THE EPISTATIC EFFECT OF THE DOUBLE MUTATION OF *ASPERGILLUS FLAVUS INDUCED* WITH SODIUM AZIDE AND POTASSIUM CHLORATE

BY

ABDUL-AZEEZ YAKUB

LSC2104187

DEPARTMENT OF PLANT BIOLOGY AND BIOTECHNOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN, BENIN CITY. NIGERIA

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CERTIFICATION

This is to certify that this work was carried out by ABDUL-AZEEZ YAKUB in the Department Of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin city, Nigeria.

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Dr. L. Eboigbe Date

(Project Supervisor)

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Prof. B. IKHAJIAGBE Date

(Head of Department)

DEDICATION

I dedicate this work to Allah the Creator, from whom all knowledge, wisdom and understanding comes from, appreciating his unlimited mercy, blessings, guidance and provisions throughout this research study.

ACKNOWLEDGEMENT

The success of this work is undoubtedly a testimony of the grace of Allah, to whom I am eternally grateful for his protection and provisions, his mercy has been ever present even before the commencement of this project.

I am most grateful to my parents Mr and Mrs Yakubu for their endless love and support, this project was fully funded by them , as is the entirety of my education.

I am very grateful to my project supervisor Dr. L. Egoigbe for his ever present guidance, supervision, sacrifices, corrections, effort and most importantly his patience. His reward can only be given by God.

I want to thank the HOD Prof. B. Ikhajiagbe for his effort in keeping the department in due order.

I also want to thank my lecturers for the academic and moral support they presented to me, during the course of my study. May God bless you all.

Special thanks to the members of my project group, who contributed to the completion of this work, in more than one way.

Finally, to my siblings and parents once again, truly expressing my gratitude to you ,would have me writing my research report by the end of the year. So instead to you all I say Jazakallahu Khairan.

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ABSTRACT

Aspergillus flavus is a fungus known for posing significant health risks in humans and animals. This study investigates the epistatic effect of double mutations induced by sodium azide and potassium chlorate in *A. flavus*. The mutants were generated were generated in culture media containing the mutagens and their epistatic interactions were observed along with their nutrient utilization and growth rates. The results of mutation resulted in significant changes in the growth rate and morphology of the mutants, compared to the wild type. The epistatic interactions between the mutations revealed both synergistic and antagonistic effects on various traits. These findings provide insights into the genetic mechanisms underlying aflatoxin production and fungal development in *A. flavus*, with implications for developing novel strategies to control aflatoxin contamination.

CHAPTER 1

INTRODUCTION

1.1 General overview of *Aspergillus flavus*

Aspergillus flavus is a facultative pathogen capable of inhabiting diverse ecological niches (Klucke et al., 2009). It demonstrates a remarkable ability to thrive in tropical regions characterized by temperatures ranging from 28°C to 37°C and a relative humidity of approximately 95% (Yu, 2012). As a saprophytic organism, it derives its energy from the decomposition of carbohydrate-rich plant detritus, thereby playing a crucial role in nutrient cycling within its environment (Abbott et al., 2009). The metabolic versatility of *Aspergillus flavus* is underscored by its production of a wide array of secondary metabolites, including aflatoxins B1 and B2, aspergillic acid, nitropropionic acid, and kojic acid (Yu, 2004). These bioactive compounds serve multiple functional roles, acting as virulence factors during host-pathogen interactions, chemical signals for communication, and adaptive mechanisms to environmental stressors. Such attributes contribute to the organism's ecological success and its ability to persist across various environmental conditions.

A flavus is of particular significance in the production of aflatoxins, which are among the most potent naturally occurring carcinogens and pose a serious threat to public health and food security. Consequently, *Aspergillus flavus* is a focal point in research pertaining to mycotoxicology, food contamination, and fungal pathogenicity. Its dual role as a saprophyte and pathogen highlights the complexity of its ecological and biological interactions, underscoring the need for a refined understanding of its behavior in both natural and anthropogenic settings.

1.2 Morphology of *Aspergillus flavus*

Accurate species identification within the *Aspergillus flavus* complex remains a challenging task due to the significant overlap in their morphological and biochemical characteristics. This complexity often makes it difficult to differentiate between closely related species within the group. *A. flavus* is commonly characterized as a filamentous mold with a velvety texture and a color spectrum ranging from yellow to green surrounded by a white circle that was eventually covered by conidia (Khan et al., 2020).

The conidiophores of *A. flavus* exhibit irregular lengths and are distinctly rough, with a pitted and spiky surface. These structures can be either uniseriate or biseriate in arrangement, a key feature in their identification. The phialides, which are the specialized cells that produce conidia, are distributed across the entire vesicle surface and point in various directions, contributing to the characteristic appearance of this species.

The conidia, or asexual spores, are typically globose to subglobose in shape, with a prominently echinulate (spiny) surface texture. They measure between 3.5 to 4.5 micrometers in diameter, a distinguishing microscopic feature that aids in species identification. Despite these defining traits, the subtle variations and overlaps with other species within the *Aspergillus flavus* complex often necessitate advanced molecular techniques for accurate identification and classification.

Based on the characteristics of the sclerotia produced, *A. flavus* isolates can be divided into two phenotypic types: the S strain produces numerous small sclerotia (average diameter 400 mm) while the L strain produces fewer, larger sclerotia (Cotty, 1989), within the S strain, some

isolates, termed SB, produce only b aflatoxins, while others, named SBG, produce both b and g aflatoxins (Cotty, 1989).

1.2.1 Taxonomic classification of *Aspergillus flavus*

Aspergillus is a large and diverse group of filamentous fungi that includes over 180 anamorphic species (Pitt et al., 2000), many of which are found in a wide range of environments across the globe. They are also species within this genus that have sexual stages, known as teleomorphs, which are classified into nine different genera based on their reproductive characteristics (Pitt and Samson, 2000). The genus Aspergillus itself is further divided into seven subgenera, each containing multiple species that share similar morphological and genetic traits. These subgenera are then broken down into smaller groups called sections, which categorize species even further based on detailed taxonomic and phylogenetic studies (Klich, 2002).

In 1729, a Florentine priest and mycologist P. A. Micheli named the genus Aspergillus as its conidiophore structure resembled that of the aspergillum, a liturgical tool used to sprinkle holy water (Bennett and Klich, 1992 as cited in Amaike, 2011). This genus has been classified multiple times depending on morphology (Samson, 1992). Aspergillus is recognized by its distinctive conidiophore. The base of the conidiophore typically forms a “T” or “L” shape where it joins the vegetative hyphae, and this structure is often referred to as the “foot cell,” though it is not an independent cell. The stipe extends from the foot cell and can range from being very short (50 µm or less) to several millimeters in length. At the apex of the stipe, a vesicle forms, which can vary in shape. In some species, phialides that produce conidia are directly from the vesicle, a configuration called uniseriate. In other species, there is an additional layer of cells, known as metulae, between the vesicle and the phialides, with these species being termed biseriate. A

distinguishing feature of Aspergillus, compared to closely related genera, is that the phialides and metulae appear simultaneously on the vesicle (Klich, 2002).

