+
1250	+	+
1280	+	+

Table 3: Molecular weights of fragments generated using primer 6 for *A. chevalieri* and *E. chevalieri*. +, band present; -, band absent.

Fungal isolate Band (bp)	*A. chevalieri*	*E. chevalieri*
630	+	+
1200	+	+
1379	+	+
1759	+	+
1890	+	+
2141	+	+

Table 4: Molecular weights of fragments generated using primer 3 for *A. nidulans and E.nidulans* +, band present; -, band absent.

Fungal isolate Band (bp)	*A. nidulans*	*E. nidulans*
720	+	+
900	+	+
1087	+	+
1239	+	-
1300	+	+
1666	+	+
1757	-	+
2080	+	+

Table 5: Molecular weights of fragments generated using primer 5 for *A. nidulans and E.nidulans* +, band present; -, band absent.

Fungal isolate Band (bp)	*A. nidulans*	*E. nidulans*
200	+	+
300	+	+
382	+	+
515	+	+
670	+	+
945	+	+
1100	+	+
1209	-	+
1380	+	+
1740	+	+
2476	+	+
2750	+	+

Table 6: Molecular weights of fragments generated using primer 6 for *A. nidulans and E.nidulans* +, band present; -, band absent.

Fungal isolate Band (bp)	*A. nidulans*	*E. nidulans*
760	+	+
800	+	+
1040	+	+
1250	+	+
1306	+	+
1500	+	+
1740	+	+
2137	+	+
2224	+	+
2495	+	+

A. chevalieri represented the isolate with the maximum number of fatty acids (thirteen out of the sixteen detected); only tridecanoic, stearic and oleic acids were undetected, followed by *E. chevalieri* (twelve detected fatty acids) and then *E. nidulans* and *A. nidulans* (each possessing ten fatty acids) (Figure 8). The detected unsaturated fatty acids were palmitoleic, oleic, elaidic, linoleic and linolenic. Palmitoleic was only detected in the cultures producing asexual conidia (*A. chevalieri*, 3.7 μg/ml and *A. nidulans*, 6.2 μg/ml) while oleic acid was only detected in the cultures producing sexual cleistothecia (*E. chevalieri*, 0.9 μg/ml and *E. nidulans*, 3.73 μg/ml).

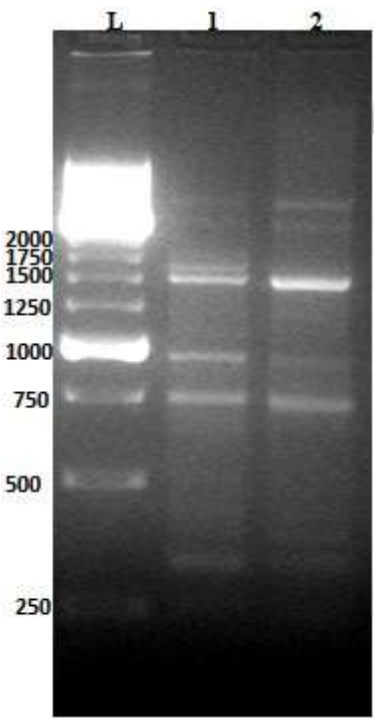

Figure 2: RAPD patterns of *A. chevalieri* (1) and *E. chevalieri* (2) using primer 3. L, 250 bp ladder.

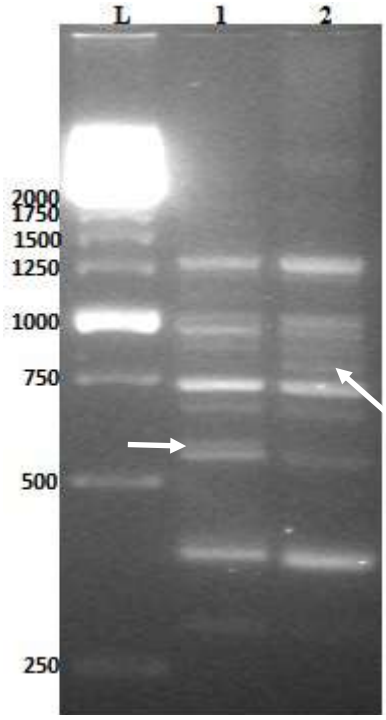

Figure 3: RAPD patterns of *A. chevalieri* (1) and *E.chevalieri* (2) using primer 5. L, 250bp ladder.

