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EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
BOTANY

ISSN 2090-3812

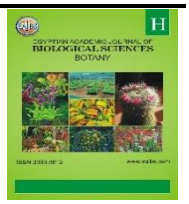
www.eajbs.com

Vol. 16 No.1 (2025)

Citation: Egypt. *Acad. J. Biolog. Sci.* (H. Botany) *Vol.16 (1) pp 117-127 (2025)*
DOI: 10.21608/EAJBSH.2025.432424

Egypt. Acad. Journal Biology. Sci., 16 (1): 117-127 (2025)

Egyptian Academic Journal of Biological Sciences
H. Botany
ISSN 2090-3812
www.eajbsh.journals.ekb.eg.



The Effect of Double Mutations in Aspergillus *flavus*

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ARTICLE INFO
Article History
Received:2/5/2025
Accepted:10/6/2025
Available:14/6/2025

***Keywords*:**
Aspergillus flavus, Double mutation, Gene, sodium azide, potassium chlorate.

ABSTRACT

The pervasive presence of *Aspergillus flavus* in agricultural systems and its dual role as a facultative pathogen and saprophyte underscore its critical impact on food safety and human well-being. A major concern stems from its production of aflatoxins, toxins that contaminate food products and present severe health risks to consumers. To better understand *A. flavus* and its toxin production, this research focused on inducing double mutations using sodium azide and potassium chlorate. We subsequently characterized the phenotypic and metabolic profiles of these mutants, evaluating their growth patterns and antifungal resistance relative to the wild-type strain. Three wildtype strains ($Aspfwt_1$, $Aspfwt_2$ and $Aspfwt_3$) isolated previously in the laboratory were subcultured and subjected to double mutation. by subjecting the strains to mutagens (Potassium Chlorate and sodium azide) twice. Thus the mutants that were phenotypically different the wild type were generated through repeated culturing on mutagen-supplemented media, sodium azide demonstrating a stronger mutagenic effect at 0.12g/ml. The experiment on double mutation led to the development of mutants that differs from the wild in phenotypic, metabolic and growth characteristics. The results exhibited that the double mutation caused a significant alteration in the fungus's growth rate, with the mutants exhibiting a higher growth rates than the wild strains. The mutation also increased the organism's resistance to fluconazole, an antifungal agent. The mutation led to the formation of auxotrophic mutants that were unable to grow on minimal medium. When two of these mutants were combined in a plate with minmum medium, the progeny of the mutants resulted in a heterokaryon, showing a wildtype phenotype through complementation. The use of double mutation in this work which revealed the complexity of the genetics of *A. flavus*, has given insight into the development of novel antifungal agent. However, the need for molecular basis for a clear understanding of the interaction between double mutation and secondary metabolites production in the fungus is highlighted.

INTRODUCTION

Aspergillus flavus, a facultative pathogen, thrives across diverse ecological niches (Klueken et al., 2009), particularly in tropical regions with temperatures from 28°C to 37°C and approximately 95% relative humidity (Yu, 2012). As a saprophyte, it contributes to nutrient cycling by breaking down carbohydrate-rich plant debris (Abbas *et al*,, 2009). The increasing prevalence of aflatoxins**,** potent naturally occurring carcinogens primarily produced by *A. flavus* (Klich, 2007), in food products is a pressing concern. This necessitates novel strategies to combat *A. flavus* contamination, noting that chromosomal abnormalities from meiotic and mitotic recombination can lead to aflatoxin-

producing strains (Kafer, 1997 The increasing presence of aflatoxins in food products has become a pressing issue of concern, necessitating the creation of innovative strategies to combat *A. flavus* contamination. *A. flavus* is the major producer of Aflatoxins, one of the most powerful naturally occurring carcinogens (Klich, 2007). However, the lack of Knowledge on understanding the genetic mechanisms behind the development of aflatoxin-producing strains hampers the development of effective countermeasures. Moreover, the challenge is made more difficult by the intricacy of accurately identifying species within the *Aspergillus flavus* complex, because of overlapping morphological and biochemical traits.