Aspergillus flavus, as described by Link in 1809, is an asexual species that produces solely spores (conidia) and overwintering sexual fruiting bodies (sclerotia). The sexual stage of A. flavus has been classified as Petromyces flavus (Horn et al., 2007).

Kingdom: Fungi

Phylum: Ascomycota

Class: Eurotiomycetes

Order: Eurotiales

Family: Trichocomaceae

Genus: Aspergillus

Species: flavus (Klich, 2007)

1.2.2 Lifecycle of Aspergillus flavus

Aspergillus flavus is a saprophytic and pathogenic fungus that may live in a variety of settings. This lifecycle is significant because it affects agriculture, food safety, and health. Understanding the life cycle and stages of *A. flavus* is critical for controlling its spread and minimizing its negative consequences. The lifecycle of *A. flavus* begins with the creation and spread of conidia, which are asexual spores. These conidia are formed on conidiophores, which are specialized structures that allow them to be released into the environment. The lightweight conidia are easily spread by air currents, water, and insect vectors, allowing the fungus to colonize a variety of

substrates, including decaying organic debris and agricultural crops like maize and peanuts (Amaike and Keller, 2011).

When conidia settle on a suitable surface, they germinate under optimal conditions of moisture, temperature, and nutrient availability. This process begins with the spores swelling and the emergence of a germ tube, initiating hyphal growth (Horn & Dorner, 1999). The germ tube extends and develops into hyphae, creating a mycelial network that infiltrates and colonizes the substrate. At this stage, the fungus releases enzymes to decompose complex organic compounds into simpler substances, which it absorbs to sustain its nourishment and growth.

Reproduction in A. flavus is primarily asexual. Conidiophores continuously produce conidia, allowing the fungus to disseminate and colonize new surroundings. However, under some conditions, *A. flavus* can reproduce sexually and develop structures known as sclerotia. These hardened, multicellular formations act as survival units in extreme situations like drought or nutrient deprivation. Sexual reproduction involves the creation of ascospores within sclerotia, which contributes to genetic variety and long-term survival (Horn et al., 2009).

Sexual reproduction in A. flavus follows a heterothallic system, meaning that mating requires two strains of opposite mating types, MAT1-1 and MAT1-2 (Moore et al., 2013). When these compatible strains interact under favorable conditions, they form ascospore-bearing ascocarps embedded within the matrix of sclerotia (Horn et al., 2009). Sclerotia are typically considered survival structures that help A. flavus endure harsh environmental conditions (Wicklow, 1987), but they also play a critical role in its reproductive cycle. In laboratory settings, when mating types are paired, ascocarp formation occurs within the sclerotia, confirming the fungus's ability to undergo sexual reproduction.

Interestingly, sclerotia can originate from a single strain of one mating type and still engage in sexual reproduction if exposed to compatible strains present in the soil. This means that *A. flavus* can acquire genetic material from native soil populations, leading to increased genetic variation.

This phenomenon was confirmed through laboratory and field experiments by Horn et al. in 2016 which demonstrated that single-strain sclerotia, when incubated on soil with natural fungal populations, could produce ascospores containing novel genetic alleles. Such findings suggest that sexual reproduction may be more common in natural environments than previously believed. The process of fertilization in *A. flavus* sclerotia remains largely unexplored, but the study suggests that conidia or other fungal propagules in the soil could serve as male gametes, fertilizing single-strain sclerotia to initiate sexual development. Laboratory experiments revealed that fertilization resulted in biparental inheritance, where progeny displayed independent chromosomal assortment and acquired new alleles from soil strains. This genetic recombination is a key factor in the high diversity observed in *A. flavus* populations, which can affect important traits such as aflatoxin production, environmental adaptability, and resistance to fungicides. However, fertilized sclerotia that were collected from laboratory crosses and applied to soil before ascocarp formation did not show evidence of acquiring new alleles from soil populations. Instead, the progeny only contained alleles from the known parental strains. This suggests that once fertilization occurs, additional genetic exchange with other strains is unlikely, possibly due to a biological mechanism that prevents further fertilization, similar to dikaryon formation in certain basidiomycetes. A crucial aspect of *A. flavus* sexual reproduction is the pattern of mitochondrial inheritance. Genetic analysis of progeny confirmed that mitochondria are inherited exclusively from the sclerotial parent, meaning that all progeny receive their mitochondrial DNA from the female partner. This uniparental inheritance ensures that mitochondria, which are

essential for cellular respiration and energy production, are passed down without recombination, maintaining genetic stability in these organelles.

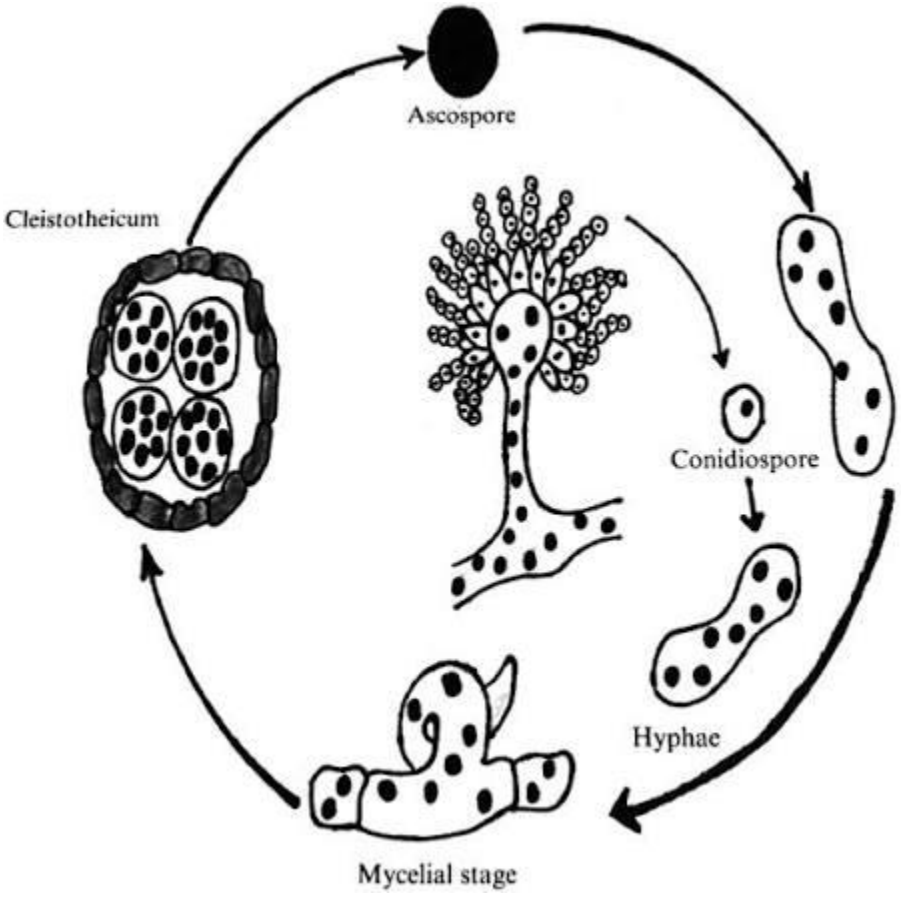

FIGURE 1.1: Life cycle of Aspergillus flavus

Source:springer

1.3 Significance in agriculture and health

1.3.1 In agriculture

Aspergillus flavus is a filamentous fungus that primarily infects oil-rich seeds of economically important crops, including maize, peanuts, cottonseed, and various tree nuts. This infection can occur both during the pre-harvest phase, when crops are still growing in the field, and during the