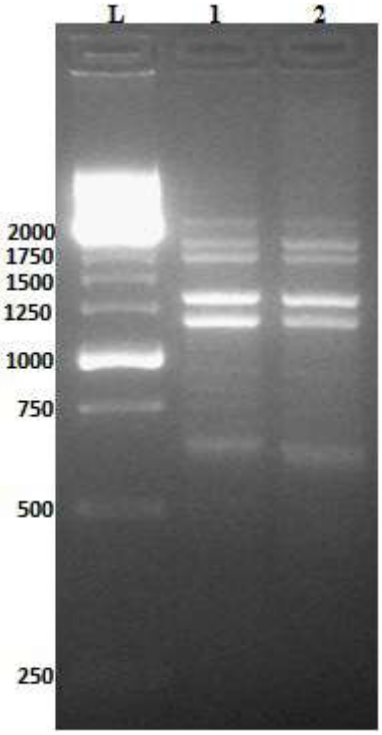

Figure 4: RAPD patterns of *A. chevalieri* (1) and *E.chevalieri* (2) using primer 6. L, 250bp ladder.

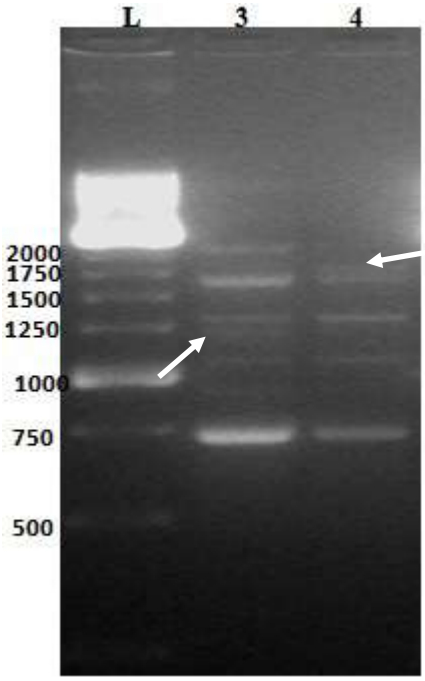

Figure 5: RAPD patterns of *A. nidulans* (1) and *E.nidulans* (2) using primer 3. L, 250bp ladder.

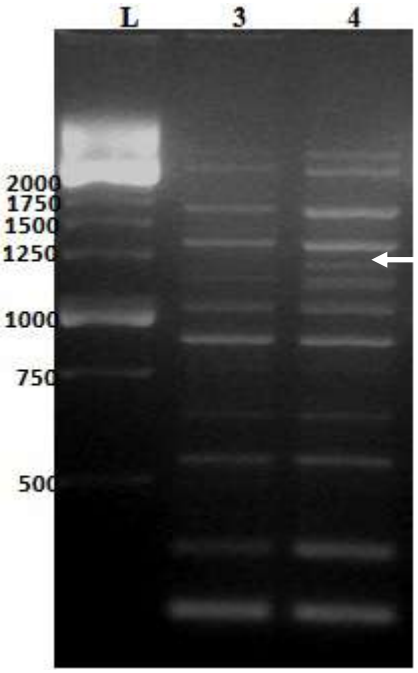

Figure 6: RAPD patterns of *A. nidulans* (1) and *E. nidulans* using primer 5. L, 250bp ladder.

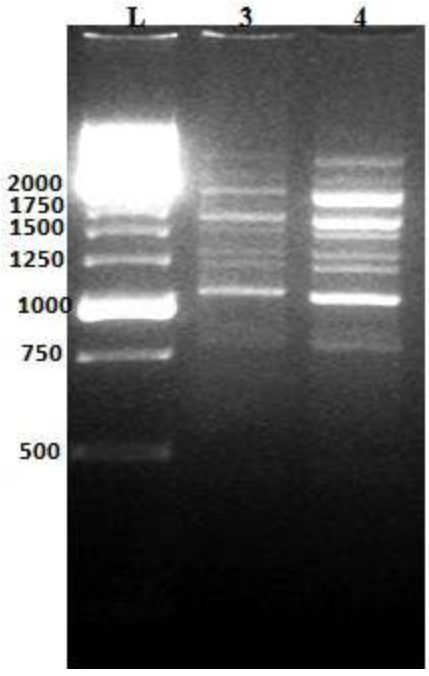

Figure 7: RAPD patterns of *A. nidulans* (1) and *E.nidulans* (2) using primer 6. L, 250bp ladder.