Mutagenesis can lead to genetic mutations resulting in the instability and decay of the primary structure of DNA (Lindahl, 1993). Mutations could be harmful and sometimes beneficial. For instance, single mutations in *Aspergillus nidulans* have been shown to confer resistance to anti-fungal agents and *Aspergillus niger* have been used to alter biochemical pathways (He *et al.*, 2014; Ullah *et al.*, 2012) Similarly, double mutations in *A. nidulans* have been used to study conidation mutants and elucidate the genetic mechanisms underlying this process (Clutterbuck, 1969; Clutterbuck, 1976; Clutterbuck, 1977; Martinelli, 1979; Walker, 2017).

Auxotrophy, a typical phenomenon in microbes as the inability to synthesize one or more essential growth factors. Auxotrophic mutants require external support for growth due to their inability to produce some specific proteins, have been generated in various *Aspergillus* species. These mutants have been used to study the genetic mechanisms underlying amino acid biosynthesis and other metabolic pathways.

The relevance of this research is to investigate the development of double auxotrophic mutants of *Aspergillus flavus,* evaluating their phenotypic, metabolic, and growth variations in comparison to the wild-type strain. In particular, we seek to answer the following querries: Can double auxotrophic mutants of *Aspergillus flavus* be produced using potassium chlorate and sodium azide as mutagenic agents? And how do the double auxotrophic mutants and the wild-type strain differ in terms of phenotypic, metabolic, and growth?. To achieve this objective, we employed a conjunction of potassium chlorate and sodium azide mutagenesis procedure, followed by phenotypic analysis, growth rate assessment, and metabolic response studies.

MATERIALS AND METHODS

Strains and Growth Media:

Aspergillus. flavus strains previously isolated in the Department of Plant Biology and Biotechnology, the University of Benin, Benin City, Edo State, Nigeria were used in this research. The growth cultures were maintained at 25 ºC. The strains were labeled as $Aspfwt_1$, $Aspfwt_2$ and $Aspfwt_3$ representing the three (3) wild strains identified as *A. flavus* on PDA using morphological characteristics by Samson *et al.* (2007). Potato Dextrose agar (PDA) was routinely used as a complex medium, Potato Dextrose Chlorate **(**PDC), Potato dextrose sodium azide (PDSA), Minimal Medium **(**MM) and fluconazole-supplemented PDA were prepared following the procedure according to Correll *et al.* (1987).

Mutagenesis, Starvation, and Susceptibility Test:

Auxotrophic mutants were generated through a two-step mutagenesis process. For the first generation, vegetative parts of the wild-type strain were cultivated on Potato Dextrose Agar (PDA) supplemented with 0.12 g/mL Potassium Chlorate (PDC) as a mutagen. The "spot streaking" technique was used for inoculation, and cultures were incubated at 30 °C until maturity. The resulting isolates were declared original auxotrophic mutants based on their inability to grow on a minimum medium compared to the wild-type strain, indicating a nutritional requirement absent in the parental strain. The second generation was developed by repeating this entire process using confirmed first-generation auxotrophs. This mutagenesis procedure generally followed the methodology described by Scriban (1988), utilizing both Potassium Chlorate and Sodium Azide as strong chemical mutagens to induce mutations in the *A. flavus* strains. To assess starvation tolerance, developing portions of both wild-type and mutant Aspergillus strains were inoculated into a minimum medium containing nitrate as the sole nutrient. Growth diameter was measured with a meter rule, and the average growth rate for each group was computed. For susceptibility testing, 20 µL of spore suspension was added

to 1 mL of sterile water, and this mixture was serially diluted up to 10-5. Subsequently, 30 µL of the fifth dilution was spread onto PDA plates supplemented with fluconazole.

Statistical Analysis:

One way ANOVA and Post-hoc analysis were performed to compare the growth rates of the wild-type (WT) and mutant (M1 and M2). Statisitcal significance was recorded at $p < 0.05$.