post-harvest phase, often due to improper storage conditions. A significant concern associated with *A. flavus* is its ability to produce a variety of secondary metabolites, including toxic compounds known as mycotoxins. These mycotoxins include cyclopiazonic acid, aflatrem, and aflatoxins, which are highly toxic and carcinogenic. Among these, aflatoxins are particularly notorious for their impact on human and animal health, as well as their potential to cause significant economic losses in agriculture and food industries. (Duran et al., 2014; Kenneth C. Ehrlich, 2014).

Maize and groundnut are highly susceptible to Aflatoxin contamination, which poses a major risk to human health. Groundnuts, in particular, can be colonized by Aspergillus spp. at various stages, from pre-harvest to storage and transport. There are three known resistance mechanisms in groundnuts: in-vitro seed colonization resistance (IVSC), resistance to pre-harvest Aflatoxin contamination (PAC), and resistance to Aflatoxin production in seeds (Spurthi et al., 2017).However, Aflatoxin is only produced in the cotyledons after fungal infection. Fungal infections in groundnuts cause various seedling and foliar diseases, such as root rot, stem rot, wilts, blight, pod rot, rust, and leaf spots. *Aspergillus flavus*, the primary Aflatoxin producer, infects seeds through sporulation, reducing germination, damaging seedlings, and lowering grain quality and market value (Jalapa et al., 2018).

1.3.2 In health

Aspergillus flavus, a type of filamentous fungus, can naturally infect certain animals, including cattle, dogs, buffalo, goats, and poultry (Pal, 2007). It is also associated with mycotic abortion and mastitis in dairy animals (Pal and Jadhav, 2013). Birds suffering from aflatoxicosis may

exhibit symptoms such as loss of appetite, weight loss, stunted growth, weakness, lethargy, depression, paralysis, and decreased egg production (Pal, 2007).

Aflatoxin M1, while less mutagenic and carcinogenic than Aflatoxin B1, has strong genotoxic properties. It can cause liver damage, lower milk production, weaken immune function, and contribute to anemia, which reduces oxygen supply to tissues (Frantisek et al., 2016). In dairy cattle, exposure to aflatoxins can lead to poor appetite and hinder growth. Research has shown the harmful impact on the liver (Sharmila et al., 2009), reproductive organs (Faisal et al., 2008), kidneys, and heart (Mohammed and Metwally, 2009; Panahrad et al., 2014). Additionally, the detection of aflatoxins in post-mortem brain tissue suggests they can cross the blood-brain barrier (Qureshi H. et al., 2015), potentially affecting mitochondrial DNA and brain cells (Bbosa et al., 2013). Some studies have also reported structural changes in the central nervous system of rodents following exposure to Aflatoxin B1 (Laag and Abdel, 2013).

Contaminated food processing can introduce aflatoxins into the general food supply, where they have been detected in pet food, human food, and livestock feed. When animals consume contaminated feed, they may pass aflatoxin residues into eggs, milk, and meat. In Pakistan, for example, high levels of aflatoxin contamination have been reported in chicken meat and eggs, likely due to tainted poultry feed (Iqbal et al., 2014).

infections) and cutaneous aspergillosis, particularly in cases where the skin has been damaged by trauma (Hedayati et al., 2007).

1.4 MUTATION

Mutagenesis refers to the process by which the genetic material of an organism is altered, leading to mutations in its DNA or RNA. These changes can arise from a variety of sources, including errors during DNA replication, exposure to external factors like chemicals or radiation, or as part of normal cellular processes. Mutagenesis plays a critical role in genetic variation and evolution, contributing to both beneficial and harmful genetic alterations. It can occur spontaneously or be induced artificially in laboratory settings for research purposes.

Spontaneous mutagenesis often results from internal processes, such as replication errors or natural chemical reactions within the cell. In contrast, induced mutagenesis occurs when external agents, such as radiation or chemical mutagens, intentionally alter the DNA structure. These induced changes can be useful for studying gene function or for generating genetic diversity in model organisms, as noted by Lindahl (1993). For example, site-directed mutagenesis is a method used to create specific genetic alterations in a controlled manner, allowing researchers to examine the effects of particular mutations on gene function or protein activity (Sambrook and Russell, 2001).

1.5 EPISTASIS

Epistasis is a type of gene interaction that occurs when one gene affects the expression of another gene. Epistasis can be classified into two main types (Phillips, 2008).

1.5.1 RECESSIVE EPISTASIS

Here one gene masks the expression of another gene. This occurs when the presence of a recessive allele at one gene locus inhibits the expression of another gene. An example of

recessive epistasis can be observed in the coat color of Labrador retrievers. The coat color of these dogs is controlled by two genes, B and E. The gene at the B locus controls the production of black pigment, while the gene at the E locus controls the distribution of black and yellow pigments (Stuber and Goodman, 1989). If an individual is homozygous recessive for the B gene, no black pigment is produced, and the coat color is entirely yellow, irrespective of the alleles at the E locus. Therefore, the recessive allele at the B locus is epistatic to the alleles at the E locus.

1.5.2 DOMINANT EPISTASIS

In this case one gene overrides the expression of another gene. This occurs when the presence of a dominant allele at one gene locus masks the expression of another gene. As in the flower color of snapdragons, color of these plants is controlled by two genes, R and C. The gene at the R locus controls the synthesis of pigments, while the gene at the C locus controls the color of the pigments. If an individual is homozygous dominant for the R gene, no matter the alleles at the C locus, the flower color will be white. Therefore, the dominant allele at the R locus is epistatic to the alleles at the C locus (Phillips, 2008).

Epistatic interactions can be classified into:

1.5.3 POSITIVE EPISTASIS

Positive epistasis occurs when the effect of one gene enhances the effect of another gene, leading to a more pronounced phenotype. For example, in a study on Arabidopsis thaliana, mutations in different genes involved in the flowering pathway were found to interact synergistically, resulting in early flowering (Kover et al., 2009).

1.5.4 NEGATIVE EPISTASIS

Negative epistasis refers to the situation where the effect of one gene masks or suppresses the effect of another gene, leading to a less severe phenotype. Negative epistasis is often observed in studies on antibiotic resistance. Multiple genetic mutations are required for bacteria to become resistant, and if these mutations occur in different genes, negative epistasis may reduce the likelihood of resistance due to their combined effects (Kover et al., 2009).