Table 7: Intracellular secondary metabolites of sexual and asexual states of the investigated aspergilla. (+) detected ; (-) not detected

Fungal isolate / **Secondary metabolite**	*A. chevalieri*	*E. chevalieri*	*A. nidulans*	*E. nidulans*
Xanthocillin	+	+	-	-
Ochratoxin A	+	+	+	+
Carlosic acid	+	+	+	+
2-carboxy-3,5-dihydroxyphenylacetylcarbinol	+	+	+	+
Schizopaltic acid	-	+	-	-
Genestic acid	-	+	-	-
Psoromic acid	-	+	-	-
Viridicatum toxin	-	-	-	+

Table 8: Extracellular secondary metabolites of sexual and asexual states of the investigated aspergilla. (+) detected ; (-) not detected

Fungal isolate / **Secondary metabolite**	*A. chevalieri*	*E. chevalieri*	*A. nidulans*	*E. nidulans*
Alectronic acid	-	-	+	-
α-collatolic acid	-	+	+	+
Chaetoglobosin A	-	+	-	+
Chaetochromin A	-	+	-	-
2-pyruvoylaminobenzamide	+	+	-	+
Rosepurpurine	+	+	-	-

Linoleic acid was detected in the four investigated isolates with lower concentrations in the sexual cultures (*E. chevalieri,* 0.1 µg/ml and *E. nidulans,* 0.2 µg/ml) than in the asexual ones (*A.chevalieri,* 1.9 µg/ml and *A. nidulans*, 2.3 µg/ml). Elaidic acid was also present in the four investigated cultures with the highest concentration being detected in *E. chevalieri* (22.9 µg/ml) while with much lower concentrations in the rest of the investigated isolates (*A. chevalieri,* 1µg/ml; *A. nidulans*, 3 µg/ml; *E. nidulans*, 0.5 µg/ml).

Antimicrobial Assay: antimicrobial potency of intra and extra *A. chevalieri; E. chevalieri; A. nidulans* and *E. nidulans* metabolites were recorded in **table (11).** Susceptibilities of pathogenic bacterial and fungal isolates to different fungal extracts were investigated by measuring their inhibitory effect on isolates growth, compared to the solvent used. In the current data show that extra-secondary metabolites extracts of asexual fungi "*A. chevalieri* and A. *nidulans*" had strong antibacterial and antifungal activities on Gram positive and filamentous fungal growth, while Gram negative bacterial isolates and yeast like fungi exhibited high sensitivity to extra-secondary metabolites extracts of sexual fungi "*E. chevalieri* and *E. nidulans*". On the other hands, intracellular metabolites of all four ascomycetes fungi tested had moderate effects against the growth of all bacterial and fungal isolates used.

Table 9: Percentage of amino acids (%) detected in the cell free extract of sexual and asexual states of the investigated aspergilla. (-) not detected

Fungal isolate / **Amino acids**	*Aspergillus chevalieri*	*Eurotium chevalieri*	*Aspergillus nidulans*	*Emercilla nidulans*
Phosphoserine	8.56	5.16	2.54	-
Taurine	-	1.00	2.05	-
Phosphoethanol amine	1.74	4.34	4.02	9.65
Aspartic acid	12.33	8.0	6.25	2.47
Theronine	-	2.25	6.29	5.34
Serine	3.9	-	1.02	-
Glutamic acid	7.77	6.07	4.89	10.25
α- amino adipic acid	1.65	-	9.25	4.25
Proline	2.44	1.56	2.00	-
Glycine	0.28	-	-	6.08
Alanine	0.83	-	5.21	-
Citruline	5.87	4.25	1.25	2.97
Valine	11.07	13.00	16.21	10.27
Cysteine	-	3.21	0.93	-
Cystathionine	6.91	8.01	5.11	2.58
Methionine	0.94	-	6.04	9.25
Isoleucine	0.86	-	-	-
Leucine	1.05	3.65	1.92	0.96
Phenyl alanine	1.25	1.78	-	-
β- alanine	4.20	2.21	2.19	3.56
β- amino iso butric	1.41	1.49	0.91	2.98
γ- amino n- butric acid	7.99	8.23	6.02	2.78
Histidine	2.17	2.89	6.09	2.01
1- methyl-histidine	1.72	-	5.16	-
Carnosine	1.56	3.20	0.25	1.59
Ornithine	0.51	-	5.02	2.01
Lysine	1.50	1.90	2.89	-
Arginine	-	2.35	14.3	2.89
γ- amino butric acid	-	-	4.78	-
Tyrosine	-	-	5.69	2.0
3- methyl histidine	-	-	1.02	-