RESULTS

Three strains of *Aspergillus flavus* were obtained from the pure culture, with green, yellow-green and olive-green spores as phenotypic traits (Fig.1). Colonies covered the entire plate within 7 days of inoculation starting from the center and extending radially. The observation of the microorganism in terms of colour and texture was consistent with the description provided by Ouattara - Sourabie (Ouattara-Sourabie *et al.*, 2011).

Aspfwt$_1$ Aspfwt$_2$ Aspfwt$_3$

Fig. 1: The strains of *Aspergillus flavus*. (Aspfwt$_1$, Aspfwt$_2$ and Aspfwt$_3$)

Mutant Development and Characteristics:

We developed mutants using Potato Dextrose Agar (PDA) supplemented with either Potassium Chlorate (PDC) or Sodium Azide. Our findings clearly indicated that Sodium Azide exhibited a significantly greater mutagenic effect compared to Potassium Chlorate. Specifically, concentrations of 0.02 g/mL or 0.12 g/mL of Sodium Azide completely inhibited the growth of the strains. In contrast, at 0.12 g/mL Potassium Chlorate, we observed the growth of chlorate-resistant strains, indicating a selection for tolerance rather than broad mutagenicity.

The mutant strains developed on PDC displayed distinct growth patterns when compared to the wild-type. While the wild-type strains exhibited typical growth, our mutants grew more slowly and showed uniform, directional expansion (Fig. 2). This observation suggests a significant alteration in their colonial morphology. The mutation process was repeated twice, yielding first and second-generation mutants.

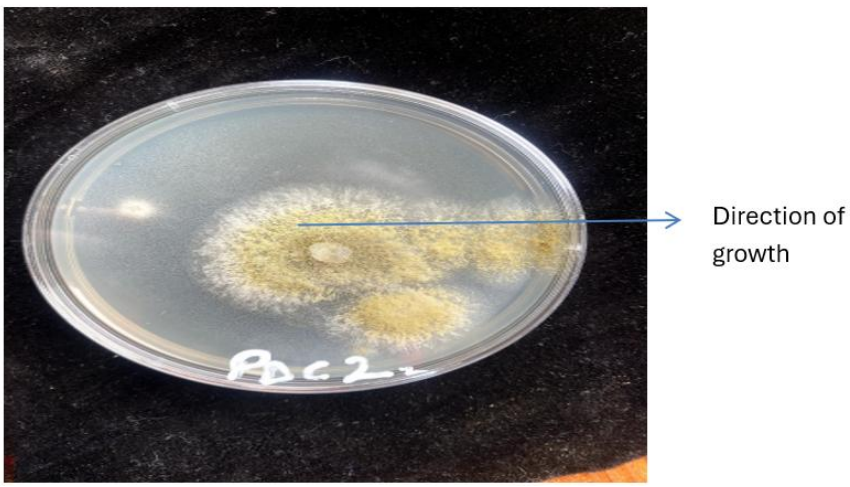

Fig. 2: Image of mutant (M_2) growing uniformly in one direction(to the right).

Microscopic analysis revealed further key differences. The first-generation mutants of strain 2 (Aspfmt2) exhibited rough cell walls, a stark contrast to the smooth, straight cell walls of the wild-type (Fig. 3). Interestingly, the second-generation mutants showed some morphological similarities to the wild-type phenotype, suggesting potential variations in the stability or expression of the induced mutations across generations.