1.6 AUXOTROPHY IN ASPERGILLUS

An auxotroph is a microorganism that cannot synthesize one or more essential growth factors and is unable to grow in fermentation media that do not contain them (Walker, 2014).

In Aspergillus, auxotrophy typically results from mutations in genes responsible for specific metabolic pathways. These mutations disrupt the synthesis of essential compounds such as amino acids, purines, or pyrimidines. For example, mutations in the arg and pyr genes, which are involved in arginine and pyrimidine biosynthesis respectively, prevent the organism from producing these compounds. As a result, external supplementation of arginine or uracil is necessary for growth (Kafer, 1977).

Auxotrophy has several important applications in research. In genetics, auxotrophic mutants are frequently used as markers in transformation experiments. These markers allow researchers to introduce wild-type alleles into mutants and restore their ability to synthesize essential compounds, enabling genetic mapping and functional studies. In metabolic engineering, auxotrophic strains are used to enhance the production of valuable metabolites. For instance, mutants of *Aspergillus oryzae* with deficiencies in lysine biosynthesis have been developed to increase lysine production (Shimizu, 2013). Additionally, auxotrophic mutants are instrumental in studying fungal pathogenesis. Mutants with disrupted metabolic pathways often show reduced

virulence, offering insights into the metabolic requirements of fungal infections. This approach has been particularly useful in understanding the pathogenicity of Aspergillus fumigatus (Amich et al., 2016).

Several auxotrophic markers are commonly used in Aspergillus research. Mutations in the niaD gene, which disrupt nitrate assimilation, prevent the fungus from using nitrate as a nitrogen source. Similarly, the pyrG gene, which encodes an enzyme involved in pyrimidine biosynthesis, is widely used as a marker in transformation studies for species such as *Aspergillus nidulans* and *Aspergillus fumigatus*.

Other markers include genes involved in the biosynthesis of amino acids like arginine (arg), histidine (his), and lysine (lys), which are frequently utilized in genetic studies.

The development of genome editing tools like CRISPR/Cas9 has made it easier to create targeted auxotrophic mutations. This has broadened the scope of their applications in both basic research and biotechnology. Auxotrophic mutants will continue to be valuable tools for advancing our understanding of fungal biology and developing innovative solutions in industrial and medical fields.

1.7 AIMS AND OBJECTIVES

Aim:

To develop auxotrophic mutants of *Aspergillus flavus*, evaluating metabolic, growth differences and epistatic interactions between both mutants

Objectives:

- **Mutant Development:**

Generate an auxotrophic mutant of Aspergillus flavus using potassium chlorate and sodium azide as mutagenic agents

- **Growth Rate Assessment:**

Compare the growth rates of the mutant and wild-type strains under identical environmental conditions.

- **Metabolic response study:**

Evaluate the metabolic response of the strains by varying substrate composition to determine its nutrient adaptability and dependency relative to the wild-type strain

- **Epistatic Interaction:**

The compatibility of both mutant strains is examined in complete and nutrient deficient medium to evaluate epistasis between both auxotrophic mutants.

CHAPTER 2

MATERIALS AND METHODS

2.1 ISOLATION OF FUNGAL STRAIN

The fungal strain, *Aspergillus flavus,* was obtained McCartney bottles of harvested *A flavus,* which were originally isolated from bread purchased from Fola Bakery, located in Ekosidin, Benin City, Edo State. The bread was intentionally left exposed to air for several days to promote fungal contamination under natural conditions.

2.2 MEDIA PREPARATION

Different culture media were prepared as follows:

2.2.1 Potato Dextrose Agar (PDA)

This medium was made by boiling 200 g of peeled and chopped Irish potatoes in 500ml of water for 30 minutes, the mixture was then strained through cheesecloth, and the filtered liquid was collected as the potato infusion. 20 g of dextrose sugar and 15 g of agar powder was added to the potato infusion and mixed. Water was added to this mixture up to 1 L (Sagar, 2022).

2.2.2 Potato Dextrose Chlorate (PDC)

To create this medium, 6 g of potassium chlorate was added to 1 L of PDA.

2.2.3 Potato dextrose sodium azide (PDSA)

This medium was prepared by adding 20.0 g of sodium azide to 1 L of PDA. This medium was also prepared at a lower concentration by adding 12 g of sodium azide to 1 L of PDA due to the

inability to have fungal growth in the previous concentration.

2.2.4 Potato dextrose sodium azide broth

2g of sodium azide is added to 20g of dextrose and 200g potato infusion. This medium was prepared as a result of the failure of *Aspergillus flavus* to grow in PDSA

2.2.5 Minimal Medium (MM)

Prepared by mixing 20 g of dextrose, 15 g of agar powder.

2.2.6 Minimal Medium + Nitrate ($MMNO_3$)

Prepared by mixing 20 g of dextrose, 15 g of agar powder, and 6 g of sodium nitrate in 1 liter of sterile water.

2.3 STERILIZATION OF MEDIA

The prepared media were sterilized using an autoclave at 121°C under a pressure of 15 psi for 15 minutes to ensure sterility.

2.4 INOCULATION PROCEDURE

After cooling of each sterilized media to 45–50°C, they were poured into sterile Petri dishes under aseptic conditions in a laminar flow cabinet and left to solidify.In an aseptic condition vegetative part of *Aspergillus flavus* inoculated using a sterile inoculating loop from bread samples on five petri dishes containing potatoes dextrose agar gel using spot streaking method. After two days fungi growth was observed but it was in a mixture culture. In order to get pure isolates, *Aspergillus flavus* strains were subcultured and three different stains were gotten (the strains were differentiated morphologically according to their colours).

2.5 MUTAGENESIS

Using an inoculating loop the vegetative parts of each strain were cultured on PDC using the spot streaking method which gave rise to the first generation of auxotrophic mutants. The procedure was repeated to develop the second generation of auxotrophic mutants. The first and second generation of mutants were labeled.

2.6 STARVATION

Using a cork borer of about 5 mL the growing section of the wild and mutant types of Aspergillus were inoculated on minimal medium. Growth diameter was taken using a meter rule and average growth rate of each groups were calculated.

2.7 HARVESTING OF SPORES

Spores of wild Aspergillus strain were harvested by flooding them with sterile water and scraping them with a sterile inoculating loop. The spore suspension were stored in sterile McCartney bottles and were refrigerated for further use.

CHAPTER 3

RESULTS

Pure culture of wild-type *A. flavus*, were obtained by in PDA. They exhibited robust growth with a radial growth rate of around 8.5 ± 0.3 mm daily colonies were green, velvety, and were heavily sporulated by day 5.