DISCUSSION:

The genus Aspergillus includes fungi of importance in the food and biotechnology industries, as well as pathogens. Aspergillus is a large genus containing more than 100 ecognized species. Teleomorphs are sexual, or perfect, states of fungi. *Aspergillus* anamorphs (imperfect states) are found in at least eight teleomorphic Ascomycete genera; however only three of these, *Eurotium*, *Neosartorya* and *Emericella*, occur in foods. All form cleistothecia. It would therefore be of major economic and medical advantage to be able to study their biochemistry as well as the inheritance of genes of interest and to bring together desirable genetic traits in the aspergilli. Unfortunately, such genetic efforts have been impeded because most Aspergillus species are only known to reproduce asexually, thus the sexual cycle cannot be used for strain improvement and inheritance studies (**Calvo *et al.*, 2001**).

Table 10: Concentration of fatty acids (μg/ml) detected in the cell free extract of sexual and asexual states of the investigated aspergilla. (-) not detected

Fungal isolate / **Fatty acid**	*Aspergillus chevalieri*	*Eurotium chevalieri*	*Aspergillus nidulans*	*Emercilla nidulans*
Butyric acid (4:0)	0.5	0.65	0.7	1.76
Caproic acid (6:0)	2.63	0.6	0.84	1.2
Caprylic acid (8:0)	0.7	1.2	0.5	0.8
Capric acid (10:0)	1.0	0.7	-	0.6
Lauric acid (12:0)	3.5	1.2	-	4
Tridecanoic acid (13:0)	-	9.28	-	-
Pentadecanoic acid (15:0)	0.7	-	-	-
Palmitic acid (16:0)	1.5	0.5	8.5	0.2
Margaric acid (17:0)	10.23	0.3	5.4	-
Stearic acid (18:0)	-	3	1.9	9.69
Arachidic acid (20:0)	0.5	-	-	-
Palmitoleic acid (16:1)	3.7	-	6.2	-
Oleic acid (18:1Δ^9 cis)	-	0.9	-	3.73
Elaidic acid(18: 1 Δ^9 trans)	1.0	22.9	3	0.5
Linoleic acid (18:2)	1.9	0.1	2.3	0.2
Linolenic acid (18:3)	3.05	-	1.2	-

In the current study, RAPD patterns of the four investigated fungal isolates, it could be observed that the maximum number of different bands was only two bands using primer 5 with *A. chevalieri* and *E. chevalieri* and primer 3 with *A. nidulans* and *E. nidulans*. Primer 5 resulted in only one band difference with *A. nidulans* and *E. nidulans*. The rest of the investigated primer-isolate combinations resulted in no band differences. This might suggest that the asexual isolates unable of cleistothecia production suffered sexual, fatty acid or secondary metabolite gene mutations as these three criteria are connected.

Regarding the oleic acid was undetected in the imperfect cultures (*A. nidulans* and *A. chevalieri*) while was of concentrations of 0.9 ppm in *E. chevalieri* and 3.7 ppm in *E. nidulans* suggesting its increased requirement in conidia formation (asexual reproduction). While, in case of linoleic acid, it was of lower concentrations in perfect cultures (0.1 ppm for *E. chevalieri* and 0.2 ppm for *E. nidulans*) than in imperfect ones (1.9 ppm for *A. chevalieri* and 2.3 ppm for *A. nidulans*) suggesting its increased demand in sexual spore production. The latter was also observed for linolenic acid which was undetected in the perfect cultures while was of concentrations of 3.05 and 1.2 ppm in *A. chevalieri* and *A. nidulans* respectively. Also, increase in stearic and decrease in palmitic acids agree with the current results were in cases with increased oleic acid (0.9 and 3.37 ppm for *E. chevalieri* and *E. nidulans* respectively) production there were also increased stearic acid (3 and 9.69 ppm for *E. chevalieri* and *E. nidulans* respectively) but decreased palmitic acid production (0.5 and 0.2 ppm for E. *chevalieri* and *E. nidulans* respectively). It could also be observed that the fatty acid profile of the asexual culture of *A. nidulans* and *A. chevalieri* was different than that of their corresponding sexual ones (*E. nidulans* and E. *chevalieri*, respectively).