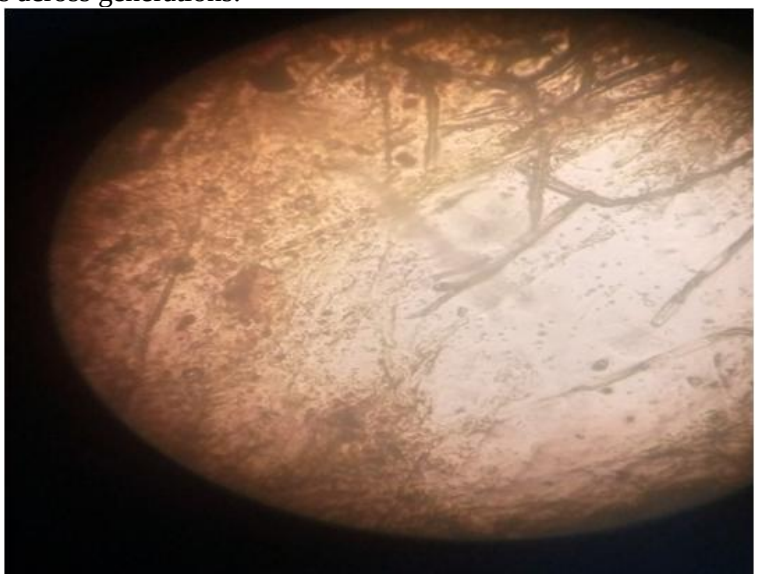

Fig. 3: Microscopic view of *Aspergillus flavus* showing the rough cell wall of M1 in Aspfmt2.

Growth Rate Under Starvation:

We measured the growth diameters of both wild-type and mutant strains consecutively for four days to assess their performance under starvation conditions. Our results, depicted in Figure 4, reveal a significant difference in the growth rates of the first and second-generation mutants (Aspfmt1 and Aspfmt2) compared to the wild-type strains. Notably, on day 4, the first-generation mutants consistently exhibited higher growth than the wild-type.

To further analyze these differences, we performed a One-Way ANOVA comparing the growth rates of the wild-type (WT) and mutant (M1 and M2) strains. The post-hoc analysis indicated significant differences in growth rates between the WT and M1 groups on all days ($p < 0.05$). Furthermore, significant differences were observed between the WT and M2 groups on Days 1, 2, and 4 ($p < 0.05$).

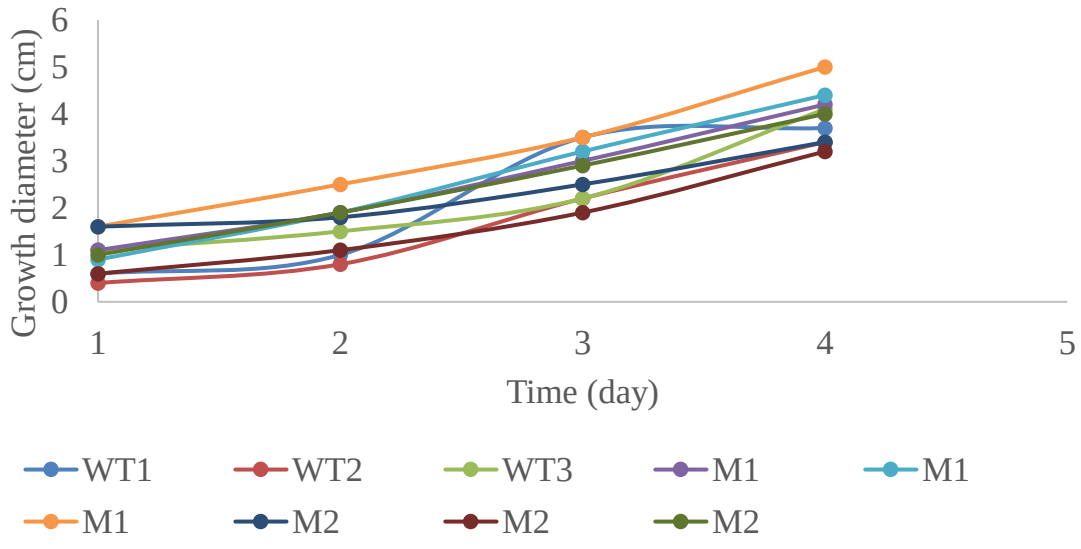

Fig. 4: Growth diameter of the wild and mutants for a period of 4 days.