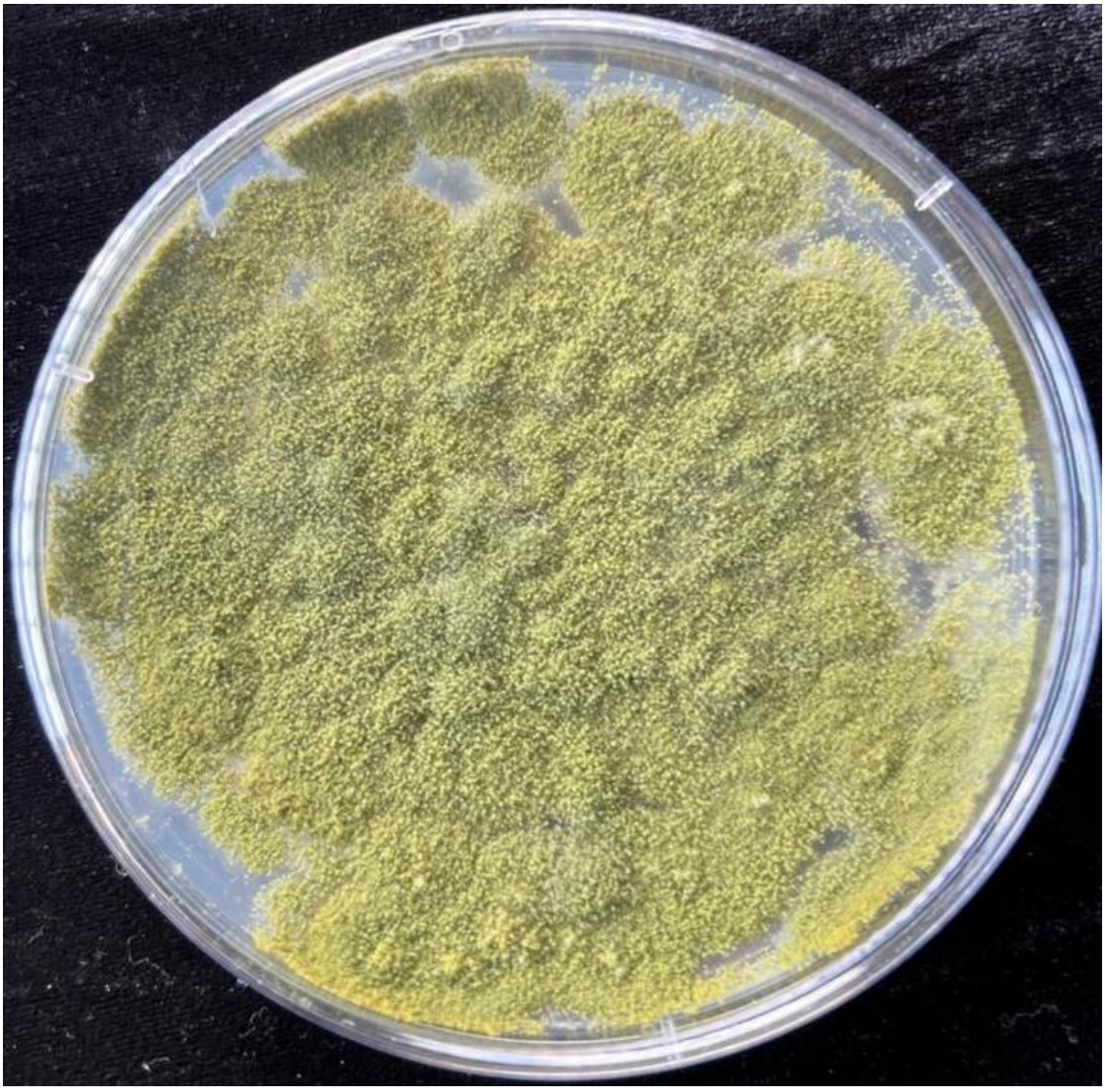

Plate 3.1: Day 5 of *Aspergilus flavus* cultured on PDA.

Azide mutant of *Aspergillus flavus* developed on PDA(after being subjected to the PDSA-broth) and chlorate mutant developed on PDC medium.

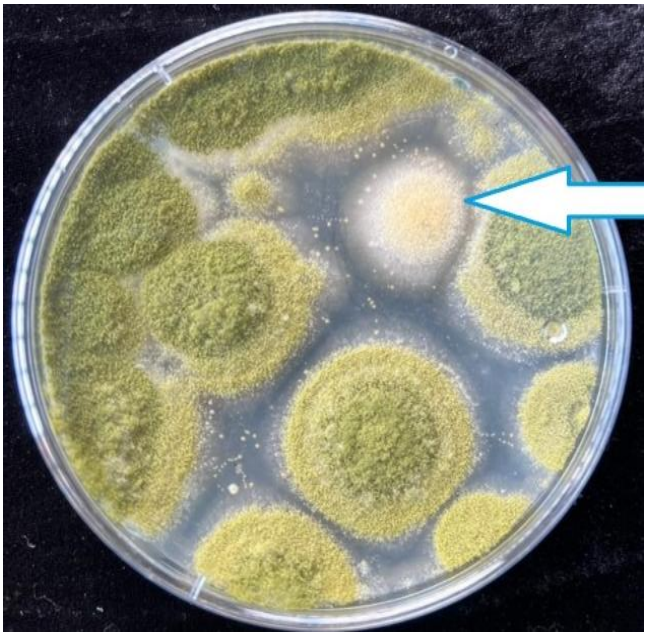

Plate 3.2: Emergence of Chlorate mutated *A. flavus* on PDC.

Plate 3.3: Emergence of azide mutant of *A. flavus* on PDA.

When co-cultured on PDA both mutants grew towards each other, with no distinct zone (margin) of inhibition at the interface, suggesting antagonistic interactions. No significant overlap was observed.