This agrees with the results of **Tsitsigiannis *et al*. (2004)** who reported that in *A. nidulans,* mutations in *ppoA* (encoding the dioxygenase PpoA contributing to the generation of 8-hydroxy linoleic acid, psiBα or 8- HODE) enhanced the ratio of asexual to sexual spore production, reflecting the role of linoleic acid in sexual spore

production. However, Δ*ppoC* (encoding the dioxygenase PpoC necessary for optimal production of 8-hydroxy oleic acid, psiBβ or 8-HOE) mutant exhibited an increase in sexual spore production reflecting the role of oleic acid in asexual reproduction. These effects on sporulation are reflected in expression levels of the sporulation-specific transcriptional regulatory genes, *brlA* and *nsdD*.

Table 11: *In vitro* antimicrobial activity of fungal intra and extra-metabolites used (measurement of inhibition zones by cm, Ext.= Extracellular metabolites; Int.= intracellular metabolites)

Fungal isolate / **Microorganisms used**	***A. chevalieri***		***E. chevalieri***		***A. nidulans***		***E. nidulans***	
	Ext.	***Int.***	***Ext.***	***Int.***	***Ext.***	***Int.***	***Ext.***	***Int.***
Gram positive bacterial isolatees								
Staphylococcus aureus	2.0	1.2	1.5	00	2.5	1.2	00	00
Bacillus subtilis	1.5	0.5	00	0.5	3.2	1.6	0.5	00
Bacillus cepatia	2.3	1.0	1.0	00	3.5	0.6	1.2	0.8
Corynebacterium	0.9	00	0.9	0.8	1.2	0.5	00	1.0
Streptococcus pyogenes	2.5	1.5	0.5	00	1.5	00	00	0.4
Streptococcus pneumoniae	3.8	0.7	00	00	2.0	00	1.5	00
Gram negative bacterial isolatees								
Escherichia coli	1.2	00	2.2	1.0	1.6	1.6	2.3	0.9
Pseudomonas aeruginosa	00	0.8	1.2	1.6	0.6	0.5	1.8	1.2
Klebsiella pneumonia	1.0	00	2.1	00	1.2	00	2.1	1.5
Serratia marcescens	1.9	1.6	0.7	0.6	1.4	0.9	1.5	0.7
Alcaligenes eutrophus	0.8	00	1.3	00	2.0	00	1.5	0.4
Salmonella typhi	1.6	00	2.2	00	0.9	0.5	2.5	0.8
Fungal isolates								
Aspergillus flavus	2.6	00	0.6	0.6	1.6	1.5	0.0	00
Aspergillus niger	2.2	1.0	0.0	0.5	1.0	1.0	0.0	00
Aspergillus fumigatus	1.5	0.5	2.6	1.0	2.2	1.2	1.6	0.5
Fusarium cladosporum	2.0	00	1.5	0.6	1.8	00	1.0	0.6
Alternaria alternata	1.2	00	0.0	00	0.0	1.5	0.5	1.0
Rhizopus	0.0	00	1.6	1.5	0.8	0.8	0.0	0.5
Syncephalastrum	1.8	0.5	0.0	0.7	1.5	0.6	1.0	0.8
Penicillium chrysogenum	1.4	0.7	2.5	00	1.1	0.4	1.3	00
Microsporum canis	0.5	00	0.0	0.5	2.0	00	0.0	00
Trichophyton	1.2	00	0.0	00	1.7	00	0.0	00
Yeast like fungi								
Candida albicans	1.2	0.5	00	1.0	1.2	00	0.7	1.0
Candida krusei	0.6	0.9	1.5	0.5	2.0	0.8	0.8	0.6
Candida glabrata	1.8	0.7	00	0.7	1.0	00	00	0.9
Cryptococcus humicola	2.0	0.8	2.8	0.9	00	0.6	1.2	1.0
Geotrichum candidum	2.2	1.2	1.5	0.8	0.6	00	1.2	0.9
Trichosporon cutameum	0.9	0.6	00	1.0	1.2	0.5	00	00

Recently, extra-metabolites of *E. chevalieri* and *E. nidulans* exhibited great potency against Gram negative bacterial isolates and yeast like fungi while intra

metabolites showed mild effects against the growth of all bacterial and fungal isolates used. That in agree with **Char *et al*., (2002) and Gao *et al*. (2012)** who we examined *in vitro* antibacterial and antifungall activities of secondary metabolites isolated from the many fungus belong to *Eurotium* sp. the results showed mild to moderate antibacterial or antifungal or both activities except *E. chevalieri* was the best of fungi tested. The ability or inability of the investigated fungal cultures to produce cleistothecia (which also resulted in different fatty acid profiles and concentrations) resulted in different intracellular and extracellular secondary metabolite profiles, where certain metabolites were detected in case of cleistothecia production which were ceased when the culture lost its ability to produce cleistothecia. It has been reported that reproduction in fungi is accompanied by developmental changes among which are changes in secondary metabolite profiles **(Brodhagen and Keller, 2006)**. Fungal secondary metabolism and sporulation are associated both temporally and functionally **(Calvo *et al*., 2001).**