Production of Secondary Metabolites Under Starvation:

Under starvation conditions, both the wild-type and mutant variants of strain 2 (Aspfwt2, M1 of Aspfmt2, and M2 of Aspfmt2) exhibited the production of unknown secondary metabolites. These metabolites were characterized by distinct pigmentation, specifically red and yellowish-green hues, as shown in Figure 5. This variation in metabolite production suggests a significant physiological response to environmental stress, particularly the limited availability of essential nutrients crucial for normal cellular growth and metabolic activity.

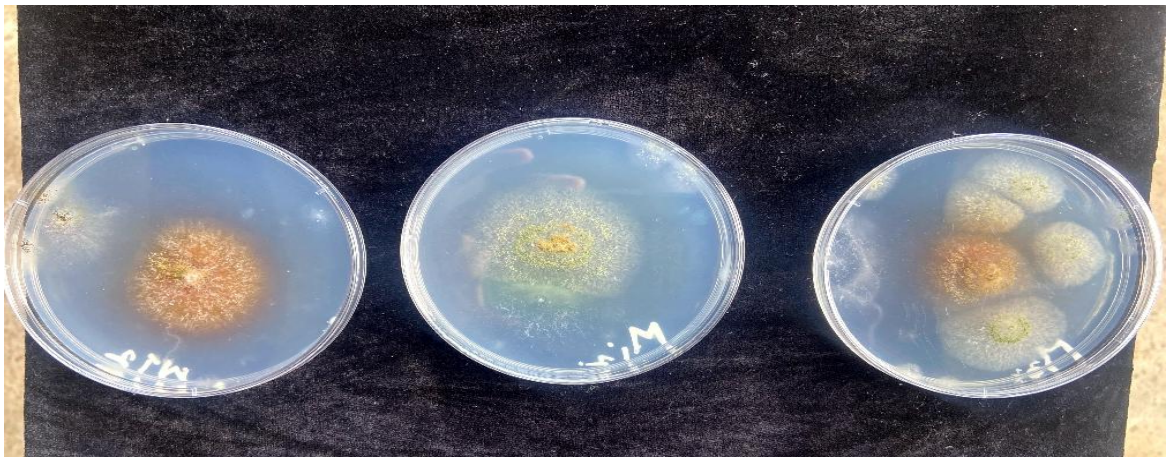

Fig. 5: Showing the production of unknown secondary metabolites by second strain of *Aspergillus flavus*

Formation of Heterokaryon:

When we deliberately paired the mutants on a Minimal medium containing sodium nitrate as the sole nutrients, M1 and M2 of $Aspfmt_2$, showed heterokaryon (Fig. 6) indicating the presence of vegetative compatible strains. Phenotypically no barrage zone was observed as mycelium from two different strains mixed to produce a heterokaryon.