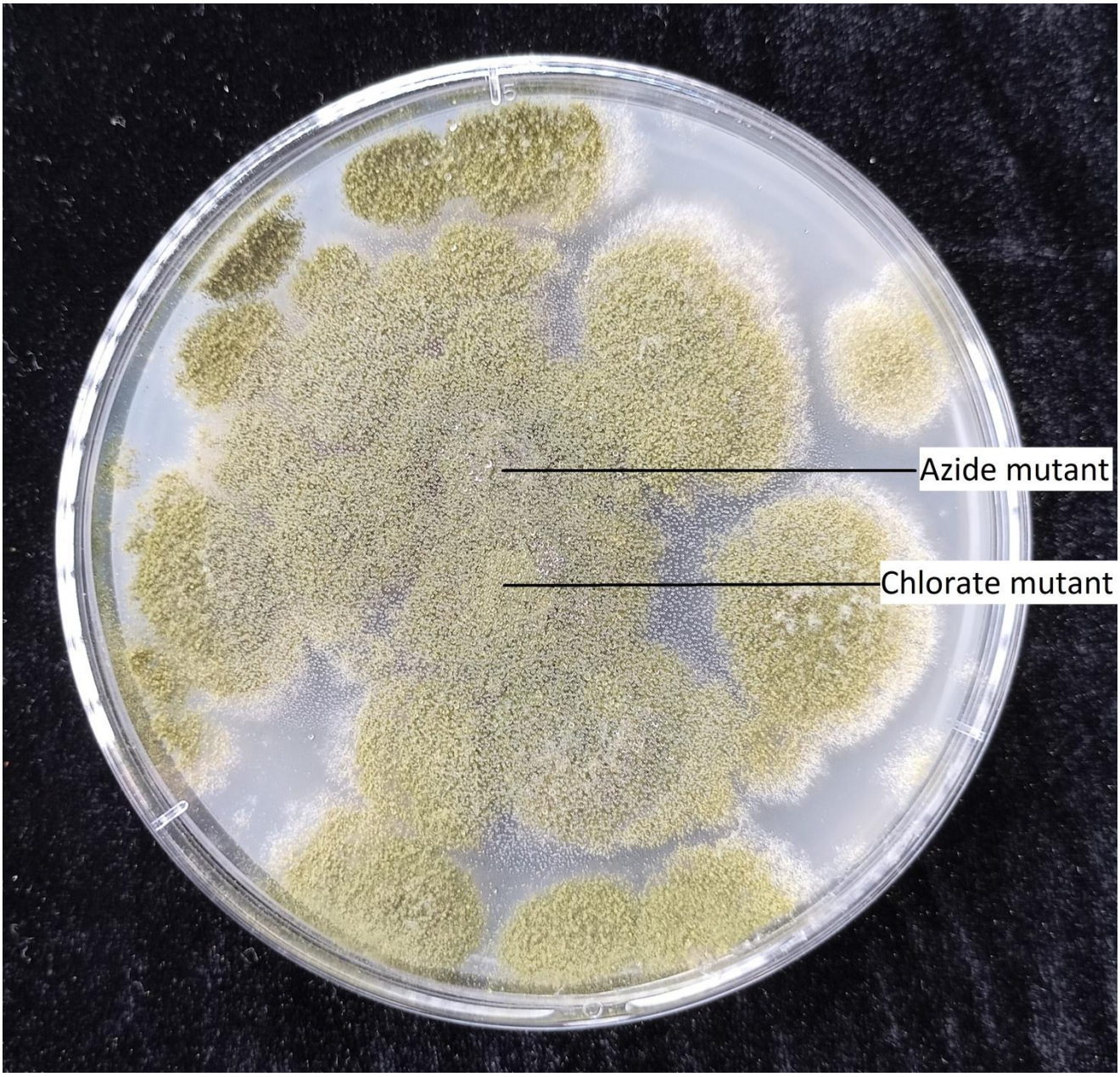

Plate 3.4: Co-culture of azide and chlorate mutants on PDA.

The mutants showed competitive inhibition when co-cultured in Minimal medium, with the potassium chlorate mutant outgrowing the sodium azide mutant, reducing its colony diameter. A clear inhibition zone was observed, indicating strong antagonism in MM.

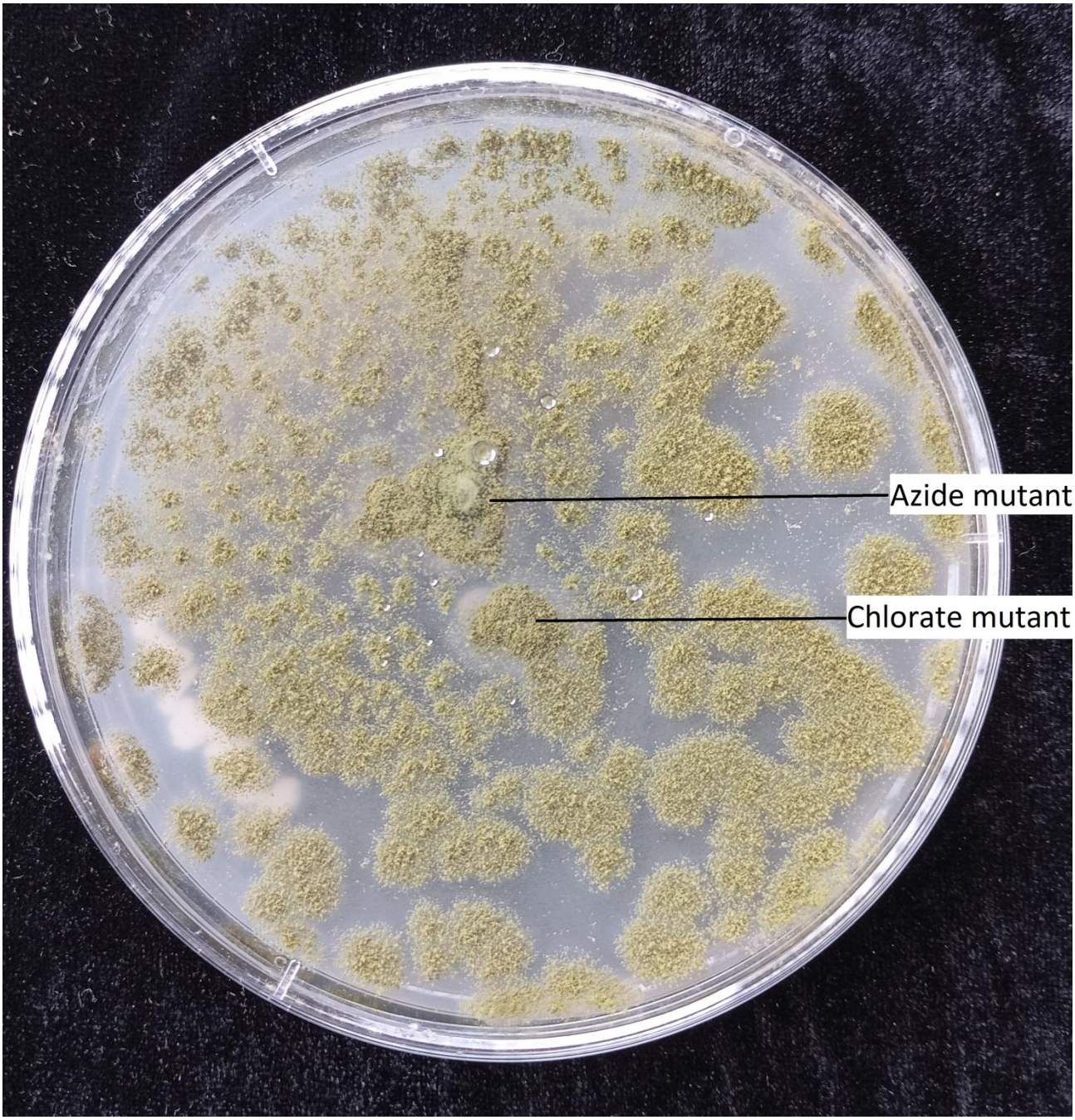

Plate 3.5: Co-culture of both mutants of *A. flavus* on minimal medium.