The accompanying changes in secondary metabolite and fatty acid profiles between the sexual and asexual cultures with RAPD profile differences agrees with the results of **Tsitsigiannis and Keller** (**2006**) who reported that deletion of *ppo* genes (affecting sexuality) affected the production of at least three different secondary metabolites in *A. nidulans*, including sterigmatocystin, the antibiotic penicillin and an octaketide, shamixanthone, where for sterigmatocystin and penicillin, these effects were reflected and supported by levels of biosynthetic gene transcription. Comparing the RAPD results with fatty acid results, it could be concluded that variations in the DNA are very low when compared to fatty acid variations, this reflects that it might be the expression of the genes that govern fungal sexuality which might be repressed or blocked, or the parts of the gene regulators are mutated so these isolates need DNA repair (differences in some of the bands with this great DNA similarity might reflect the possibility of some mutations).

Hence mutations in fatty acid synthesis, secondary metabolite synthesis and/or sexuality genes as well as differences in the promoter sequence of either genes (or gene clusters) might be responsible for the ceasing of fungal sexual reproduction especially that it has been recently reported that sexual genes exist in the formerly thought asexual forms **(Dyer, 2007).**

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الانشطه البيولوجيه و التغيرات الجوهريه بين عزلات فطريه تنتمى الى جنس اسكوميسيدس

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لقد تم الانتباه الي التكاثر الجنسى للفطريات لاهميتها وايضا دراسه العلاقات بين الفطريات التى تاخذ اطوار متنوعه لنفس الفطر. فى هذه الدراسه تم مقارنه الوزن الجزيئي للماده الوراثيه و الاحماض الامينيه و الاحماض الدهنيه و ايضا المواد الايضيه لاربع عزلات فطريه تنتمى لجنس و احد اسكوميسيدس وهم اسبراجيللس نتيولانت و اسبراجيللس شيفيلايري و الاجناس التى تتكاثر جنسيا لها و هم اميرسيلاه نتيولانت و ايروشييم شيفيلايري. وايضا تم دراسه مقارنه بين مقدره تلك العزلات الاربعه كمضادات بكتريه و فطريه على العديد من العزلات الممرضه منها عزلات بكتريه سالبه و اخرى موجبه لصبغه جرام وايضا عزلات فطريه منها الخمائر. واظهرت نتائج هذا البحث اختلافات بين العزلات الفطريه التى لها مقدره على التكاثر الجنسى ولا جنسى حيث كانت نسبه حمض اوليك اقل عند اسبراجيللس نتيولانت و اسبراجيللس شيفيلايري عنها عند نظيراتهم اميرسيلاه نتيولانت و ايروشييم شيفيلايري و بخلاف حمض لينولينك كانت نسبه بالعزلات التى لا تتكاثر جنسياُ اعلى. و ايضا كان هناك تغيرات واضحه فى الوزن الجزيئي للماده الوراثيه فكان الاختلاف بين اسبراجيللس وايروشييم شيفيلايري فقط عند الوزن الجزيئى ٥٥٩ و ٧٩٠ عند استخدام بريمر ٥ بينما الاختلاف بين اسبراجيللس و اميرسيلا ه نتيولانت كان عند الوزن الجزيئي ١٢٣٩ و ١٧٥٧ عند استخدام بريمر ٣ و ايضا عند ١٢٠٩ عند استخدام بريمر ٥. وكذلك لوحظ اختلافات بين تسب الاحماض الامينيه و المركبات الايضيه بين العزلات التى لها مقدره على التكاثر الجنسى و نظيرتها لما يدل على احتياجتها الى نسب معينه لتكوين الكليستو ثيثيه و الاجزاء الجنسيه. وكان النشاط البيولوجى لهم كمضادات بكتريه وفطريه ايضا مختلف حيث ان العزلات الجنسيه اكثر تاثيراُ على بكتريا السالبه لصبغه جرام و الخمائر بينما العزلات التى تتكاثر لا جنسياُ فقط كانت مقدرتها على تثبيط نمو البكتريا الموجبه لصبغه جرام و الفطريات الخيطيه عاليه.