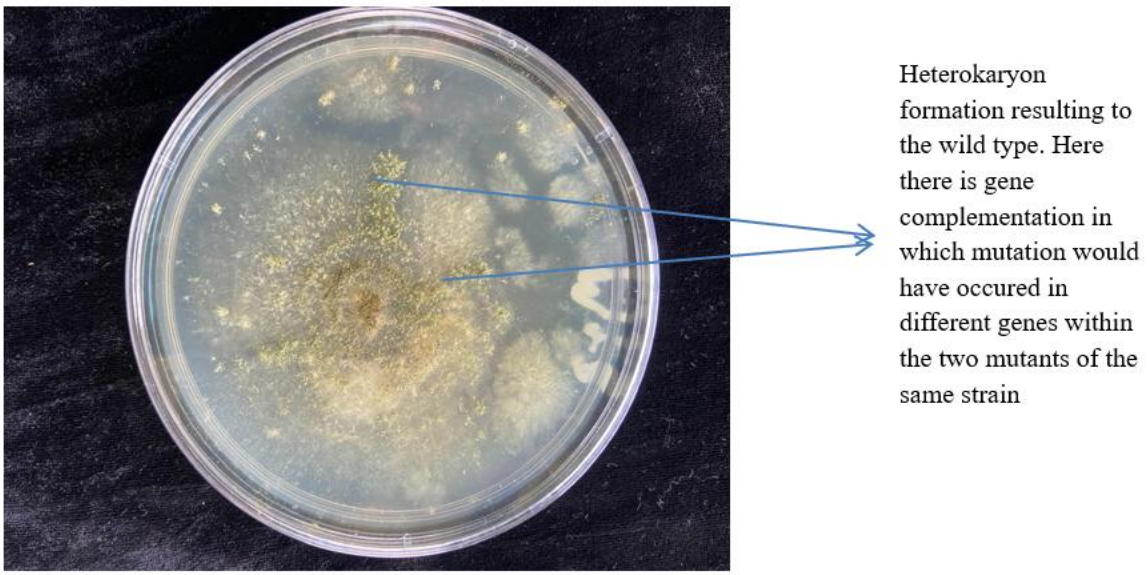

Fig. 6: Showing formation of heterokaryon by the second generation of mutants (M2) in $Aspfmt_2$.

Susceptibility Test:

Susceptibility test was conducted to show the reaction of the mutants compared with the wild strains to anti-fungal drug (fluconazole). The results revealed variations in growth rates between the groups. In day 7, the mutant of the first (M1) and second (M2) generation of strain1 and 3 respectively outgrew the wild-type strains indicating that the mutants are more resistant to fluconazole (Fig. 7).

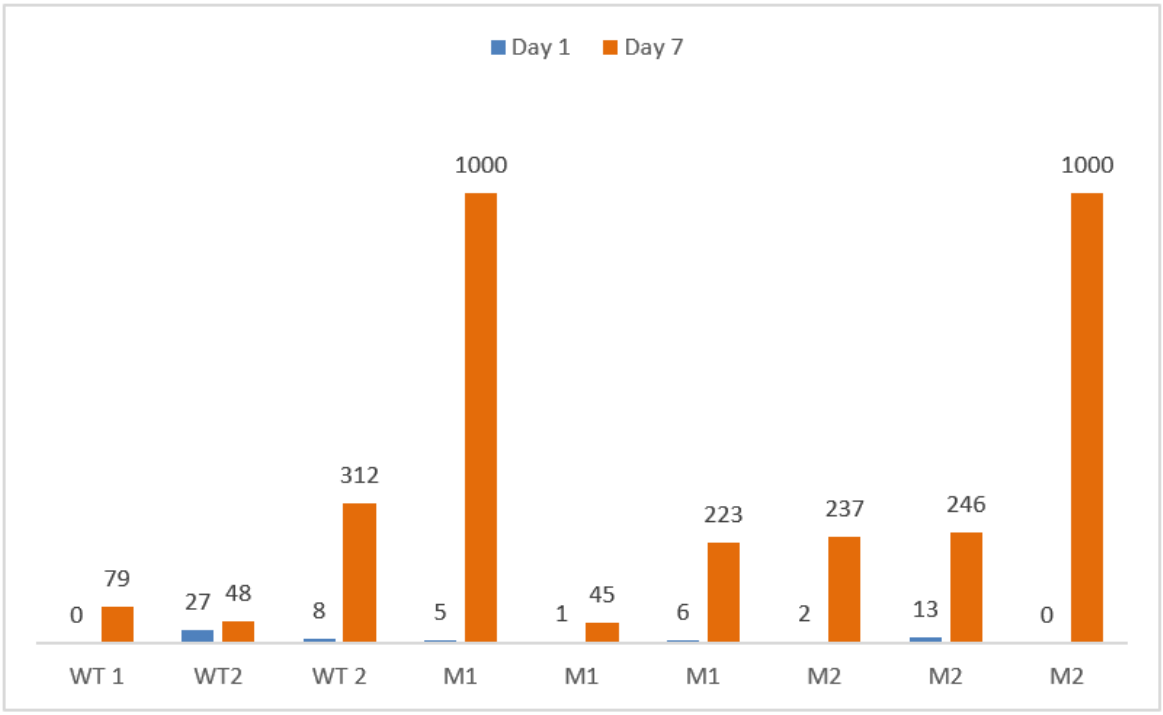

Fig. 7: Graphical representation of susceptibility in all three strains of *Aspergillus flavus*.