Both mutants were cultured seperately on $MMNO_3$, and proved they could utilise nitrate as a nutrient suppliment by growing in this medium.

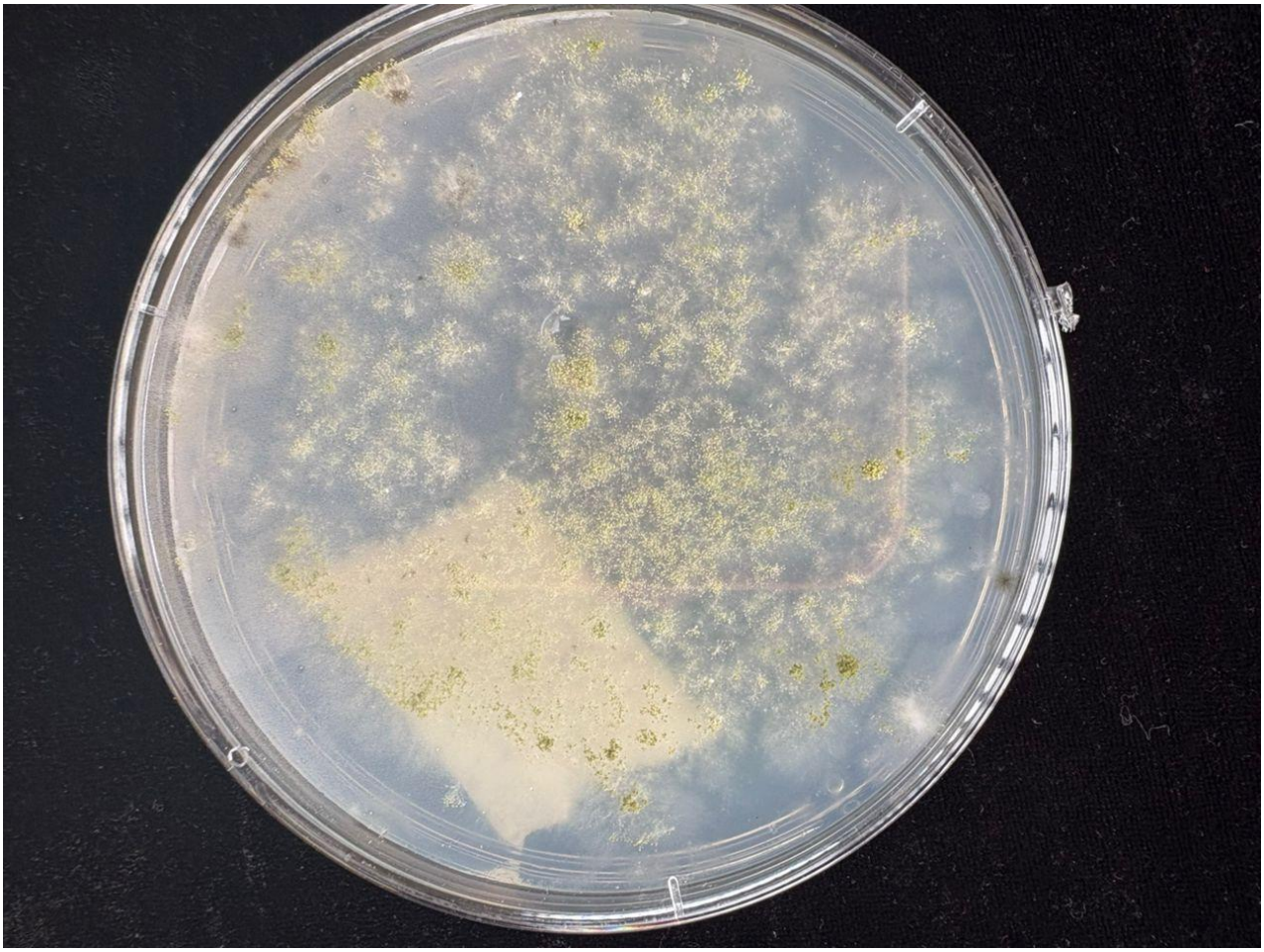

Plate 3.6: Chlorate mutant cultured on Minimal medium.

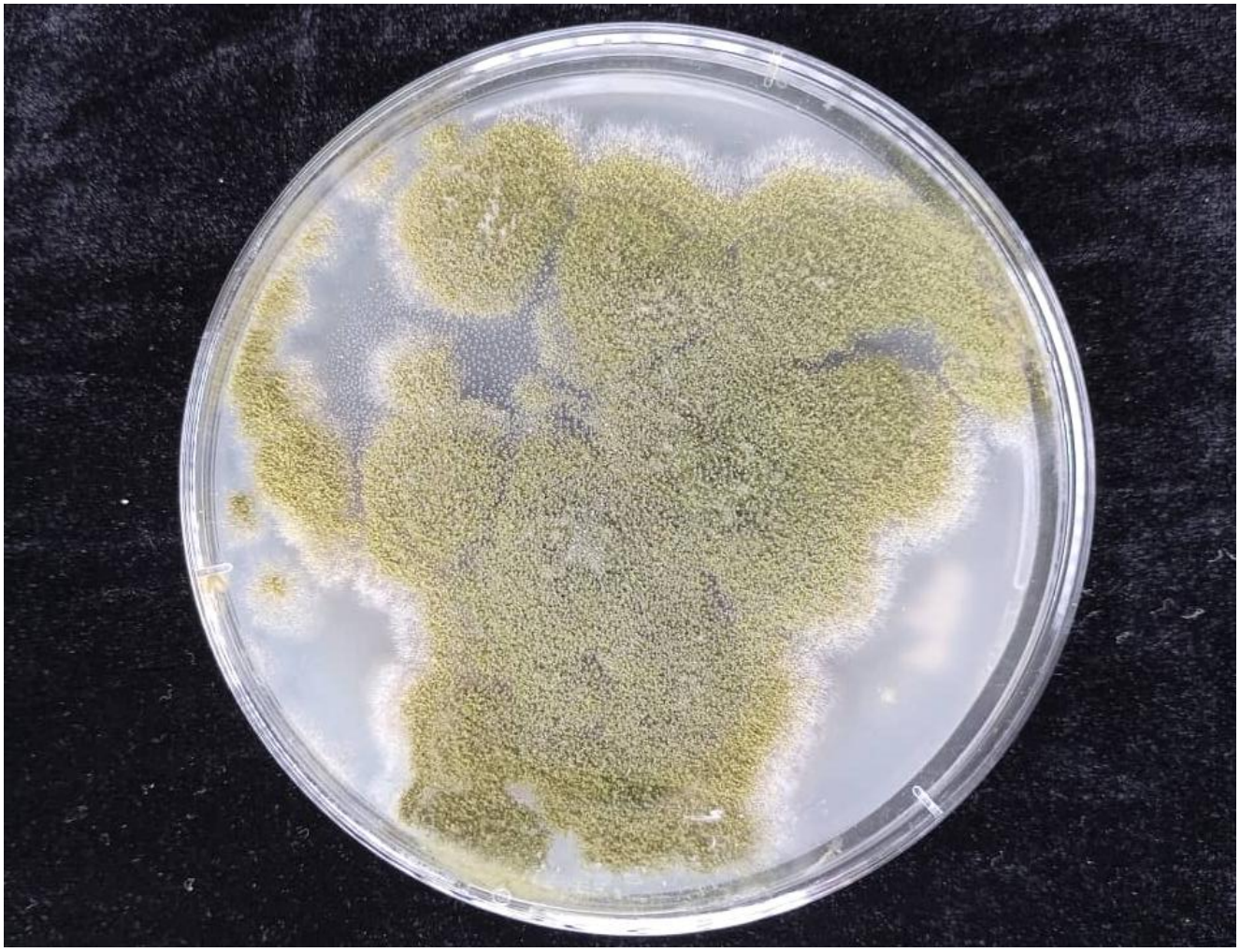

Plate 3.7: Azide mutant cultured on Minimal medium.