DISCUSSION

This study investigated the impact of double mutation in *Aspergillus flavus*, focusing on its effects on growth, susceptibility, and metabolite production. Our findings revealed that the double mutation profoundly affects the fungus's growth rate, susceptibility to fluconazole, and genetic recombination.

Both wild-type and mutant strains demonstrated the ability to produce secondary metabolites. However, we were unable to ascertain the specific effect of the double mutation on secondary metabolite production. This remains a crucial area for future research. We hypothesize that the gene responsible for secondary metabolite production in the wild-type may not have been among those mutated. It's also important to consider that secondary metabolite production is influenced by both genetic and environmental factors (Lohmar *et al*., 2019). The complexity of this process is further highlighted by studies on the functions and molecular mechanisms of genes involved in A. flavus development and aflatoxin biosynthesis (Tumukunde *et al*., 2021).

The double mutation resulted in an auxotrophic mutant, which were unable to synthesize essential nutrients. However, the effect of the double mutation became so clear when we combine the two mutants on Minimal medium that led to the production of heterokaryon. The effect of the second mutation may have created a room for a better genetic recombination, such that two different genes in transposition, resulted to a prototype of the wild strains through complementation (Fig.6) (Eboigbe, 2018). Complementation is a genetic process where two mutants with different genes can form a heterokaryon, exhibiting a wild-type or near-wild-type phenotype (Stinson and Loparo, 2021). Heterokaryon formation is a full proof that two different genes are mutated

Our susceptibility tests, as shown in Fig. 7, revealed that the mutants are more resistant to fluconazole than the wild-type strain. While the exact mechanisms behind this increased resistance are unknown, it's recognized that fluconazole resistance in Candida albicans is often associated with

ERG11 mutation (Urbanek *et al*., 2022). It's possible a similar mechanism is at play in Aspergillus flavus, a hypothesis supported by our double mutation experiment (Paul *et al*., 2019). The outcome of mutational events can be unpredictable, but in this case, the double mutation has led to increased resistance, and the underlying mechanism warrants further investigation.

Regarding growth, we measured growth diameter for both wild-type and mutant strains over four days. By day 4, the growth rates of the first-generation mutants were consistently higher than the wild-type, suggesting that these double mutations can significantly impact the fungus's growth rate. Interestingly, the second-generation mutants did not maintain the same accelerated growth rate as the first generation by day 4; their growth was more similar to the wild-type. This might indicate that the initial mutational event had a more potent effect than subsequent mutations. Similar studies have shown that growth parameters like temperature and nutrient availability can significantly influence the growth rate of Aspergillus flavus (Sun *et al*., 2016).

In conclusion, this research demonstrates that the double mutation has significant effects on *Aspergillus flavus,* including altered growth rates, increased resistance to fluconazole and alteration in the expression of specific genes. On account of secondary metabolite production a specific gene mutation would be necessary in other to clearly underpin the production pathway; this invariably will involve molecular studies in the nearest future.

Declarations:

Ethical Approval: This study did not involve human or animal subject. Therefore, ethical consideration was not applicable. Data used for this study were obtained secondarily for public databases, without participants' identifier information. Therefore, informed consent was not applicable.

Conflict of interest: The authors declare no conflict of interest.

Authors Contributions: I hereby verify that all authors mentioned on the title page have contributed significantly to the idea and planning of the research, has carefully read the work, attested to the veracity and correctness of the data and its interpretation, and has given their approval for submission.

Funding: No funding was received.

Availability of Data and Materials: All the required data are included in the manuscript.

Acknowledgements: I wish to acknowledge the Department of Plant Biology and Biotechnology where the work was done.

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