When co-cultured on $MMNO_3$ the azide mutant completely surpressed the growth of the chlorate mutant.

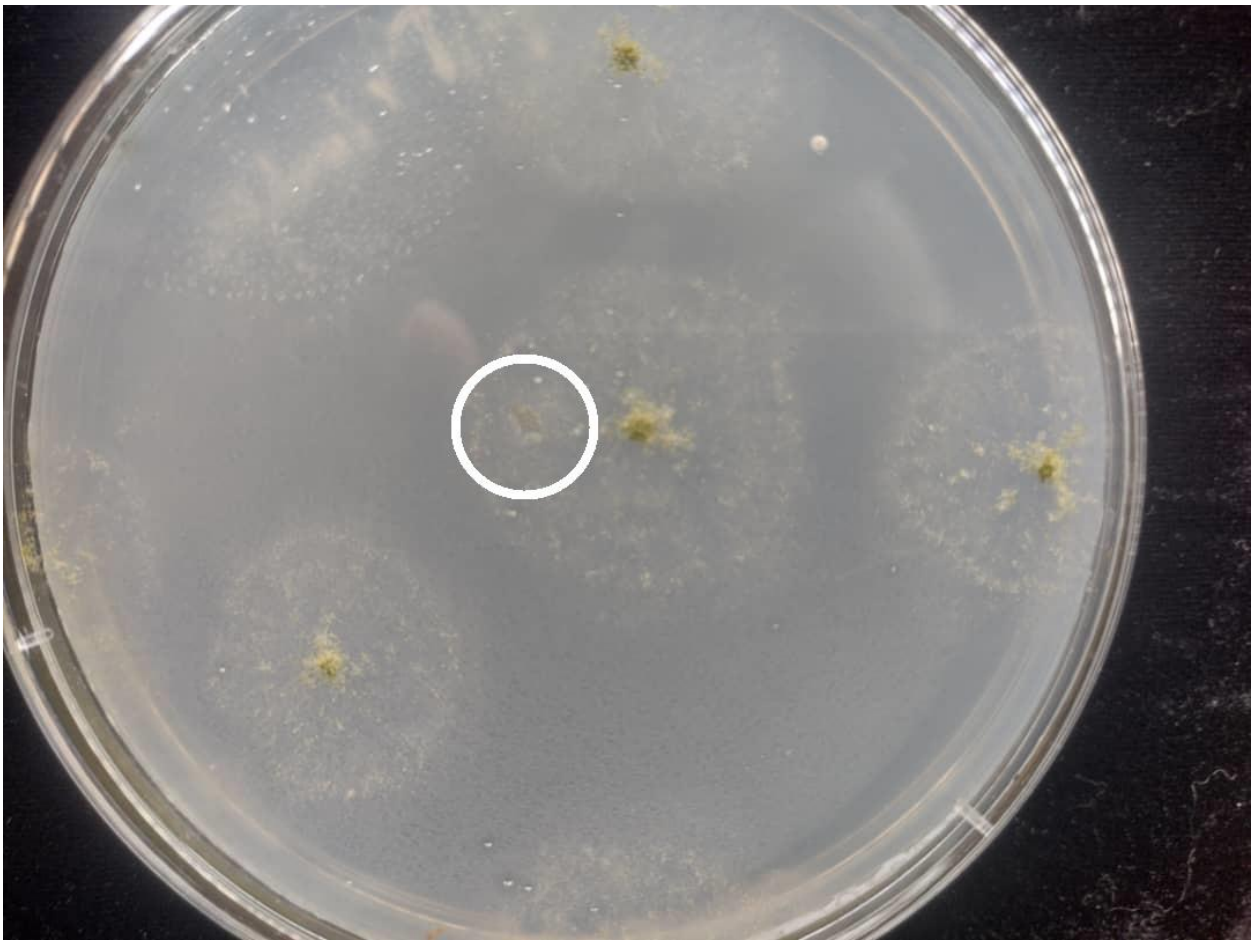

Plate 3.8: Azide and chlorate mutants cultured on $MMNO_3$.

CHAPTER 4

DISCUSSION

This study investigated the epistatic effect between sodium azide and potassium chlorate mutants of *Aspergillus flavus*. Sodium azide was shown to be a more powerful mutagen than potassium chlorate, as its initial application completely inhibited growth in its medium, until a different method was applied, highlighting its stronger impact. The mutations primarily affected the hyphal wall, while their effects on spores were less significant, indicating varying levels of structural susceptibility.

The auxotrophic mutants of *Aspergillus flavus*, induced by sodium azide and potassium chlorate, exhibited contrasting growth behaviours, when co-cultured in complete medium (PDA), incomplete medium (MM) and in Minimal medium without sodium nitrate. In PDA, which provides all essential growth factors (Walker, 2014), the mutants displayed robust growth and high compatibility, with colonies merging seamlessly.

This suggests metabolic complementation, where the azide mutant likely compensates for the chlorate mutant's deficiency, possibly by supplying a critical metabolite (Fincham, 1966; Pontecorvo, 1953). In contrast, growth in MM was scanty, with clear boundaries between

colonies, indicating no complementation, likely due to nutrient scarcity preventing metabolite sharing (Glass & Kaneko, 2003).

These differences stem from epistatic interactions, where the azide mutant's gene expression masks the chlorate mutant's defects in PDA but not in MM, where both mutants prioritize survival over interaction (Meidanis & Zanettini, 2016).

Cultured seperately, both mutants proved they could utilize nitrate by growing on MMNO3, but when co-cultured in the medium, the azide mutant completely suppressed the growth of the chlorate mutant of *A. flavus*, displaying dominant epistasis.

This study investigates the epistatic effect of double mutations induced by sodium azide and potassium chlorate in *A. flavus*. The epistatic interactions between the mutations revealed both asynergistic and antagonistic effects on various traits. These findings provide insights into the genetic mechanisms underlying aflatoxin production and fungal development in *A. flavus*, with implications for developing novel strategies to control aflatoxin contamination

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